Brown spider dermonecrotic toxin directly induces nephrotoxicity

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Abstract

Brown spider (Loxosceles genus) venom can induce dermonecrotic lesions at the bite site and systemic manifestations including fever, vomiting, convulsions, disseminated intravascular coagulation, hemolytic anemia and acute renal failure. The venom is composed of a mixture of proteins with several molecules biochemically and biologically well characterized. The mechanism by which the venom induces renal damage is unknown. By using mice exposed to Loxosceles intermedia recombinant dermonecrotic toxin (LiRecDT), we showed direct induction of renal injuries. Microscopic analysis of renal biopsies from dermonecrotic toxin-treated mice showed histological alterations including glomerular edema and tubular necrosis. Hyalinization of tubules with deposition of proteinaceous material in the tubule lumen, tubule epithelial cell vacuoles, tubular edema and epithelial cell lysis was also observed. Leukocytic infiltration was neither observed in the glomerulus nor the tubules. Renal vessels showed no sign of inflammatory response. Additionally, biochemical analyses showed such toxin-induced changes in renal function as urine alkalinization, hematuria and azotemia with elevation of blood urea nitrogen levels. Immunofluorescence with dermonecrotic toxin antibodies and confocal microscopy analysis showed deposition and direct binding of this toxin to renal intrinsic structures. By immunoblotting with a hyperimmune dermonecrotic toxin antiserum on renal lysates from toxin-treated mice, we detected a positive signal at the region of 33–35 kDa, which strengthens the idea that renal failure is directly induced by dermonecrotic toxin. Immunofluorescence reaction with dermonecrotic toxin antibodies revealed deposition and binding of this toxin directly in MDCK epithelial cells in culture. Similarly, dermonecrotic toxin treatment caused morphological alterations of MDCK cells including cytoplasmic vacuoles, blebs, evoked impaired spreading and detached cells from each other and from culture substratum. In addition, dermonecrotic toxin treatment of MDCK cells changed their viability evaluated by XTT and Neutral-Red Uptake methodologies. The present results point to brown spider dermonecrotic toxin cytotoxicity upon renal structures in vivo and renal cells in vitro and provide experimental evidence that this brown spider toxin is directly involved in nephrotoxicity evoked during Loxosceles spider venom accidents.

Keywords: Brown spider; Dermonecrotic toxin; Cytotoxicity; Kidney; MDCK cells

Introduction

Accidents involving spiders of the genus Loxosceles (brown spiders) have been reported in North America, Latin America, Europe, Middle East and other parts of Asia, Africa and Australia (Futrell, 1992; da Silva et al., 2004). In the USA, the range of Loxosceles spiders extend from southeastern Nebraska to southernmost Ohio and south into Georgia and most of Texas. Brown spiders inhabit also Arizona, Nevada, New Mexico, Utah and southern California (Futrell, 1992; da Silva et al., 2004;
Vetter and Bush, 2002). Envenomation caused by brown spider gives rise to dermonecrotic lesions with gravitational spreading (the hallmark of bites) and systemic manifestations such as renal failure, disseminated intravascular coagulation and intravascular hemolysis (Futrell, 1992; da Silva et al., 2004). Systemic involvement is less common than skin injuries, but it may also be the cause of complications and death.

The mechanisms by which Loxosceles spider venom causes its lesions are currently under investigation. The venom is a mixture of proteic toxins enriched with molecules of low molecular mass (5–40 kDa) (Mota and Barbaro, 1995; da Silva et al., 2004; da Silveira et al., 2002). Several toxins have been identified and well characterized biochemically in Loxosceles venom. These include alkaline phosphatase, ribonuclease phosphohydrolase, hyaluronidase, serine proteases, metalloproteases and sphingomyelinase-D (Feitosa et al., 1998; Futrell, 1992; da Silva et al., 2004; Veiga et al., 2001a). Metalloproteases named Loxolysin A (20–28 kDa) and Loxolysin B (32–35 kDa) have gelatinolytic, fibronectinolytic and fibrinogenolytic activities and can play a role in hemostatic disturbances occurring after envenomation such as injury of blood vessels, hemorrhage into the dermis, imperfect platelet adhesion and defective wound healing (Feitosa et al., 1998; da Silva et al., 2004; Zanetti et al., 2002). The hyaluronidase toxin degrades hyaluronic acid and chondroitin sulfate residues from proteoglycans and could be putatively involved in the gravitational spreading of dermonecrosis and as systemic spreading factor (Futrell, 1992; da Silva et al., 2004; Young and Pincus, 2001). The sphingomyelinase-D (30–35 kDa), also called dermonecrotic toxin, is the best biochemically characterized molecule identified in the venom of different Loxosceles species. This toxin, as a native molecule or as recombinant variants, can induce dermonecrosis, platelet aggregation and experimental hemolysis (Cunha et al., 2003; Kalapothakis et al., 2002; Pedrosa et al., 2002). Recently, de Castro et al. (2004) identified a family of low molecular mass (5.6–7.9 kDa) insecticidal toxins in the Loxosceles intermedia venom. The authors postulated that these molecules might contribute to the toxicity of the venom. Other activities produced by unidentified toxins have been described in the venom. These include hydrolytic activities in the protein core of a heparan sulfate proteoglycan from vessel endothelial cells, entactin and basement membranes (Veiga et al., 2000, 2001a, 2001b). The mechanisms by which these activities play a role in the noxious effects of the venom have not been fully determined.

Brown spider venom contributes directly or indirectly to cytotoxic activities upon different cells. The venom has hemolytic activity on erythrocytes (Futrell, 1992; Williams et al., 1995) and causes platelet aggregation (Futrell, 1992; Veiga et al., 2000). The venom has a direct inhibitory effect on neutrophil chemotaxis in vitro (Majestik et al., 1977). On the other hand, it can induce a strong indirect dysregulated endothelial-cell-dependent neutrophil activation (Patel et al., 1994), which seems to play a role in dermonecrotic injuries evoked after envenomation (Futrell, 1992; da Silva et al., 2004). This last hypothesis is strengthened by histopathological findings from rabbits that were experimentally exposed to the venom (Elston et al., 2000; Ospedal et al., 2002) and from histological analysis of human patients after brown spider bites (Futrell, 1992; da Silva et al., 2004; Yiannias and Winkelmann, 1992). Additionally, the venom has a cytotoxic effect upon cultured human umbilical vein endothelial cells (Patel et al., 1994) and rabbit aorta endothelial cells (Veiga et al., 2001a).

Renal disorders evoked by brown spider venom have been earlier reported from clinical data of victims (Futrell, 1992; Lung and Mallory, 2000; da Silva et al., 2004). Venom nephrotoxicity was further demonstrated by histopathological findings from crude-venom-treated mice. Histological analysis of kidneys showed hyalinization and erythrocytes in the Bowman’s space, glomerular collapse, tubular epithelial cell cytotoxicity and deposition of proteinaceous material within the tubular lumen. Immunofluorescence demonstrated the deposition and binding of venom toxin(s) along the tubular and glomerular structures (Luciano et al., 2004). We report here the direct effect of a recombinant dermonecrotic toxin obtained from a cDNA library of L. intermedia gland venom. The recombinant molecule was able to reproduce the nephrotoxicity evoked by crude venom. This toxin may account for renal injury associated with envenomation by Loxosceles spiders.

Methods

Reagents. Polyclonal antibodies to L. intermedia dermonecrotic toxin (LiRecDT) were produced in the rabbit as described by Luciano et al. (2004). Hyperimmune IgGs were purified from serum using Protein–A Sepharose (Amersham Biosciences, Piscataway, USA) as recommended by the manufacturer. Fluorescein-conjugated anti-rabbit IgG was purchased from Sigma, St. Louis, USA. Crude venom from L. intermedia was extracted from spiders captured in the wild as described by Feitosa et al. (1998).

cDNA library construction. Two hundred adult L. intermedia spiders were submitted to venom extraction by electrostimulation (15 V applied to the cephalothorax) to stimulate mRNA production. After 5 days, venom glands were collected, and mRNA was purified using the FastTrack 2.0 mRNA Isolation Kit, according to the manufacturer’s protocol (Invitrogen, Carlsbad, USA). The cDNAs were synthesized from 4.3 μg mRNA using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning, linked to SalI adaptors, NotI digested and linked to pre-cut NotI–SalI pSPORT1 vector, using the
method recommended by the manufacturer (Invitrogen). *Escherichia coli* DH5α cells were transformed with ligation reaction and then plated on LB agar plates containing 100 μg/ml ampicillin.

cDNA library screening. Randomly chosen colonies were inoculated in 5 ml LB broth containing 100 μg/ml ampicillin, grown overnight at 37 °C (with aeration), and the DNA plasmid was purified by alkaline lysis method using QIAprep Spin Miniprep Kit following the manufacturer’s protocol (QIAGEN, Valencia, USA). Purified plasmids were sequenced on both strands using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). Reactions were carried out on an ABI 377 automatic sequencer (Applied Biosystems), and the primers used to sequence were T7 promoter and SP6 promoter. The nucleotide sequences were analyzed using Genetyx-Mac v7.3 software (Software Development, Tokyo, Japan). The putative protein products from cDNA sequences were compared to GenBank protein databases at NCBI (Altschul et al., 1997).

Recombinant protein expression. The cDNA corresponding to the putative mature dermonecrotic protein was amplified by PCR. The forward primer used was 30 Rec sense (5'-CTCGAGCCAGTAATCGTCGGCCTATA-3') designed to contain an *Xho*I restriction site (underlined) plus the sequence related to the first seven amino acids of mature protein. The reverse primer used was 30 Rec antisense (5'-CGGGATCCCTATTTCTTGAATGTCAC-CCA-3'), which contains a *Bam*HI restriction site (underlined) and the stop codon (bold). The PCR product was cloned into pGEM-T vector (Promega, Madison, USA). The pGEM-T vector containing the mature protein encoding cDNA was then digested with *Xho*I and *Bam*HI restriction enzymes. The excised insert was gel purified using QIAquick Gel Extraction Kit (QIAGEN) and subcloned into pET-14b (Novagen, Madison, USA) digested with the same enzymes. The correct construct was confirmed by sequencing. The recombinant construct was expressed as fusion protein, with a 6× His-tag at the N-terminus and a 13 amino acid linker including a thrombin site between the 6× His-tag and the mature protein (N-terminal amino acid sequence before the mature protein: MGSSHHHHHHH-SGLVPRGSHMLLE). pET-14b/L. *intermedia* cDNA construct was transformed into One Shot *E. coli* BL21(DE3) pLysS competent cells (Invitrogen) and plated on LB agar plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. A single colony was inoculated into 50 ml LB broth (100 μg/ml ampicillin and 34 μg/ml chloramphenicol) and grown overnight at 37 °C. A 10 ml portion of this overnight culture was grown in 1 L LB broth/ampicillin/chloramphenicol at 37 °C until the OD at 550 nm reached 0.5. IPTG (isopropyl β-D-thiogalactoside) was added to a final concentration of 0.05 mM, and the culture was induced by the incubation for additional 3.5 h at 30 °C (with vigorous shaking). Cells were harvested by centrifugation (4000 × g, 7 min), and the pellet was frozen at −20 °C overnight.

Protein purification. Cells were disrupted by thawing, and the harvested cell was pasted in 40 ml of extraction buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme). Lysed material was centrifuged (20,000 × g, 20 min), and the supernatant was incubated with 2 ml Ni-NTA agarose beads for 1 h at 4 °C (with gentle agitation). The suspension was loaded to a column, and the packed gel was exhaustively washed with the appropriate buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole) until the OD at 280 nm reached 0.01. The recombinant protein was eluted with 20 ml of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 250 mM imidazole), and 1 ml fractions were collected and analyzed by 15% SDS-PAGE under reducing conditions. Fractions were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 8.0, containing 200 mM NaCl.

Animals. Adult Swiss mice weighing approximately 25–30 g and adult rabbits weighing approximately 4 kg from the Central Animal House of the Federal University of Paraná were used for in vivo experiments with crude venom and *LiRecDT*. All experimental protocols using animals were performed according to the “Principles of Laboratory Animal Care” (NIH Publication no. 85-23, revised 1985) and “Brazilian Federal Laws”.

*LiRecDT* administration. For the evaluation of the dermonecrotic effect, 10 μg of *LiRecDT* diluted in PBS was injected intradermally into a shaved area of rabbit skin. Dermonecrosis was checked 24 h after injection as previously described by Veiga et al. (2000). Purified *LiRecDT* (samples of 1 mg of proteins/kg of mice) were diluted in PBS (150 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.3). These samples were injected intraperitoneally (to quicken and to make uniform the release of toxin into circulation) in a volume of 100 μl in each mouse. Intradermal injections induced similar animal manifestations, however, the time for this observation was greater and more variable (between 24 and 72 h, data not shown). The animals were divided into two groups, a control group and a test group. The control group consisted of five animals receiving only PBS, and the test group consisted of five animals receiving *LiRecDT*. During the experimental procedures, the envenomation was repeated 3 times, completing a number of 15 animals in the control group and 15 animals receiving *LiRecDT*. All animals were kept under the same experimental conditions.

Blood and urine collections and laboratory analyses. Blood samples (directly from the heart) were obtained from mice anesthetized with ketamine (Agribands, Paulinia, Brazil) and acepromazine (Univet, São Paulo, Brazil). Urine samples were obtained from mice submitted to soft massage.
on the abdominal region and collected using a micropipette. Blood urea nitrogen and urinalysis were determined using standardized techniques and reagents as described by Henry (2001).

**Gel electrophoresis and immunoblotting.** Lysed renal cells were obtained from treatment of kidneys with a lysis buffer (50 mM Tris–HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 1 mM CaCl₂, 1 mM phenylmethanesulfonyl fluoride, 5 mM EDTA and 2 μg/ml aprotinin) for 15 min at 4 °C. The extract was clarified by centrifugation for 10 min at 13,000 × g. Protein content was determined by the Coomassie blue method (BioRad, Hercules, USA) as described by Bradford (1976). Renal extracts (100 μg of proteins) or purified LiRecDT (2 μg) were submitted to SDS-PAGE under non-reducing conditions. For protein detection, gels were stained with Coomassie blue. For immunoblotting, proteins were transferred to nitrocellulose filters overnight as described by Towbin et al. (1979) and immunoassayed using hyperimmune purified IgG that reacts to dermonecrotic toxin as described in Reagents. The molecular mass markers used were acquired from Sigma.

**Histological methods for light microscopy.** Rabbit skin and kidneys (mouse) were collected from animals anesthetized with ketamine (Agribands) and acepromazine (Univet) and then fixed in “ALFAC” fixative solution (ethanol absolute 85%, formaldehyde 10% and glacial acetic acid 5%) for 16 h at room temperature. After fixation, samples were dehydrated in a graded series of ethanol before paraffin embedding (for 2 h at 58 °C) (Drury and Wallington, 1980). Then, thin sections (4 μm) were processed for histology. Tissue sections were stained by hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) (Beautler et al., 1995; Culling et al., 1985).

**Kidney sections and MDCK cells immunofluorescence assays.** For immunofluorescence microscopy, kidney tissues were fixed with 2% paraformaldehyde in PBS for 30 min at 4 °C, incubated with 0.1 M glucose for 3 min and blocked with PBS containing 1% BSA for 1 h at room temperature. Histological sections were incubated for 1 h with specific antibodies raised against dermonecrotic toxin (2 μg/ml) as described in Reagents. The sections were washed three times with PBS, blocked with PBS containing 1% BSA for 30 min at room temperature and incubated with fluorescein-conjugated anti-rabbit IgG secondary antibodies (Sigma) at room temperature for 40 min. For antigen competition assay, the immunofluorescence protocol was the same as described above, except that the hyperimmune IgG to dermonecrotic toxin was incubated previously for 1 h with 10 μg/ml of LiRecDT diluted in PBS. Then, the mixture was incubated with renal sections identically as above. Alternatively, MDCK cells were replated on glass coverslips (13 mm diameter) for 48 h. Cells were then incubated with 10 μg/ml of LiRecDT for 8 h at 37 °C under a humidified atmosphere plus 5% CO₂. In the control group, the medium contained adequate amount of PBS. Cells were washed five times with PBS and fixed with 2% paraformaldehyde in PBS for 3 min at 4 °C. Cells on coverslips were incubated with 0.1 M glycine for 3 min, washed with PBS and then blocked with PBS containing 1% BSA for 30 min at room temperature (25 °C). The cells were then incubated with primary antibodies (2 μg/ml in PBS–1% BSA) for 1 h at room temperature. For antigen competition assay, the immunofluorescence protocol was the same as described above. After washing three times with PBS, the cells were subsequently incubated with secondary antibodies conjugated with fluorescein isothiocyanate. After washing, samples were mounted with Fluormont-G (Sigma) and observed under a fluorescence confocal microscope (Confocal Radiance 2,100, BioRad, Hercules, USA) coupled to a Nikon-Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division, Melville, USA).

**Cell culture conditions.** The cell line used in this study was MDCK (Madin Darby canine kidney epithelial cells—ATCC no. CCL-34). Cells were maintained in liquid nitrogen with a low number of passages. After thawing, cells were grown in monolayer cultures in DMEM-F12 medium containing penicillin (10,000 IU/ml) and supplemented with 10% fetal calf serum (FCS). The cultures were kept at 37 °C in a humidified atmosphere plus 5% CO₂. Release of cells was performed by treating with a 2 mM solution of ethylenedianiinetetaacetic acid (EDTA) in cation-free/PBS and 0.05% trypsin for a few minutes. After counting, the cells were then resuspended in an adequate volume of medium supplemented with FCS, allowed to adhere and grow for 24 h. Cells were then evaluated in the presence or absence of LiRecDT (10 μg/ml and 50 μg/ml). During the experiment, the plates were photographed at 8 and 24 h using an inverted microscope (Leica-DMIL, Wetzlar, Germany), and changes in cell morphology were evaluated.

**Cell cytotoxicity assays.** Cytotoxicity assays were carried out on 96-well plates (TPP, Trasadingen, Switzerland) using MDCK cells, which are excellent models for in vitro cytotoxicity evaluation (Bonham et al., 2003). Cells (5 × 10⁵ cells/well) were plated and allowed to adhere and grow for 24 h before incubation with LiRecDT at concentrations of 10, 25, 50, 100 and 200 μg/ml for 24 and 48 h in hexaplicate. After toxin incubation, the measurement of toxicity was performed by estimation of viability by Neutral-Red Uptake (Merck, Darmstadt, Germany) and XTT formazan-based assays (Sigma) as described by Freshney (2000) and Petrick et al. (2000). The same experimental conditions were used with control group except that the medium contained adequate amounts of vehicle (PBS) rather than LiRecDT. Cell viability of control group (absence of LiRecDT) was normalized to 100%.
Fig. 1. Molecular cloning and expression of a functional recombinant dermonecrotic protein. (A) Nucleotide sequence of cloned cDNA for *L. intermedia* dermonecrotic toxin and its deduced amino acid sequence. In the protein sequence, the predicted signal peptide is underlined. Arrows show the annealing positions for primers 30 Rec sense and 30 Rec antisense. The asterisk corresponds to the stop codon. (B) SDS-PAGE analysis of recombinant dermonecrotic toxin expression stained by Coomassie blue dye. Lanes 1 and 2 show respectively *E. coli* BL21(DE3)pLysS cells collected by centrifugation (and resuspended in SDS-PAGE gel loading buffer) before and after 3.5 h induction with 0.05 mM IPTG. Lanes 3 and 4 depict supernatant of cells lysates obtained by freeze thawing in extraction buffer before and after incubation with Ni-NTA agarose beads, respectively. Lane 5 shows eluted recombinant protein. Molecular mass markers are shown on the left.
Statistical analysis. Statistical analysis of biochemical parameters and viability data were performed using analysis of variance (ANOVA) and the Tukey test for average comparisons GraphPad InStat program version 3.00 for Windows 2000. Mean ± standard error of mean (SEM) values were used. Significance was determined as $P \leq 0.05$.

Results

Molecular cloning and expression of a recombinant dermonecrotic toxin from the L. intermedia venom gland

Initially, we cloned and expressed a recombinant isoform of the dermonecrotic toxin (sphingomyelinase-D family), from a cDNA library of L. intermedia gland venom. The recombinant protein was expressed as an N-terminal 6×His-tag fusion protein in E. coli BL21(DE3)pLysS cells and purified from soluble fraction of cell lysates by Ni²⁺-chelating chromatography, resulting in 24 mg/l of culture. The deduced protein from cDNA sequences resembles those obtained from several dermonecrotic toxins (sphingomyelinase-D family) of different Loxosceles spider species (Barbaro et al., 1996; Binford et al., 2005; Cisar et al., 1989; Pedrosa et al., 2002). BLAST search at NCBI exhibited 99% amino acid identity to L. intermedia dermonecrotic toxins (Kalapothakis et al., 2002, Tambourgi et al., 2004). Fig. 1A shows the cloned cDNA sequence and its deduced amino acid sequence. The longest cDNA sequence obtained is a 1139 pb molecule coding for a mature protein of calculated 31,239 Da and an isoelectric point at the region of 7.21. The cDNA revealed a 26 amino acid signal peptide before mature protein, as predicted by Nilsen’s algorithm (Nilsen et al., 1997). As shown in Fig. 1B, purified recombinant toxin has SDS-PAGE mobility under reduced conditions at the region of 34 kDa.

Histopathological changes in rabbit skin induced by LiRecDT

To evaluate the functionality of dermonecrotic recombinant toxin, aliquots of crude venom and LiRecDT (10 μg) were injected intradermally into shaved areas of rabbit skin. The dermonecrotic lesions were checked 24 h

Fig. 2. Macroscopic and microscopic changes of rabbit skin exposed to crude venom and LiRecDT. Macroscopic visualization of dermonecrosis into the skin of a rabbit intradermally injected with 10 μg crude venom (A) and 10 μg purified LiRecDT (C). Light microscopic analysis of skin sections stained with HE exposed to crude venom (B) and LiRecDT (D) (magnification 400×). Massive inflammatory cell accumulation within blood vessels in the dermis is shown for both treatments.
after injections. Figs. 2A and B depict respectively macroscopic lesion and light microscopic analysis of biopsy from skin that received crude venom (collection of inflammatory cells in the blood vessels which represent a hallmark of dermonecrotic loxoscelism). Figs. 2C and D show the skin, which received LiRecDT. The results pointed to functionality of recombinant dermonecrotic toxin.

Histopathological findings in kidneys from mice that received L. intermedia recombinant dermonecrotic toxin

To ascertain the renal damage evoked by the dermonecrotic toxin of *Loxosceles* spider venom, mice were exposed i.p. to LiRecDT for 6 h. All animals exposed to LiRecDT showed alterations including lethargy, shivering and stretch attend postures suggesting physical discomfort. Light microscopic analyses of renal biopsy specimens (as depicted in Fig. 3) revealed remarkable alterations including diffuse glomerular edema, focal collapse of glomerular basement membrane, diffuse erythrocytes in the Bowman’s space, diffuse hyalinization with proteinaceous material within the proximal and distal tubule lumen and diffuse vacuolar degeneration of proximal and distal tubular epithelial cells. Interestingly, neither leukocyte accumulation nor marginalization or infiltration was detected in the kidney vessels and structures.

Laboratory investigations after administration of LiRecDT

With the intent to confirm histopathological findings following LiRecDT treatment, we additionally determined

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Fig. 3. Light microscopic analysis of kidneys from LiRecDT-treated mice. Sections of kidneys from mice treated with LiRecDT were stained with HE or PAS and analyzed by light microscopy. (A) Details of tubule structure (arrows) showing accumulation of proteinaceous material within the proximal and distal tubules lumen (arrowheads) (magnification 600×) (HE). (B) Details of tubule structure (arrows) showing vacuoles in the epithelial cells and vacuolar degeneration of proximal and distal tubules (arrowheads) (magnification 600×) (HE). (C) Cross-section of glomerulus (arrow) with intra-glomerular erythrocytes (arrowheads) in the Bowman’s space (magnification 600×) (HE). (D) Glomerular cross-section (arrow) showing collapse of basement membranes (arrowheads) (magnification 400×) (PAS). (E) Glomerular cross-section showing edema (arrows) (magnification 400×) (HE). (F) Renal blood vessel (arrows). Note the absence of leukocytes accumulated in and around the vessel (magnification 400×) (HE).
such biochemical parameters as urinalysis and serum urea comparing LiRecDT-treated mice with control group. Serum urea was significantly increased in LiRecDT-treated mice (51.83 mg/dl ± 8.72 mg/dl) compared to control group (19.24 mg/dl ± 1.33 mg/dl). Similarly, hematuria and urine alkalization were evidenced in treated animals compared to control. These findings together with histopathological analyses supported the idea of nephrotoxicity following LiRecDT exposure.

Evidence that LiRecDT binds to intrinsic renal components

With the aim of demonstrating that the LiRecDT interacts and binds directly to kidney structures, we investigated renal biopsies from LiRecDT-treated and control mice by immunofluorescence using an antibody that reacts with the dermonecrotic toxin. As shown in Fig. 4A, the antibody reaction produced a positive signal in renal biopsies from LiRecDT-treated mice but did not react with

![Figure 4A](image)

**Fig. 4. Direct binding of LiRecDT to kidney structures.** (A) Confocal immunofluorescence microscopy analysis of kidney sections from LiRecDT-treated mice. Cross-sectioned kidneys immunostained with antibodies against LiRecDT. (1) Section of a kidney from control group, which did not receive LiRecDT. (2 and 3) Kidney sections from LiRecDT-treated animals showing regions rich in glomeruli (arrows) with lower positivity and tubules (arrowheads) higher positivity for binding of LiRecDT. (4) Antigen competition assay in which antibodies to LiRecDT were previously incubated with LiRecDT in solution, and then a kidney section from LiRecDT-treated mouse was exposed to the reaction mixture under identical conditions to those described above. A great decrease in immunolabeling is shown, confirmed LiRecDT as “planted antigen” in kidney structures from toxin-treated mice (magnification 400×). (B) Renal extract from LiRecDT-treated mice (lanes 1 and 2), renal extract from control mice (absence of LiRecDT treatment) (lane 3) or purified LiRecDT (lane 4) was separated by 10% SDS-PAGE under non-reducing conditions. The gel was stained by Coomassie blue dyc (lane 1) or transferred to a nitrocellulose membrane that was immunoreacted with antibodies against LiRecDT (lanes 2–4). Molecular protein standard masses are shown on the left of the figure.
the samples from normal mice. To confirm antibody specificity, we repeated the same immunofluorescence approach, this time incubating the antibody with LiRecDT (10 μg) in a solution and then exposing renal biopsies from LiRecDT-treated mice to this mixture (antigen competition assay). Results supported the direct binding of LiRecDT on the renal structures of glomerulus and tubules. Additionally, to strengthen this evidence, renal lysates from LiRecDT-treated mice were electrophoresed and immunoblotted with hyperimmune purified IgG which reacts to dermonecrotic toxin. As shown in Fig. 4B, we were able to detect a positive signal at the region of 33–35 kDa in the LiRecDT-treated lysate compared to an absence in the control lysate, confirming immunofluorescence results and identifying the dermonecrotic toxin as a direct and “planted antigen” bound to kidney structures.

Effect of LiRecDT on the morphology and viability of epithelial kidney cells

To ascertain the direct cytotoxicity of LiRecDT on renal cells and to support the histopathological findings, which indicated renal injuries after LiRecDT treatment, experiments

![Cytotoxicity assays](image-url)

**Fig. 5. Cytotoxicity assays. Effect of LiRecDT on the morphology of tubular epithelial cells.** (A) MDCK cells exposed to LiRecDT were observed in an inverted microscope. The cytoplasm of the cells becomes vacuolated in a toxin concentration and time exposure manner. Identically, cell spreading appears to be impaired, and detachment from the substrate was observed. Analyses were performed at 8 and 24 h after LiRecDT exposure. Concentrations of purified toxin in culture medium were 10 μg/ml and 50 μg/ml (10 and 50) respectively. Control cells were analyzed in the absence of toxin (c). Cytotoxic effect of LiRecDT on MDCK epithelial cells analyzed by dye uptake (Neutral-Red uptake) (B) and formazan produced (XTT-based assay) (C). LiRecDT cytotoxic effect was determined after 24 and 48 h at indicated concentrations of purified toxin. Experiments were performed in hexaplicates, and values given are the mean ± SEM. Significance is defined as *P < 0.05 and **P < 0.01.
on morphology and cellular viability were performed using MDCK epithelial cells. As depicted in Fig. 5A, LiRecDT treatment induced appearance of blebs and cytoplasmic vacuolization, caused defective cell spreading and detached cells from each other and culture substratum which enhanced in a time- and toxin-dependent manner. As shown in Fig. 5B, experiments on the cellular viability (XTT and Neutral-Red Uptake) indicated a significant alteration of MDCK cell viability when compared to control cells. These in vitro experiments strengthen the idea of dermonecrotic toxin nephrotoxicity.

The LiRecDT binds to MDCK epithelial cells

With the objective of corroborating the direct cytotoxicity of dermonecrotic toxin upon renal cells, we looked for the direct binding of LiRecDT on MDCK cells in culture. For this purpose, toxin-treated and control cells were submitted to an immunofluorescence experiment using an antibody, which reacts with dermonecrotic toxin. As shown in Fig. 6, LiRecDT binds to MDCK cells, producing an immunofluorescent pattern of deposition on the cell surface.

Discussion

Loxoscelism, the term representing accidents and envenomation involving spiders of Loxosceles genus (brown spider), has been reported world-wide (Futrell, 1992; da Silva et al., 2004). The clinical features of brown spider bites are an image of necrotic skin lesions which can also be accompanied by a systemic involvement including weakness, vomiting, fever, convulsions, disseminated intravascular coagulation, intravascular hemolysis and renal failure. Severe systemic loxoscelism is much less common than the cutaneous form, but it may be the cause of clinical complications and even death after envenomation (Futrell, 1992; da Silva et al., 2004). Reactions to brown spider bites are influenced by the victim’s health, degree of obesity and
The nephrotoxic effects of *Loxosceles* spider venom are demonstrated based on the clinical and laboratory features observed in some victims, which can include elevated creatine kinase levels, hematuria, hemoglobinuria, proteinuria and shock (Bey et al., 1997; França et al., 2002; Lung and Mallory, 2000; Williams et al., 1995). Additionally, Luciano et al. (2004) were able to show deposition and binding of brown spider venom toxins along the tubular and glomerular structures and a consequent cytotoxicity in renal tissue of mice. Hematological disturbances such as hemolytic anemia and disseminated intravascular coagulation, as well as nephrotoxicity secondary to complications of dermonecrotic lesions have been postulated as pathological processes that may lead to renal failure (Futrell, 1992; Lung and Mallory, 2000; Williams et al., 1995). However, there is no direct experimental evidence confirming such a hypothesis. In addition, histopathological findings after envenomation of mice (an animal model which does not develop dermonecrotic lesions caused by *Loxosceles* spider venom) (Futrell, 1992; da Silva et al., 2004), together with the binding of venom toxins to renal structures and the apparent absence of hemoglobin in the proteinaceous materials inside the Bowman’s space and tubules detected after venom exposure (Luciano et al., 2004), strongly support a direct nephrotoxicity activity of venom toxins and the hypothesis of “planted toxins” to intrinsic components of renal structures. This idea corroborates with several reports that evidence “planted antigens” including viral, parasitic products, bacteria and drugs such as etiological agents to renal injuries (Cotran et al., 1999).

The presence of a 30 kDa protein in renal lysates from crude-venom-treated mice was indicative of dermonecrotic toxin involvement in renal injuries caused by *Loxosceles* venom (Luciano et al., 2004). This assumption was also supported by the cytotoxic activity of dermonecrotic toxin upon erythrocytes and platelets (Futrell, 1992; da Silva et al., 2004) and by its lethal activity (Barbaro et al., 1996). In this study, we cloned, expressed and purified a recombinant isoform of the dermonecrotic toxin from *L. intermedia* gland venom. This recombinant toxin showed functionality as observed by dermonecrosis and an inflammatory response upon rabbit skin, which was similar to those activities evoked by crude venom. *LiRecDT* is one of several dermonecrotic toxin isoforms present in the venom (Tambourgi et al., 2004). It seems that each isoform of dermonecrotic toxin causes noxious activities, and the effect induced by crude venom represents a family synergism. Additionally, venom toxins other than the dermonecrotic family can contribute to dermonecrosis size and intensity. Metalloproteases described in the venom (Feitosa et al., 1998), hyaluronidases (Futrell, 1992; Young and Pincus, 2001; da Silva et al., 2004) and even complications from secondary infections (Monteiro et al., 2002) could be involved in dermonecrosis.

The glomerular and tubular damage observed in the *LiRecDT*-intraperitoneal-treated mice were similar to those induced by crude venom (Luciano et al., 2004). The azotemia detected by the increase in serum urea and such biochemical urine changes as alkalization and hematuria together with histopathological studies followed *LiRecDT* localization of the bite among other factors (Sams and King, 1999; da Silva et al., 2004; Vetter and Bush, 2002).
exposure evidenced the nephrotoxicity caused by the dermonecrotic toxin.

In contrast to the cutaneous lesion in which polymorphonuclear leukocytes play an essential role in the pathogenesis (an aseptic coagulative tissue necrosis) (Elston et al., 2000; Futrell, 1992; Ospedal et al., 2002; da Silva et al., 2004), the renal injury evoked by dermonecrotic toxin is not associated with inflammatory changes as in immune complex nephritis which is caused by deposition of exogenous antigens following some bacterial and viral infections (Cotran et al., 1999; Tisher and Brenner, 1994). In situ immune complex deposition can also be discarded because biopsies were collected 6 h after LiRecDT exposure, mouse pre-immune serum did not react with crude venom or recombinant dermonecrotic toxin (which could suggest natural immunoglobulins to this toxin) and immunofluorescence using anti-mouse IgG was negative in biopsies from toxin-treated mice (data not shown).

Additionally, immunoblotting analysis of renal lysates from LiRecDT-treated mice using dermonecrotic toxin antibodies identified the presence of this toxin at the 33–35 kDa region as direct ligand of renal intrinsic structures. This, together with the immunofluorescence results of toxin-treated renal biopsies, confirmed this protein as an “exogenous planted antigen” along the kidney structures.

The glomerular barrier function is dependent on the molecular mass of proteins (molecules with mass lower than 70 kDa are more permeable than larger proteins), as well as molecular charge of proteins (anionic molecules tend to be less permeable and are repulsed by anionic moieties present within the renal structures) (Cotran et al., 1999; Farquhar, 1991). We postulated based on the physicochemical properties of LiRecDT (33–35 kDa and pi 7.2), together with its water solubility, that these properties account for the binding of this molecule to kidney structures and consequent nephrotoxicity.

Dermonecrotic toxin nephrotoxicity was additionally proved by confocal immunofluorescence with antibodies that react to this molecule and by using MDCK epithelial cells in culture, which demonstrated the direct toxin binding on the cell surface. In addition, experiments using MDCK cells treated in culture with LiRecDT showed a potent toxic activity, particularly in the disturbance of cell morphology, which induced the appearance of vacuoles in cytoplasm, changed their spreading aspect and caused defective cell–cell and culture substratum adhesion (as shown by Collares-Buzato et al. (2002) using snake venom toxins).

Likewise, the toxin also inhibited cellular viability in a concentration- and time-dependent manner, further demonstrating a toxin direct cytotoxicity. The venom concentration to which victims would be exposed following envenomation depends on such factors as size and sex of spiders (females inject more venom than males). The total venom volume injected is about 4 μl and contains 65–100 μg of proteins (Sams et al., 2001; da Silva et al., 2004). In fact, even 10 μg and 25 μg of toxin have shown cytotoxicity upon MDCK cells and dermonecrosis into the rabbit skin. These low experimental LiRecDT concentrations resemble the toxin levels that would be seen after a typical Loxosceles envenomation. The molecular mechanism by which dermonecrotic brown spider toxin causes renal injuries is currently unknown. Since mice were used (an animal model which does not develop dermonecrotic lesions) (Futrell, 1992; da Silva et al., 2004), we can rule out nephrotoxicity in vivo secondary to complications of dermonecrosis. This conclusion is similarly corroborated by the toxin direct cytotoxicity upon MDCK cells in vitro. Likewise, some reports have indicated the participation of a serum amyloid P plasma component of adult animals but not from fetal plasma on the dermonecrotic toxin-dependent platelet activation and aggregation (Gates and Rees, 1990). Since our data indicated dermonecrotic toxin cytotoxicity on MDCK cells in the presence of fetal calf serum (culture medium), this last hypothesis can be discarded.

Experiments using endothelial cells treated in culture with brown spider venom show a potent endothelial cell agonist activity of the venom (dermonecrotic toxin), which induces endothelial cell release of macrophage colony-stimulating factor and interleukine-8, causing an exacerbated inflammatory response (Patel et al., 1994). Tambourgi et al. (1998) postulated that renal damage induced by Loxosceles spider venom putatively could be mediated by cytokine mediators. On the basis of direct dermonecrotic toxin cytotoxicity upon MDCK cells in vitro, the absence of inflammatory leukocytes in the renal histological analysis, but with a marked damage to kidney structures, we can consider this hypothesis with restriction.

On the basis of the above results, we have identified a toxin responsible for cellular and pathological alterations which causes nephrotoxicity after accidents involving Loxosceles spiders.

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References


from venoms of Loxosceles spiders: evolutionary insights from cDNA sequences and gene structure. Toxicol 45, 547–560.


