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Identification of a Direct Hemolytic Effect Dependent on the Catalytic Activity Induced by Phospholipase-D (Dermonecrotic Toxin) From Brown Spider Venom

Daniele Chaves-Moreira,[1] Olga M. Chaim,[1,2] Youssef B. Sade,[1] Kátia S. Paludo,[1,3] Luiza H. Gremski,[1,2] Lucélia Donatti,[1] Juliana de Moura,[4] Oldemir C. Mangili,[5] Waldemiro Gremski,[1,6] Rafael B. da Silveira,[7] Andrea Senff-Ribeiro,[1] and Silvio S. Veiga[1*]

[1] *Department of Cell Biology, Federal University of Paraná, Curitiba, Paraná, Brazil*
[2] *Department of Biochemistry, Federal University of São Paulo, São Paulo, Brazil*
[3] *Medical Clinic Discipline, Department of Medicine, Federal University of São Paulo, São Paulo, Brazil*
[4] *Department of Basic Pathology, Federal University of Paraná, Curitiba, Paraná, Brazil*
[5] *Department of Physiology, Federal University of Paraná, Curitiba, Paraná, Brazil*
[6] *Catholic University of Paraná, Health and Biological Sciences Institute, Curitiba, Paraná, Brazil*
[7] *Department of Structural, Molecular Biology and Genetics, State University of Ponta Grossa, Ponta Grossa, Paraná, Brazil*

ABSTRACT

Brown spiders have world-wide distribution and are the cause of health problems known as loxoscelism. Necrotic cutaneous lesions surrounding the bites and less intense systemic signs like renal failure, DIC, and hemolysis were observed. We studied molecular mechanism by which recombinant toxin, biochemically characterized as phospholipase-D, causes direct hemolysis (complement independent). Human erythrocytes treated with toxin showed direct hemolysis in a dose-dependent and time-dependent manner, as well as morphological changes in cell size and shape. Erythrocytes from human, rabbit, and sheep were more susceptible than those from horse. Hemolysis was not dependent on ABO group or Rhesus system. Confocal and FACS analyses using antibodies or GFP-phospholipase-D protein showed direct toxin binding to erythrocytes membrane. Moreover, toxin-treated erythrocytes reacted with annexin-V and showed alterations in their lipid raft profile. Divalent ion chelators significantly inhibited hemolysis evoked by phospholipase-D, which has magnesium at the catalytic domain. Chelators were more effective than PMSF (serine-protease inhibitor) that had no effect on hemolysis. By site-directed mutation at catalytic domain (histidine 12 by alanine), hemolysis and morphologic changes of erythrocytes (but not the toxin's ability of membrane binding) were inhibited, supporting that catalytic activity is involved in hemolysis and cellular alterations but not toxin cell binding. The results provide evidence that *L. intermedia* venom phospholipase-D triggers direct human blood cell hemolysis in a catalytic-dependent manner. J. Cell. Biochem. 107: 655–666, 2009.

KEY WORDS: BROWN SPIDER; VENOM; PHOSPHOLIPASE-D; CATALYSIS; HEMOLYSIS

Medical incidents caused by brown spiders (*Loxosceles* genus) are characterized by skin injuries with urticarial reaction, edema, erythema, and a necrotic lesion that may spread gravitationally as well as systemic manifestations like malaise, weakness, nausea, headache, myalgia, fever, acute renal failure, disseminated intravascular coagulation, and intravascular hemolysis. Systemic reactions may be seen in a minority of cases, but they can be severe and occasionally fatal in some patients [da Silva et al., 2004; Swanson and Vetter, 2006].

Several case reports have described intravascular hemolysis following brown spider bites [Williams et al., 1995; de Souza et al., 2008]. On the other hand, many studies have indicated the hemolytic activity upon red blood cells of *Loxosceles* venom under laboratory conditions. It seems that animal species display differential

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*Correspondence to: Silvio S. Veiga, Department of Cell Biology, Federal University of Paraná, Jardim das Américas, Curitiba, Paraná 81531-990, Brazil. E-mail: veigass@ufpr.br
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susceptibility to venom-induced hemolysis. Human and swine erythrocytes are more susceptible to lysis than those from rabbit and guinea pig [Futrell, 1992]. The laboratory features observed in victims may include hemoglobinuria, proteinuria, and bilirubin in the urine [Williams et al., 1995; Lung and Mallory, 2000; de Souza et al., 2008]. Some authors suggest that the nephrotoxicity following brown spider accidents may reflect hematologic disturbances like intravascular hemolysis and disseminated intravascular coagulation, which may lead to renal failure [Futrell, 1992; da Silva et al., 2004]. The *Loxosceles* venom is crystalline and minute in volume (a few microliters). It is enriched in proteic toxins with an electrophoretic protein profile in the regions between 20 and 40 kDa, and venom from both female and male animals triggers noxious effects [Futrell, 1992; da Silva et al., 2004]. The whole venom contains several biochemically well characterized molecules, including hyaluronidases [Barbaro et al., 2005; da Silveira et al., 2007a], astacin-like metalloproteases [Feitosa et al., 1998; da Silveira et al., 2007c], low molecular mass insecticidal peptides [de Castro et al., 2004], and phospholipases-D or named dermonecrotic toxins [Machado et al., 2005; Chaim et al., 2006; da Silveira et al., 2006, 2007b; Kalapothakis et al., 2007; Appel et al., 2008].

The dermonecrotic toxin is the best-known molecule found in different brown spider venom. Several studies identified this toxin as contributing to the major deleterious effects of spider bites, including hemolysis [Forrester et al., 1978], platelet aggregation [Kurpiewski et al., 1981], vessel hyperpermeability [da Silveira et al., 2006], nephrotoxicity [Chaim et al., 2006; Kusma et al., 2008], and typical dermonecrotic lesions [Futrell, 1992; da Silva et al., 2004]. This toxin has been found in the venoms of *Loxosceles recluse* [Futrell, 1992], *L. gaucho* [Barbaro et al., 1992, 1994], *L. laeta* and *L. intermedia* [Barbaro et al., 1994], *L. rufescens* [Young and Pincus, 2001], and *L. boneti* [Ramos-Cerrillo et al., 2004], supporting its species conservation and biological importance. Previous studies have characterized dermonecrotic toxin as a sphingomyelinase-D molecule based on its ability to hydrolyze sphingomyelin into choline and acylsphingosine phosphate [Futrell, 1992]. Nevertheless, based on additional biochemical analysis, the term sphingomyelinase-D has been replaced by phospholipase-D to represent a more accurate and broader denomination. The toxin hydrolyzes not only sphingomyelin but also lysoglycerophospholipids to generate ceramide 1-phosphate (C1P) or lysophosphatidic acid (LPA) [Lee and Lynch, 2005]. By using current molecular biology and proteomic techniques, it has been demonstrated that the dermonecrotic toxin phospholipase-D is a member of a larger family of related molecules displaying similar molecular masses as well as amino acid, immunological, and biological homologies [Kalapothakis et al., 2007]. Studies corroborate and strengthen the idea of the existence of an intraspecies family of phospholipase-D toxins and suggest that the deleterious activities induced by these toxins reflect a synergistic mechanism for different toxin isoforms found in the whole venom.

The mechanism by which the venom causes hemolysis is currently under investigation. This effect is calcium- and complement-dependent but antibody-independent, and hemolysis is induced by native and recombinant phospholipase-D toxins [Futrell, 1992; da Silva et al., 2004; Ribeiro et al., 2007].

Herein, we compare a recombinant active phospholipase-D [Chaim et al., 2006] and a recombinant phospholipase-D with site-directed mutation in the catalytic domain [Kusma et al., 2008] and report the direct involvement of the catalytic domain of this toxin in hemolytic activity. These results strengthen previous data reporting the participation of dermonecrotic toxins in red blood cell lysis and suggest a direct molecular mechanism dependent on the catalytic activity of phospholipase-D for this event.

MATERIALS AND METHODS

REAGENTS

Polyclonal antibodies to whole venom toxins and phospholipase-D toxin were produced in rabbits as previously described [Luciano et al., 2004; Chaim et al., 2006]. Hyperimmune IgGs were purified from serum using a mixture of Protein-A and Protein-G Sepharose beads (GE Healthcare Life Sciences, Uppsala, Sweden) as recommended by the manufacturer. Phenylmethylsulfonyl fluoride, EDTA, EGTA, 1,10-phenanthroline, and fluorescein-conjugated anti-rabbit IgG were purchased from Sigma (St. Louis). Whole venom from *L. intermedia* was extracted from spiders captured from the wild as described by Feitosa et al. [1998].

RECOMBINANT PROTEIN CLONING AND EXPRESSION

The venom gland cDNA library was previously built [Chaim et al., 2006; da Silveira et al., 2006]. The GenBank data deposition information for *L. intermedia* cloned LiRecDT1 cDNA is DQ218155. The cDNA corresponding to the mature phospholipase-D LiRecDT1 protein was amplified by PCR. The forward primer used was 30Rec sense (5′-CTCGAGGCAGGTAATCGTCGGCCTATA-3′) and was designed to contain an *Xho* I restriction site (underlined) plus the sequence related to the first seven amino acids of the mature protein. The reverse primer was 30Rec antisense (5′-CGGGATCC-TTATTTCTTGAATGTCACCCA-3′), which contains a *Bam*H I restriction site (underlined) and a stop codon (bold). The PCR product was cloned into a pGEM-T vector (Promega, Madison). The pGEM-T vector containing the cDNA encoding the mature protein was then digested with *Xho* I and *Bam*H I restriction enzymes. The excised insert was gel purified using a QIAquick Gel 74 Extraction Kit (Qiagen, Valencia) and subcloned into a pET-14b vector (Novagen, Madison) digested with the same enzymes. The mutated toxin LiRecDT1 H12A was obtained by a Megaprimer PCR method, which was performed with rounds of PCR to introduce a site-directed mutagenesis in the LiRecDT1 sequence [Kusma et al., 2008]. Briefly, the first round introduced the site-directed mutagenesis into the first histidine amino acid residue of the catalytic domain using the reverse primer P1H12A (5′-ATTTACCATGGCCCCCATGATC-3′), which was designed to contain the codon substitute for alanine plus the sequence related to the other original amino acids of mature protein. The recombinant protein GFP-LiRecDT1 was obtained by subcloning the previously constructed LiRecDT1 [Chaim et al., 2006] and the sequence of enhanced green fluorescence protein (GFP) into pET-14b using a Blunt-Cut-Cut strategy at *Nde* I site of pET-14b and two *Bam*H I sites (between LiRecDT1, GFP and the vector). The resulting construct was a fusion protein with a 6x His-Tag at the N-terminus, the sequence of mature LiRecDT1, and the sequence of

GFP at the C-terminus. All recombinant constructs (LiRecDT1, LiRecDT1 H12A, GFP-LiRecDT1) were expressed as fusion proteins, with a 6x His-Tag at the N terminus and a 13 amino acid linker (including a thrombin site) between the 6x His-Tag and mature protein (N-terminal amino acid sequence before the mature protein: MGSSHHHHHHSSGLVPRGSHMLE). pET-14b/*L. intermedia* cDNA constructs were transformed into One Shot *E. coli* BL21(DE3)pLysS-competent cells (Invitrogen, Carlsbad) 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 incubation for an additional 3.5 h at 30°C (with vigorous shaking). Cells were harvested by centrifugation (4,000*g*, 7 min), and the pellet was frozen at −20°C overnight.

RECOMBINANT PROTEIN PURIFICATION

Cell suspensions were thawed and additionally disrupted by six cycles of 10 s of sonication at low intensity. Lysed materials were centrifuged (20,000*g*, 20 min), and the supernatants were incubated with 1 ml Ni^{2+}-NTA agarose beads for 1 h at 4°C with gentle agitation. The suspensions were loaded into 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. Recombinant proteins were eluted with 10 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 12.5% SDS–PAGE under reducing conditions. Fractions were pooled and dialyzed against phosphate-buffered saline (PBS).

ERYTHROCYTES

Adult rabbits weighing approximately 3 kg from the Central Animal House of the Federal University of Paraná were used for blood collection and erythrocyte preparation. Sheep erythrocytes were purchased from Newprov (Pinhais, Brazil). Horse erythrocytes were obtained from adult animals of Jockey Club (Curitiba, Brazil). Human erythrocytes were obtained from normal donors. All experimental protocols using animals were performed according to the "Principles of laboratory animal care" (NIH Publication no 85–23, revised 1985), "Brazilian Federal Laws," and ethical committee agreement number 245 of the Federal University of Paraná. In the case of humans, the blood collection procedure was authorized by the ethical committee agreement of the Federal University of Paraná.

PREPARATION OF ERYTHROCYTES

After blood was harvested with acid EDTA Na_2 5% and a 21-gauge needle (BD Plastipak, Franklin Lakes) on the day of each experiment, the platelet-rich plasma and buffy coat were removed by aspiration after centrifugation at 200*g* for 15 min. Packed erythrocytes were washed three times with Ringer Solution for morphological observations (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$, pH 7.4, 300 mOsm/kg H_2O) or Tris buffer Sucrose (TBS) for hemolysis assays (250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 280 mOsm/kg H_2O). Washed erythrocytes were resuspended in the appropriate buffer at a concentration of 5×10^8 cells/ml.

DETERMINATION OF HEMOLYTIC ACTIVITY

The hemolysis assay was performed as described by Harris and Phoenix [1997]. Washed red blood cells (10^8 cells) were added to each Eppendorf tube containing the appropriate concentration of venom/toxin in TBS buffer (serial dilution: 0.025, 0.25, 2.5, and 25 μg/ml). For this assay, samples were performed in pentaplicate along with negative (in presence of the appropriate amount of TBS only) and positive (red blood cells in distilled water and 0.1% (v/v) Triton X-100) controls. After 24 h of incubation with gentle agitation, controls and samples were centrifuged at 4°C (3 min at 200*g*) and the absorbances of supernatants read immediately at 550 nm (ELISA ELX 800 Auto Reader, Meridian Diagnostics, Inc., USA). Absorbance values were converted to percent hemolysis using the absorbance values of the positive control as 100% lysis. In order to evaluate the time course of hemolysis, a series of experiments was performed with a selected concentration (25 μg/ml) of LiRecDT1 and LiRecDT1 H12A. The entire assay was carried out with normal TBS and tested against a normal osmolarity control (without toxin) in pentaplicate at five different time periods (0, 4, 8, 16, and 24 h). Supernatants were processed at each time interval as described above.

OBSERVATION USING LIGHT MICROSCOPY

Human erythrocytes were treated with whole venom (5 μg/ml), recombinant toxin LiRecDT1 (5 μg/ml), and recombinant mutated toxin LiRecDT1 H12A (25 μg/ml) for 0, 4, 8, 16, and 24 h, then fixed with 1% glutaraldehyde, prepared as described by Udden [2005], and then their morphology was observed with a light microscope (Leica-DMIL, Wetzlar, Germany). Control cells were incubated with Ringer Solution. Photomicrographs correspond to digital images of cells originally magnified at 400×.

OBSERVATION USING SCANNING ELECTRON MICROSCOPY

Ultrastructural analysis of erythrocytes was performed according to Chung et al. [2007]. After 8 h of treatment with 5 μg/ml whole venom, 5 μg/ml LiRecDT1 and 25 μg/ml LiRecDT1 H12A, cells were washed with Ringer Solution. Control cells were incubated with only Ringer Solution. Fixation was performed with a 2% glutaraldehyde solution for 1 h at 4°C, and the erythrocytes were attached onto a coverslip coated with poly-L-lysine at room temperature for 30 min. The coverslip was rinsed with Ringer Solution, dried at room temperature, and sputter-coated with gold. Coverslips were observed on a scanning electron microscope (JEOL, Tokyo, Japan).

FLOW CYTOMETRY

Human erythrocytes were incubated for 24 h with 25 μg/ml of whole venom, LiRecDT1, and LiRecDT1 H12A (data not shown). The cell suspensions were analyzed for erythrocyte counts and size distributions using a Flow Cytometer (BD FACSCalibur System, NJ). Control cells (with Ringer Solution only) were used for comparison.

Analysis of GFP-LiRecDT1 treatment was also evaluated via flow cytometry. Washed erythrocytes (5×10^6 cells) were incubated with 25 μg/ml of fluorescent recombinant toxin (24 h, 37°C) in a total volume of 400 μl of Ringer Solution. The samples were then diluted to 1 ml in Ringer Solution, and light scatter and fluorescence channels were analyzed. Background fluorescence (cell and buffer) and non-specific binding of GFP to erythrocytes were evaluated, and acquisition was gated considering these data.

PHOSPHATIDYLSERINE EXPOSURE

Annexin-V-FITC (Invitrogen) binding experiments were performed as described by Wood et al. [1996] using a flow cytometer (BD FACSCalibur System). Human erythrocytes were incubated for 4 h with 25 μg/ml of whole venom, LiRecDT1, and LiRecDT1 H12A (data not shown). Control cells were treated with Ringer Solution only. Erythrocytes were diluted (5×10^6 cells) to a final volume of 0.5 ml in an annexin-V binding buffer, pH 7.4 (containing 10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM glucose, 5 mg/ml BSA, and 2.5 mM $CaCl_2$). Annexin-V-FITC was added to a concentration of 0.1 μM to the red blood cells, and samples were incubated for 15 min at room temperature in the dark. After incubation, an aliquot of the sample was taken for flow cytometric analysis. Cells were analyzed by forward scatter, and Annexin-V fluorescence intensity was measured in fluorescence channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The percentage of Annexin-V positive cells and their mean fluorescence intensity (in arbitrary units) were calculated using CellQuest Pro software for Macintosh. Data from 100,000 events were collected and analyzed.

CONFOCAL IMMUNOFLUORESCENCE

Peripheral blood (1 ml) was washed and diluted in Ringer Solution containing 10 mM glucose and 1 mg/ml BSA, pH 7.4. BSA was used in order to preserve the biconcave shape of normal erythrocytes. This assay was performed according to Kalfa et al. [2006]. Erythrocytes were incubated with whole venom, LiRecDT1, and LiRecDT1 H12A for 8 h (25 μg/ml), and 100 μl of the cell suspension was applied onto a poly-L-lysine coated coverslip for adhesion. This procedure allows cells to be treated while attached to coverslips, thereby minimizing modification of the cell shape from repeated centrifugations. Unbound cells were removed by washing with Ringer Solution, and adherent blood cells were fixed on coverslips with 0.1% glutaraldehyde in Ringer Solution (10 mM glucose, 1 mg/ml BSA, pH 7.4) for 10 min at 4°C. To ensure complete neutralization of background unspecific fluorescence, cells were then incubated in block solution buffer (Ringer Solution containing 10 mM glucose and 1 mg/ml BSA, 0,1 M glycine, 5% serum fetal bovine, and 0.05% sodium azide) at room temperature for 1 h. The samples were stained for toxins using indirect immunofluorescence with antibodies at a 1:1,000 dilution (anti-venom and anti-LiRecDT1) and incubated for 2 h. After washing with buffer, slides were incubated for 1 h with secondary FITC-conjugated anti-rabbit IgG (1:250). Coverslips were mounted on glass slides using Fluoromount-G (Southern Biotechnology, Birmingham). Samples were observed using the confocal fluorescence microscope (Confocal Radiance 2,100, BioRad, Hercules) coupled to a Nikon-Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division, Melville, USA).

LIPID RAFT ANALYSIS OF TOXIN-TREATED ERYTHROCYTES

For localization of lipid rafts on the erythrocyte cell surface, the Vybrant Alexa-Fluor 594 Lipid Raft Labeling Kit was used as recommended by the manufacturer (Invitrogen). Briefly, red blood cells treated with (25 μg/ml) whole venom and recombinant toxins for 8 h at 37°C or control cells (absence of toxins) were incubated with Cholera toxin subunit B (CT-B) (conjugate Alexa 594) for 10 min at 4°C. The cells were then gently washed three times with chilled Ringer Solution containing 10 mM glucose and 1 mg/ml BSA, pH 7.4. Crosslinking of CT-B labeled lipid rafts was performed with chilled anti-CT-B antibody (1:200 dilution) for 15 min at 4°C. Cells were washed, fixed, and blocked as described above for immunofluorescence. The samples were visualized using a fluorescence confocal microscope (Confocal Radiance 2100, BioRad) coupled to a Nikon-Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division).

EFFECT OF ENZYMATIC INHIBITORS

The recombinant toxin LiRecDT1 (25 μg/ml) was incubated for 30 min at 4°C with 1 mM EDTA, or 1 mM EGTA, or 1 mM 1,10-phenanthroline, or 1 mM PMSF. Washed erythrocytes were added and incubated for 24 h at 37°C with gentle agitation. Also a control group without toxin (TBS only) was examined. Supernatants were read at each time interval as previously described in Determination of Hemolytic Activity Section.

STATISTICAL ANALYSIS

Statistical analyses of hemolytic assays were performed using analyses of variance (ANOVAs) and the Tukey test for average comparisons via GraphPad InStat program version 3.06 for Windows. Mean ± SEM values were used. Significance was determined as $P \leq 0.05$.

RESULTS

DIRECT HEMOLYSIS INDUCED BY RECOMBINANT PHOSPHOLIPASE-D FROM BROWN SPIDER VENOM

Brown spider venom induces hemolysis in patients and experimental animal models exposed to the venom. Venom activity is attributed to native dermonecrotic toxins that can reproduce this effect in a serum complement-dependent manner when purified from whole venom [Futrell, 1992; da Silva et al., 2004; Swanson and Vetter, 2006]. Herein, we provide additional data suggesting direct hemolysis activity caused by phospholipase-D (dermonecrotic toxin) expressed as a recombinant toxin from a cDNA library of the *L. intermedia* venom gland [Chaim et al., 2006]. Figure 1A depicts hemolysis of human red blood cells incubated in serum-free medium with different concentrations of toxin at 37°C. As observed, hemolysis is present in a dose-dependent manner. Additionally, Figure 1B shows experiments performed after different times of exposure to the recombinant toxin. Hemolysis is evident in a time-dependent manner. As shown, the above-mentioned results

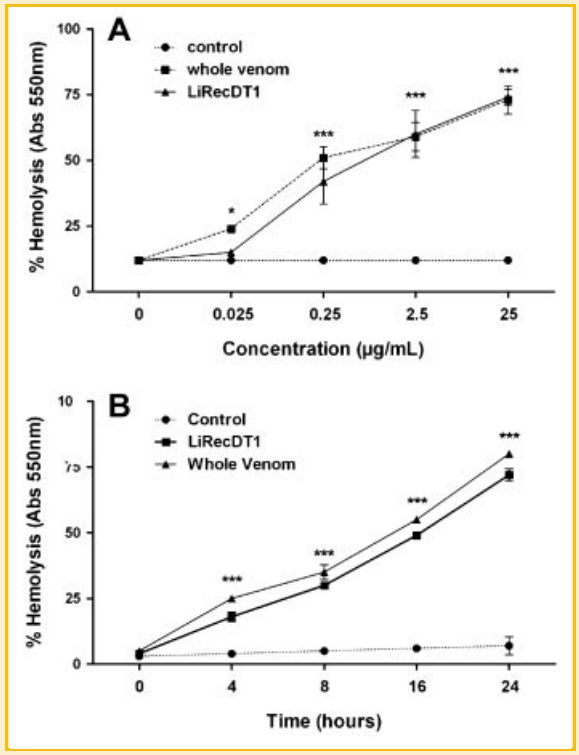

Fig. 1. Recombinant phospholipase-D from brown spider venom induces direct hemolysis in human erythrocytes. A: Human erythrocytes suspended in TBS were incubated with different concentrations of *L. intermedia* whole venom, with a LiRecDT1, or in the absence of venom toxins (negative control), for 24 h at 37°C. B: Erythrocytes were incubated with 25 µg/ml of whole venom, with LiRecDT1, or in the absence of toxins (negative control) over five different time periods (0, 4, 8, 16, and 24 h). The absorbances of supernatants were read at 550 nm, and the percentage of hemolysis was determined using the absorbance values induced by distilled water as 100% of hemolysis (positive control). Results represent averages of five experiments ± SEM. $^{*}P \leq 0.05$ or $^{***}P \leq 0.001$.

support the direct hemolytic activity of phospholipase-D toxin on human erythrocytes.

MORPHOLOGICAL ALTERATIONS OF HUMAN ERYTHROCYTES INDUCED BY RECOMBINANT PHOSPHOLIPASE-D

To ascertain cytotoxicity and the direct hemolytic effect evoked by phospholipase-D (dermonecrotic toxin) from *Loxosceles* spider venom as described above, human erythrocytes were exposed to recombinant toxin for different times and then analyzed via an inverted microscope. As depicted in Figure 2A, morphological changes were present in erythrocytes exposed to toxin but not the negative control. These findings included mainly alterations in cell size and shape in a concentration- and time-dependent manner. Poikilocytosis of erythrocytes treated with toxin included a change from biconcave disks flattened and depressed in the center (control group) to spherical and rounded cells. Additionally, erythrocytes exposed to the toxin were scanned by electron microscopy for changes. Figure 2B illustrates the alterations detected. Toxin treatment of erythrocytes led to the appearance of spherocyte, stomatocyte, and knizocyte forms, which were accompanied by a cell size decrease in a time-dependent manner. Finally, erythrocytes were examined by flow cytometry showing FSC ("forward scatter") and SSC ("side scatter") after 24 h of incubation with 25 µg/ml of recombinant toxin. As depicted in Figure 2C, toxin treatment induced cell lysis and debris formation and thus strengthened our hypothesis of direct toxin hemolytic activity.

THE DIRECT LYSIS OF ERYTHROCYTES CAUSED BY PHOSPHOLIPASE-D DEPENDS ON THE ANIMAL SPECIES EVALUATED

With the objective of corroborating the above-described results, we next analyzed the direct hemolytic activity of recombinant phospholipase-D on washed red blood cells from different animal species. Washed erythrocytes from human, rabbit, sheep, and horse were exposed to purified recombinant phospholipase-D and studied for lysis. As depicted in Figure 3, erythrocytes from different animal species did not suffer phospholipase-induced hemolysis in a similar manner. It seems that there is differential susceptibility of red blood cells to the enzyme. Additionally, human erythrocytes from donors classified as A, B, or O blood groups as well as Rh (+) or Rh (−) were exposed to recombinant toxin and evaluated for hemolysis. Hemolysis induced by phospholipase-D was not dependent on either ABO group antigens or the Rhesus system. Erythrocytes from all blood groups tested suffered a similar percentage of hemolysis; supporting the idea that hemolysis triggered by brown spider venom phospholipase-D was not dependent on factors linked to ABO markers or the Rhesus system (data not shown). Erythrocytes from sheep, human, and rabbit were more easily lysed than cells from horse, which were apparently more resistant to lysis. These data confirmed the above-described results that phospholipase-D is capable of direct lysis of erythrocytes and suggested the possibility that membrane constituents may play a role in venom phospholipase-D activity, because cytoplasmic membranes from erythrocytes of different species have differences in lipid composition.

EVIDENCE THAT RECOMBINANT PHOSPHOLIPASE-D BINDS TO THE HUMAN RED BLOOD CELL MEMBRANE

We intended to demonstrate that there are sites of attachment of phospholipase-D on the erythrocyte membrane. Since the enzyme is also a sphingomyelinase and can thus putatively bind to sphingomyelin (a phospholipid constituent of the outer erythrocyte membrane), we investigated human erythrocytes treated with phospholipase-D by an immunofluorescence reaction using an antibody that reacts with phospholipase-D. As shown in Figure 4A, the antibody reaction produced a positive signal in erythrocytes. Additionally, washed erythrocytes were incubated with a recombinant fusion toxin GFP-LiRecDT1 using GFP as a negative control. Cells were evaluated by flow cytometry. As depicted in Figure 4B, recombinant fusion toxin bound to erythrocytes whereas signal for GFP alone was negative. Additionally, human erythrocytes were treated with GFP-LiRecDT1 and visualized by confocal microscopy (Fig. 4C). Our results support the direct binding of phospholipase-D to the membrane of human erythrocytes and suggest the possible enzyme catalytic domain-dependent manner of hemolysis.

TOXIN BINDING ON THE ERYTHROCYTE SURFACE STIMULATES CYTOPLASMIC MEMBRANE LIPID REORGANIZATION

Once we detected the binding of the toxin to the surface of erythrocytes, we next analyzed the direct activity of phospholipase-D toxin on the lipid organization of the cytoplasmic membrane. For this purpose, washed erythrocytes treated with recombinant toxin were incubated with annexin-V and analyzed by flow cytometry. As evidenced by Figure 5A, erythrocytes following toxin treatment were positive for the binding of annexin-V. This finding suggests the exposure of negatively charged phosphatidylserine at the cell surface as well as the reorganization of membrane lipid components. Moreover, venom phospholipase-D-treated erythrocytes were incubated with Cholera toxin B subunit (a lipid raft marker) and evaluated through confocal microscopy. As shown by Figure 5B, our results evidence a reorganization of rafts following phospholipase-D toxin exposure. Exposure to this toxin apparently induces an aggregation of the lipid rafts.

THE CATALYTIC ACTIVITY OF PHOSPHOLIPASE-D PLAYS A ROLE IN HEMOLYSIS

To ascertain the mechanism by which phospholipase-D (dermonecrotic toxin) of *Loxosceles* spider venom triggers its noxious effects on erythrocytes and further to analyze the involvement of this enzyme's catalytic activity (which is dependent on magnesium ion coordination at the catalytic site) [Murakami et al., 2005]. Human erythrocytes were exposed to recombinant toxin in the presence of inhibitors like phenylmethylsulfonyl fluoride, 1,10-phenanthroline, EDTA, and EGTA and then analyzed for hemolysis. As depicted in Figure 6A, all three divalent ion chelators (EGTA, EDTA, and 1,10-phenanthroline) significantly inhibited hemolysis evoked by phospholipase-D. These findings contrast with those from phenylmethylsulfonyl fluoride treatment, a serine protease inhibitor that had no effect on hemolysis. EDTA has a higher affinity for magnesium ions and inhibited hemolysis more efficiently than other divalent metal chelators. Additionally, we incubated human

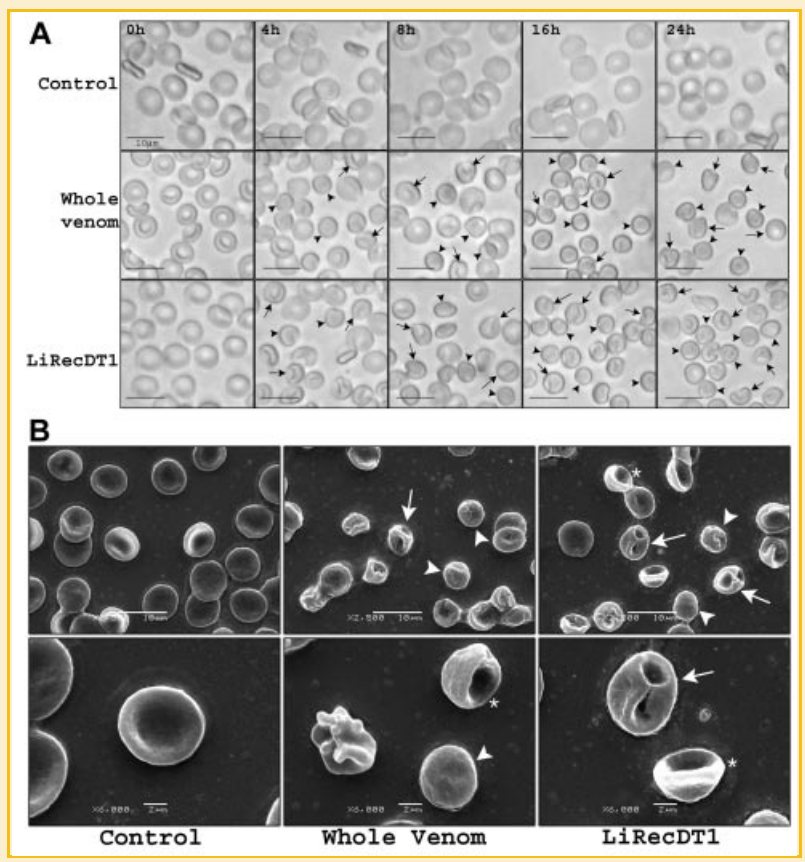

Fig. 2. Recombinant phospholipase-D treatment of human erythrocytes causes morphological alterations of cells. A: Human erythrocytes suspended in Ringer Solution were incubated with 5 μg/ml of *L. intermedia* whole venom, with LiRecDT1, or in the absence of toxins (control) for different times (0, 4, 8, 16, and 24 h) and then observed with an inverted microscope. Morphological changes of erythrocytes were observed in a time-dependent manner. Changes included the appearance of spherical and rounded cells (arrows point to stomatocytes-like cells and arrowheads show spherocytes-like cells); these contrasted with control cells that were biconcave disks flattened and depressed in the center. B: Additionally, cells were also observed by scanning electron microscopy. Venom or LiRecDT1 treatments of erythrocytes led to the appearance of spherocytes (arrowheads), stomatocytes (asterisks), and knizocytes (arrow) as well as a decrease in cell size. Scale bars are shown at the left of the figures. C: Washed erythrocytes were exposed to whole venom or LiRecDT1 (25 μg/ml) and (after 24 h of exposure) analyzed by flow cytometry to measure cell size and internal complexity through forward angle (FSC) and 90° side angle (SSC) light scatters. Two gates were created in the dot plots to distinguish intact cells (right gate) from lysed cells or debris (left gate). The number of cells with decreased size, number of lysed cells, or amount of debris visibly increased following venom or toxin treatment. Control cells were analyzed in the absence of toxins. The figure shows one representative from three trials.

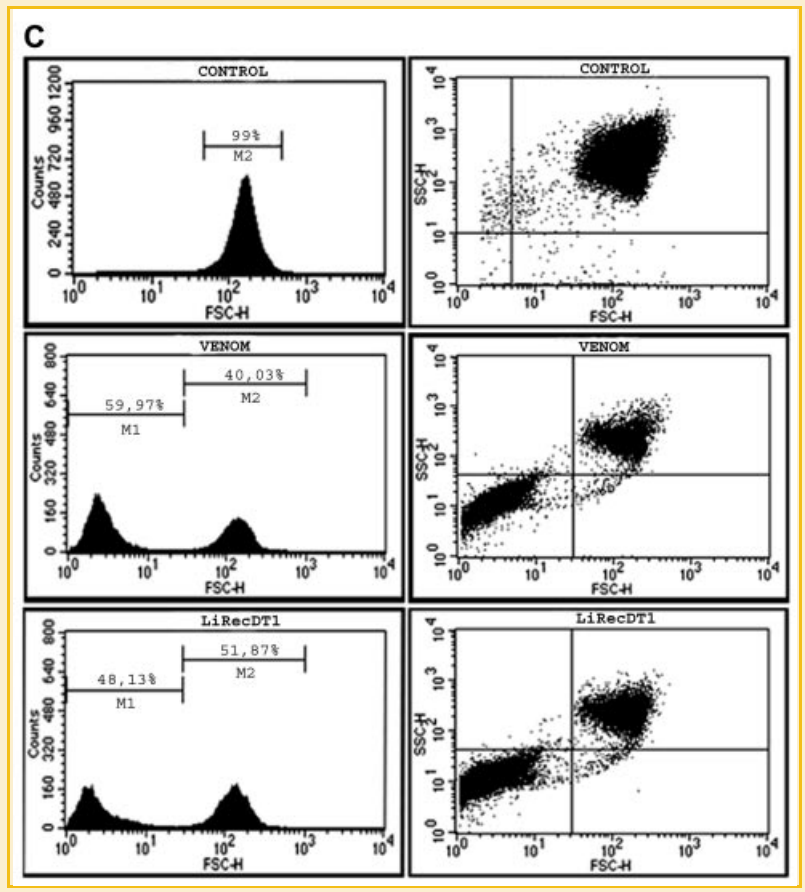

Fig. 2. (*Continued*)

erythrocytes in the presence of recombinant toxin (wild-type) or a mutated isoform in which we used site-directed mutagenesis at the catalytic domain to substitute a histidine residue at position 12 for an alanine residue (LiRecDT1 H12A) [Kusma et al., 2008]. As shown in Figure 6B, the mutated toxin did not cause significant hemolysis compared to the wild-type molecule. Additionally, it did not induce morphological changes in washed erythrocytes and despite its ability to bind to them. These findings strengthen the hypothesis that directed phospholipase-D hemolytic activity is dependent on the catalytic activity of the toxin.

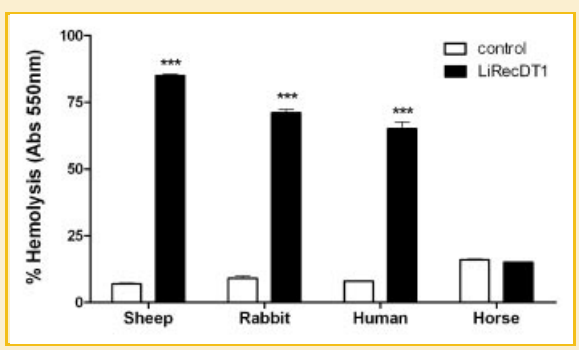

Fig. 3. Hemolysis caused by brown spider recombinant phospholipase-D depends on the animal species evaluated. Washed erythrocytes from sheep, rabbit, human, and horse were suspended in TBS, exposed to 25 μg/ml of LiRecDT1 for 24 h at 37°C, and evaluated for hemolysis. Results represent averages of five experiments ± SEM. $^{***}P \leq 0.001$.

DISCUSSION

Experimental studies with animal venoms have been observed that hemostatic disturbances, such as hemolytic and hemorrhagic events, are frequently associated with enzymatic activity of venom constituents. The serum complement system has long been described to participate in the hemolysis induced by brown spider venom. It seems that spider envenomation induces the activation of an alternative complement pathway that facilitates complement-mediated hemolysis [Futrell, 1992; da Silva et al., 2004; Swanson and Vetter, 2006].

The hemolytic effect induced by *Loxosceles* spider venom is demonstrated based on the clinical and laboratory features observed in accident victims. These features include elevated creatine kinase levels, hematuria, hemoglobinuria, proteinuria, and shock [Williams et al., 1995; Lung and Mallory, 2000; França et al., 2002]. Additionally, nephrotoxicity is supported by animal experimental

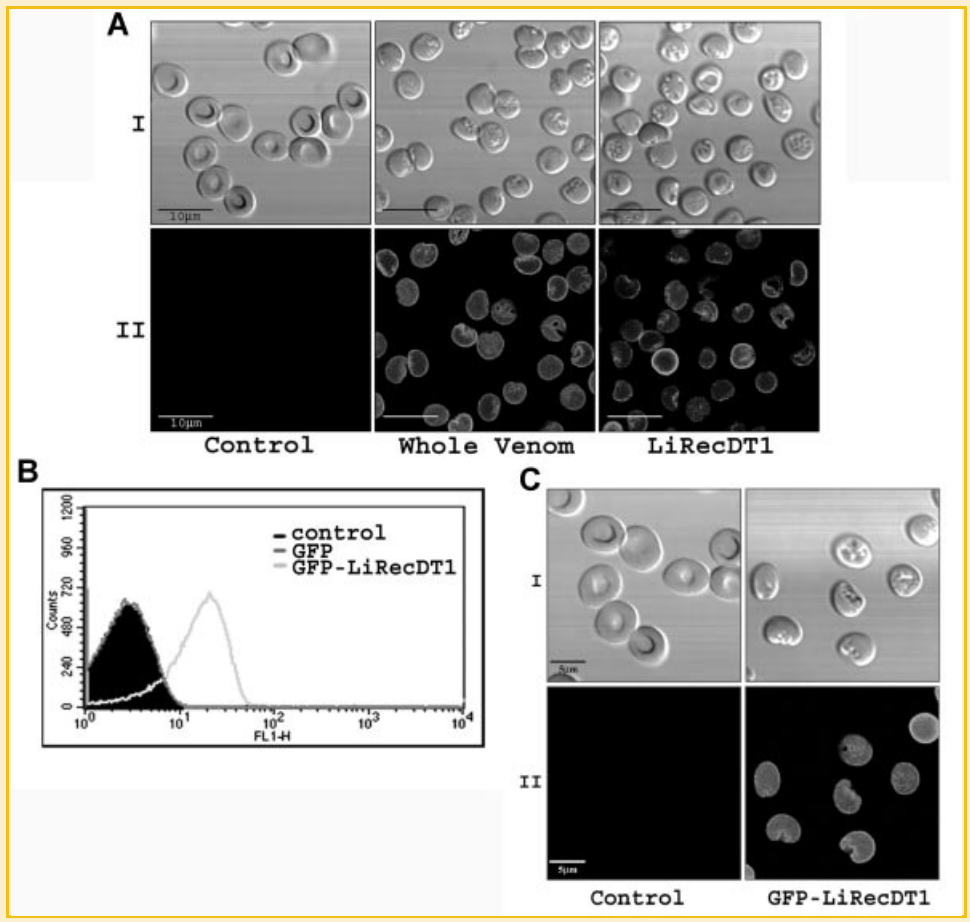

Fig. 4. Brown spider venom recombinant phospholipase-D binds to the human red blood cell membrane. A: Confocal microscopy was used to analyze human erythrocytes following (25 μg/ml) whole venom or LiRecDT1 exposure for 8 h at 37°C. Cells were immunostained with antibodies against LiRecDT1. For a negative control, cells were processed identically in the absence of toxin treatments. (I) Cells observed by Differential Interference Contrast (DIC), (II) Cells analyzed through immunofluorescence. B: Human erythrocytes were exposed under conditions identical to those described above to a recombinant fusion toxin GFP-LiRecDT1, purified GFP alone, or the absence of treatments (negative control) and analyzed by cell cytometry. Our results suggest the specific binding of the fusion protein to erythrocytes. C: Human erythrocytes were treated with a recombinant fusion toxin GFP-LiRecDT1, and negative control cells were not exposed to venom toxins. Cells were observed by confocal microscopy. Scale bars are shown at the left of the figure.

protocols. Animal models confirmed clinical data from accident patients by showing renal lesions following venom exposure [Luciano et al., 2004] and a direct nephrotoxicity evoked by the dermonecrotic (phospholipase-D) toxin [Chaim et al., 2006].

Previous studies have characterized dermonecrotic toxin as a sphingomyelinase-D molecule based on its activity to hydrolyze phospholipid sphingomyelin into choline and acylsphingosine-phosphate [Futrell, 1992]. By hydrolyzing phospholipids that generate ceramide 1-phosphate or lysophosphatidic acid, it is postulated that dermonecrotic toxin activates signaling pathways in different cells and causes pathophysiological changes like the inflammatory response, platelet aggregation, and increased blood vessel permeability [Anliker and Chun, 2004; Moolenaar et al., 2004; Tokumura, 2004; Lee and Lynch, 2005].

In this study, we used two recombinant isoforms of the phospholipase-D toxin from *L. intermedia* venom gland: a wild-type molecule and a molecule with a single amino acid mutation (H12A) at the catalytic site of the toxin [Murakami et al., 2005, 2006]. Our experimental data support the direct involvement of phospholipase-D activity in the hemolytic activity evoked by brown spider venom. Our first results demonstrated a direct hemolytic effect of wild-type recombinant phospholipase-D on human erythrocytes. Toxin-dependent hemolysis occurs in a concentration- and time-dependent manner, supporting the specificity of the reaction. Toxin cytotoxicity was additionally confirmed by changes of erythrocytes over time following toxin exposure; such changes were observed by inverted microscopy, scanning electron microscopy, and cell cytometry and included poikilocytosis and anisocytosis. Moreover, the hemolysis observed after exposure of human red blood cells to the toxin is not dependent on the ABO or Rhesus systems, since washed erythrocytes from groups A, B, and O as well as Rh positive and negative samples were lysed in a similar way following toxin treatment (data not shown). These findings support the hypothesis that this reaction did not depend on blood group compatibility. Nevertheless, direct hemolysis is dependent on animal species, since human, sheep, and rabbit erythrocytes were

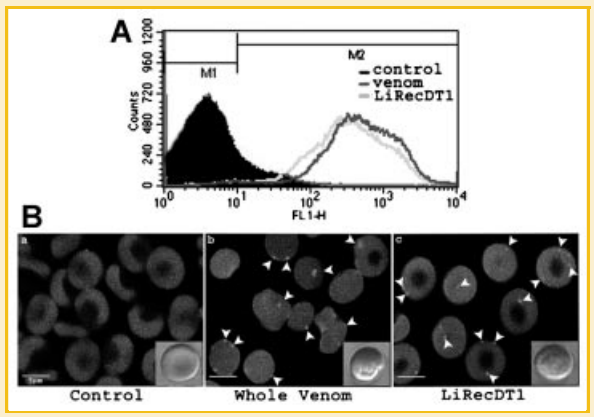

Fig. 5. Brown spider recombinant phospholipase-D binding to the erythrocyte surface stimulates cytoplasmic membrane lipid reorganization. A: Human erythrocytes suspended in Ringer solution were incubated for 4 h with 25 μg/ml of LiRecDT1. For a negative control, cells were maintained only with Ringer Solution. Following treatments, cells were incubated with annexin-V-FITC and analyzed through cell cytometry. The results indicate the binding of annexin-V and exposure of phosphatidylserine at the cell surface after whole venom and LiRecDT1 treatments. B: Also, lipid rafts were visualized through confocal microscopy with Cholera toxin subunit B-Alexa-Fluor 594 and anti-Cholera toxin subunit B rabbit serum. Arrowheads indicate for reorganization in the distribution of lipid rafts on the erythrocyte cell surface, as observed by aggregates of membranes. On the bottom of figures are shown details of the DIC cell analyses (*). The scale bar is shown at the left of the figure.

lysed but erythrocytes from horse were less severely lysed after toxin treatment. These results suggest that cell surface constituents regulate the susceptibility of erythrocytes to phospholipase-D lysis, since erythrocytes from different animal species have differing compositions of the extracellular lipoprotein monolayer. Interestingly, the concentration of sphingomyelin (a putative substrate for venom phospholipase-D) differs in the erythrocytes of these four tested animals. Lipid analyses of red blood cells reported 19.5% of sphingomyelin to human cells [Ingraham et al., 1981], 50% to sheep [Ochi et al., 2004], and 20% to rabbit [Ochi et al., 2003]. Erythrocytes in these animals were more susceptible to phospholipase-dependent lysis than those in horse, which has 14% of sphingomyelin [Ochi et al., 2003].

Through confocal immunofluorescence microscopy using antibodies to phospholipase-D toxin [da Silveira et al., 2006], we were able to detect an interaction of the toxin with the human erythrocyte cell surface. This result demonstrates the existence of sites of attachment for phospholipase toxin on human erythrocyte cell membranes and supports the hypothesis that the morphological and structural changes, as well as hemolysis induced by the toxin, occur as a result of toxin binding to erythrocyte membranes as an early step of process. Additionally, the interaction of venom phospholipase-D with erythrocyte cell membranes was supported by changes in the phospholipid content, as shown by the binding of annexin-V following toxin treatment. This finding indicates the exposure of phosphatidylserine, a negatively charged and inner monolayer molecule that translocates to the outer surface of the erythrocyte after phospholipase-D exposure. Phosphatydilserine exposure at the cell surface can induce several physiopathologic consequences including enhancement of coagulative reactions, recognition of apoptotic cells by phagocytes and blood cell attachment to endothelium [Closse et al., 1999]. Toxin binding also causes lipid plasma membrane reorganization, as visualized by Cholera toxin B binding. It has previously been shown that increases in ceramide levels following sphingomyelinase activation are one of the mechanisms that promote PS exposure in erythrocytes [Lang et al., 2005].

We next evaluated the effects of protein inhibitor molecules on toxin-dependent hemolysis and identified that divalent metal chelators like EDTA, EGTA, and 1,10-phelanthroline efficiently inhibited human blood cell lysis. However, the serine protease inhibitor phenylmethylsulfonyl fluoride did not block erythrocyte lysis. These results support the idea that a divalent metal ion is necessary for the biological activity of toxin in hemolysis. They are in agreement with literature data suggesting that phospholipase-D from brown spider venom contains a magnesium coordination site that plays an essential role in enzyme catalysis as a catalytic domain [Murakami et al., 2005, 2006]. This is interesting since EDTA, which has the greatest affinity for magnesium ions relative to the other tested chelators, was more efficient in hemolysis inhibition. Together, the above findings suggest that the catalytic activity of phospholipase-D plays an essential role in the direct hemolysis induced by this toxin. Experimental cell lysis has been shown to be related with phospholipase A_2 activity from *Bothrops jararacussu* snake venom [Soares et al., 2002].

The involvement of the catalytic site of venom phospholipase-D toxin in hemolysis was finally proved using a site-directed mutated isoform of phospholipase-D. In the catalytic domain of this isoform, a histidine (a basic and positively charged amino acid) residue at position 12 was replaced by a residue of alanine (non-polar amino acid) [da Silveira et al., 2006; Murakami et al., 2006]. Human red blood cells treated with the mutated toxin (even at concentrations higher than those used for the wild-type molecule) over time showed no hemolysis. Likewise, the mutation of the toxin also blocked the sphingomyelinase-D activity of this enzyme, demonstrating that sphingomyelin molecules on the cell surface of erythrocytes are involved in the lysis of cells. Additionally, these findings confirm the involvement of the catalytic domain in phospholipase activity that plays a role in direct hemolysis. The evidence shown here indicates that LiRecDT1 H12A can interact with the erythrocyte membrane, but this does not result in morphological changes. These findings are in agreement with our previous results from biopsies of kidney and renal cells [Kusma et al., 2008]. They strongly suggest that the catalytic activity of this enzyme, and not its interaction with the cell membrane, is essential for the hemolytic effects observed here. Treatment of erythrocytes with mutated toxin does not change the lipid organization, as visualized by annexin-V and Cholera toxin B binding (data not shown).

What is the mechanism by which phospholipase-D toxins from brown spider venom cause the direct hemolysis of cells? We postulate that the direct correlation between phospholipase-D activity and hemolysis is due to the fact that brown spider venom phospholipases can generate ceramide 1-phosphate, which is known to induce pathological responses like inflammation and platelet

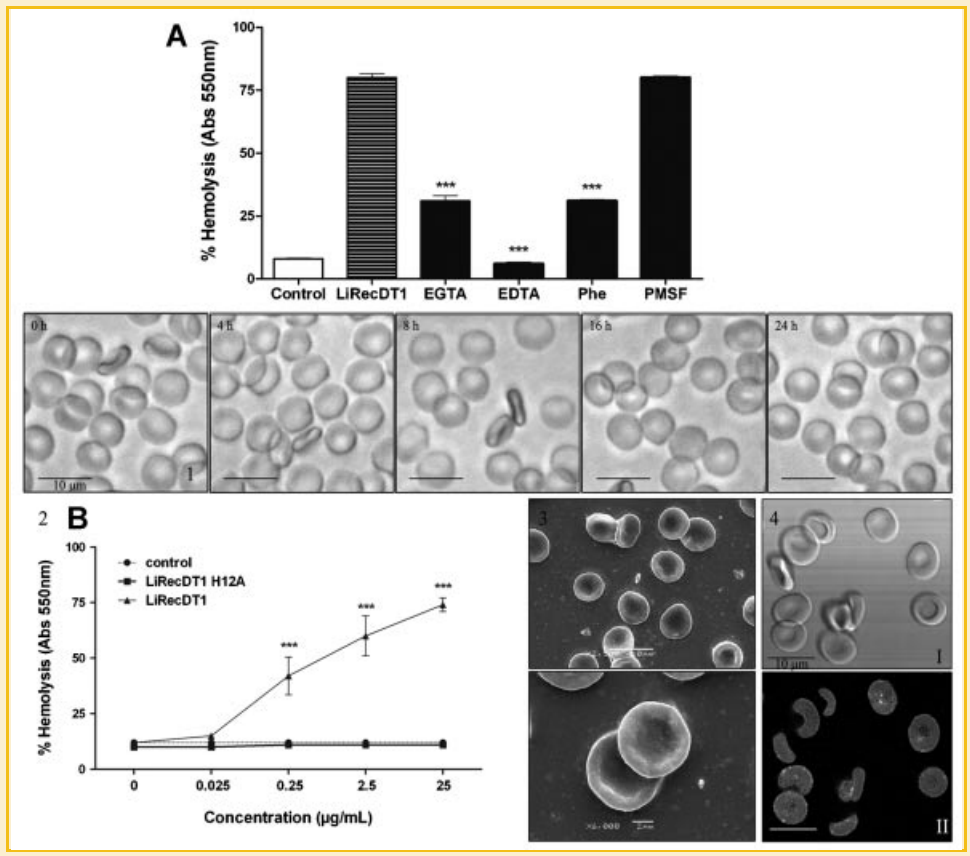

Fig. 6. Brown spider venom phospholipase-D catalysis plays a role in blood cell lysis. A: LiRecDT1 (25 µg/ml) was incubated with either EGTA, EDTA, 1,10-phenanthroline, or PMSF or alone for 30 min. Additionally, washed human erythrocytes suspended in TBS were exposed to LiRecDT1 and inhibitors for 24 h at 37°C and evaluated for hemolysis. Controls for hemolysis were performed using washed erythrocytes without toxin. B: (1) Human erythrocytes suspended in Ringer solution were visualized at light inverted microscope post-incubation with 25 µg/ml of site-directed mutated isoform of recombinant phospholipase-D H12A (LiRecDT1 H12A) for different times (0, 4, 8, 16, and 24 h). (2) Human erythrocytes were suspended in TBS and incubated with LiRecDT1 H12A for 24 h at 37°C. Hemolysis percentage was determined as described in Materials and Methods Section. (3) Cells were then observed with a scanning electron microscopy 8 h following toxin exposure. No morphological changes of erythrocytes were seen following treatment with the LiRecDT1 H12A even at the later assay times. (4) Confocal immunofluorescence microscopy was used to analyze human erythrocytes following of LiRecDT1 H12A exposure for 8 h at 37°C. Cells were immunostained with antibodies against LiRecDT1. (I) Cells observed by DIC. (II) Cells analyzed by immunofluorescence. Scale bars are shown at the left of the figure.

aggregation [Anliker and Chun, 2004; Moolenaar et al., 2004; Lee and Lynch, 2005].

Thus, by generating lipid mediators from red blood cells, venom phospholipase-D stimulates structural changes of the cytoplasmic membrane (such as the exposure of negatively charged phospholipids like phosphatidylserine). This then renders cell membranes more susceptible to physiological stress and finally contributes to hemolysis. In addition, the lipid mediators generated by the action of venom phospholipase-D on cell membranes can directly bind to human red cell membranes. Thus this toxin causes noxious effects in cells that finally evoke lysis. *Clostridium perfringens* α-toxin, which exhibits phospholipase-C (PLC) and sphingomyelinase (SMase) activities, has already been studied as a hemolytic agent [Sakurai et al., 2004]. PLC induces the hemolysis of sheep erythrocytes, and this activity is linked to the activation of sphingomyelin metabolism and the formation of sphingosine 1-phosphate [Ochi et al., 2004]. It was recently shown that hemolysis caused by α-toxin requires the participation of an endogenous sphingomyelinase [Oda et al., 2008].

Many of the morphological alterations observed herein (e.g., shrinkage, membrane blebbing, and phosphatidylserine exposure at the outer membrane leaflet) are characteristic of suicidal death of erythrocytes (eryptosis) [Lang et al., 2005]. One of the established signaling pathways that converge to trigger eryptosis involves phospholipase A_2-mediated release of platelet-activating factor, which activates a sphingomyelinase and leads to the formation of ceramide. The enhanced ceramide levels associated with increased cytosolic Ca^{2+} lead to membrane scrambling and subsequent phosphatidylserine exposure [Lang et al., 2007]. We can postulate that ceramide resulting from venom/toxin activity on erythrocyte membrane sphingomyelin leads to phosphatidylserine exposure. This suggestion is supported by recent work that shows the vertical movement of ceramide from the outer to the inner leaflet membrane. This ceramide was generated using an exogenous bacterial sphingomyelinase that hydrolyzes sphingomyelin at the cell surface [Mitsutake and Igarashi, 2007].

Based on the above results, we cannot rule out the possibility that the serum complement system plays a role in the hemolytic activity

of brown spider venom phospholipase-D, as previously suggested by literature data [Futrell, 1992; da Silva et al., 2004; Swanson and Vetter, 2006]. Instead of this, however, it is rational to speculate that the direct hemolytic effect induced by phospholipase together with complement-dependent hemolysis represent a synergism for the same noxious event described following accidents with brown spiders.

The data described herein indicate that the catalytic activity of phospholipase-D from brown spider venom plays a role in the direct hemolytic activity of this toxin, and they therefore provide insights into loxoscelism and contribute to the possibility of therapy based on the inhibition of phospholipase toxin catalysis.

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