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A C-TYPE LECTIN FROM *Bothrops jararacussu* VENOM CAN ADHERE TO EXTRACELLULAR MATRIX PROTEINS AND INDUCE THE ROLLING OF LEUKOCYTES

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ABSTRACT: Purification of a lectin from *Bothrops jararacussu* venom (BjcuL) was carried out using agarose-D-galactose affinity gel. MALDI-TOF gave a major signal at *m/z* 32028, suggesting the presence of a dimmer composed of two identical subunits. Divalent cations were required for the lectin activity, as complete absence of such ions reduced hemagglutination. BjcuL was more effective at neutral pH and showed total loss of activity at pH values below 4.0 and above 9.0. Its agglutinating activity remained stable at 25°C until 60min, but increased when at 35°C for at least 15min. Adhesion assays to extracellular matrix (ECM) glycoproteins showed that the biotinylated lectin (0.039–5.0μg/100μl) was capable of binding to fibronectin and vitronectin in a dose-dependent manner. The binding was partially inhibited in the presence of D-galactose. BjcuL (1.25–10μg/30μl) potential was investigated for leukocyte rolling and adhesion to endothelial cells in living microvessels using intravital microscopy, which showed that it induced a dose-dependent increase in rolling and adherence of leukocytes, acting directly on endothelial cells of postcapillary venules. The specific association between lectins and their ligands, either on the cell surface or on the ECM, is related to a variety of biological processes. The complementary characterization of BjcuL, shown here, is useful to further understand the venom effects and as a background for future investigation for therapeutic strategies.

KEY WORDS: venoms, fibronectins, vitronectin, snakes, intravital microscopy, leukocytes.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Bothrops species, known as jararacas, are widely found from the South to the Northeast of Brazil and also in Bolivia, Paraguay and Northern Argentina (2). In Brazil, they are responsible for approximately 70% of the snakebites in humans, which are lethal in 0.3% cases. *Bothrops jararacussu* venom is composed of a complex mixture of proteins and bioactive peptides, especially PLA_2, proteases, and C-type lectins (Ca^{2+}-dependent), which play different roles in local and systemic injury processes that characterize bothropic envenomation (25).

Lectins are non-enzyme and non-immune proteins capable of binding specifically and non-covalently to carbohydrates (13), rendering them important in cell-cell and cell-molecule recognition processes. Most of the C-type lectins of Viperidae venoms have been described as disulfide-linked dimers of two homologous polypeptides of ~14kDa. They have erythrocyte-agglutinating activity and other properties such as adhesion to plasmatic proteins (28), mitogenic activity on lymphocytes (24), binding to platelet receptors inducing either activation or inhibition of platelet aggregation (22), and reduction of renal flow and glomerular filtration rate (16). The lectin from *B. jararacussu* venom (BjcuL) has been purified and characterized as a C-type galactoside-binding lectin (4,29). Cells from human metastatic breast cancer and human ovarian carcinoma were shown to adhere weakly to BjcuL, reducing the viability of these and other tumor cell lines (5). BjcuL has been reported to induce edema and increase vascular permeability in mouse hind paws, and was recognized by commercial antivenom raised against a pool of *Bothrops* venoms (29).

The activation of the immune response reported for BjcuL is expected to be due to an interaction of the lectin with glycoconjugates, provoking important specific cellular responses in the formation of innate or acquired immunity. Snakebites are known to cause characteristic local reactions such as severe edema and redness, followed by necrosis (15) and accumulation of leukocytes at the bite site (8,9), which are attributed to metalloproteinases and phospholipases (6). Nevertheless, other components of the venom can also contribute to such stimulation, resulting in an inflammatory response (26).

In the current study, we report an improvement to the BjcuL isolation method and a further characterization of its hemagglutinating properties. We also demonstrated the

capacity of the lectin to interact *in vitro* with fibronectin and vitronectin and to promote *in vivo* migration of leukocytes.

MATERIALS AND METHODS

Venom and Reagents

Bothrops jararacussu venom was obtained from Butantan Institute, São Paulo, Brazil. Fibronectin and vitronectin from human plasma were gifts from Dr. Sílvio Sanches Veiga (18,32).

Lectin Purification

BjcuL was purified as previously described (4), with modifications: crude venom was suspended in CTBS buffer (NaCl 150mM, Tris-HCl 20mM, $CaCl_2$ 5mM), pH 7.5, to a final concentration of 20mg/ml and applied to a 5ml agarose-D-galactose column (Pierce, USA). After 4h agitation at 4°C, elution was carried out with the same buffer. The retained material was eluted with 100mM lactose in the buffer and this fraction was extensively dialyzed against distilled water for complete removal of the sugar. Carbohydrate content in the dialysis water was tested by the method of Dubois *et al*. (7). Protein concentrations were determined (21) using bovine serum albumin as standard.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed (20) on 20% SDS-polyacrylamide gels (12X10cm, 0.75mm thick), under natural and reducing conditions, with β-mercaptoethanol. A low molecular weight protein kit was used for standardization (Gibco BRL, USA).

MALDI-TOF Spectrometry

BjcuL was analyzed by MALDI-TOFy using a Reflex IV mass spectrometer (Bruker Daltonics, Karlsruhe, Germany). The sample was applied onto the target plate using the dry droplet technique, in which 1µl sample containing 1µg total protein in 0.1% trifluoroacetic acid (TFA) was mixed with 1µl matrix (20mg/ml sinapinic acid in 0.1% TFA, 40% acetonitrile). The sample was allowed to dry and was then washed twice with 5µL of 0.1% aq. TFA. The spectrum was obtained in linear mode.

Hemagglutination Assays

Agglutination assays were conducted using untreated human erythrocytes. These were washed three times with 0.9% aq. NaCl and suspended at a concentration of $1.0X10^7$cells/ml. Tests were carried out in hemolysis tubes by titration of the lectin (50μg/ml) in 150mM aq. NaCl, followed by addition of the erythrocyte suspension at a volume corresponding to 10% of that of the lectin solution. Tubes were gently shaken and incubated at 22ºC for 1h. Agglutination was evaluated by the naked eye and graded from 0 (negative) to +4. The hemagglutination titer is calculated as the reciprocal of the multiple of the dilution giving a positive reaction. Controls for all titrations were prepared by substituting lectin for saline solution. The test for divalent cation requirements was carried out as described above; however, $CaCl_2$, $MgCl_2$ or $MnCl_2$ alone, at indicated concentrations, were added to NaCl solution. The pH range within which the glycoprotein exhibits optimum binding to human erythrocytes was determined by titration of the lectin samples in the following buffers: 50mM sodium citrate-phosphate (pH 3.0–5.0), 50mM sodium acetate (pH 4.0–5.0), 50mM sodium phosphate (pH 6.0–7.5) and 50mM Tris-HCl (pH 8.0–9.0), all containing 150mM NaCl and 20mM $CaCl_2$. The lectin thermal stability was estimated by incubating the samples at increasing temperatures up to 80ºC for 15, 30, 45 and 60min, followed by spontaneous cooling to 25°C and titration with saline solution (150mM NaCl/20mM $CaCl_2$).

ECM Protein Adhesion Assay

BjcuL (2mg) was diluted in 50mM $NaCO_3$ (1ml), pH 8.6, and mixed with 74µl of Sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL, USA) solution (1mg/ml). After 40min incubation in the dark, the mixture was ultrafiltrated through a Centricon YM3 membrane (Amicon Division, W.R. Grace & Co., Beverly, MA). For adhesion assays, wells of 96-well flat bottom plates (MaxiSorp, FluoroNunc, Roskilde, Denmark) were coated with 100μl of fibronectin or vitronectin (0.25μg/well) in 0.1M $NaHCO_3/Na_2CO_3$, pH 9.5, overnight at 4°C. Excess protein was removed by washing the wells three times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (washing buffer), followed by blocking with 10% fetal bovine serum (FBS) in PBS for 1h. After

incubation, wells were washed (3X) and biotinylated lectin was added (2.5–0.04µg/well). For a negative control, lectin was replaced by the washing buffer; for a positive control, KM+ (a lectin isolated from *Artocarpus integrifolia* seeds) was used (10µg/well). After 2h incubation, wells were washed (5X) followed by 30min incubation with avidin-peroxidase (0.4µg/well). Then, wells were washed again (7X), and lectin adhesion to immobilized proteins was revealed after 30min incubation with benzenediamine *O*-phenylenediamine substrate, 50µl/well, in the dark. The reaction was stopped by addition of 2M H_2SO_4 (50µl/well) and colorimetrically quantified by absorbance reading at 490nm on a plate reader (Tecan, Sunrise Remote, Austria). For inhibition assays, BjcuL was preincubated with different concentrations of carbohydrates for 1h at room temperature and allowed to adhere to immobilized proteins. Results were expressed as the mean ± standard deviation. Statistical analyses were carried out using ANOVA and Tukey test.

Microcirculatory Alterations

Observations of leukocyte interactions in the mouse cremaster muscle venules were performed as previously described (27,31). Mice were anesthetized with an ip injection of sodium pentobarbital (50mg/kg body weight), placed on a water-heated bed (at 37°C), and their cremaster muscle was exposed for topical application of lectin (1.25, 2.5, 5 and 10µg/30µl). Control experiments were performed by applying 30µl PBS under otherwise identical conditions. Muscle preparations were observed in a triocular microscope (Axioskope, Carl-Zeiss) and analyzed with an image analyzer software (KS 300, Kontron). Images were obtained using an X10/025 longitudinal distance objective/numeric aperture and 1.6 optovar. A five-minute observation period was recorded before the lectin application in order to analyze the dynamics in the control tissue. Experiments were carried out for up to 30min. A 5µg dose was used to evaluate rolling and adherent leukocytes at 10, 20, and 30min.

RESULTS

Purification and Characterization of *B. jararacussu* Venom Lectin

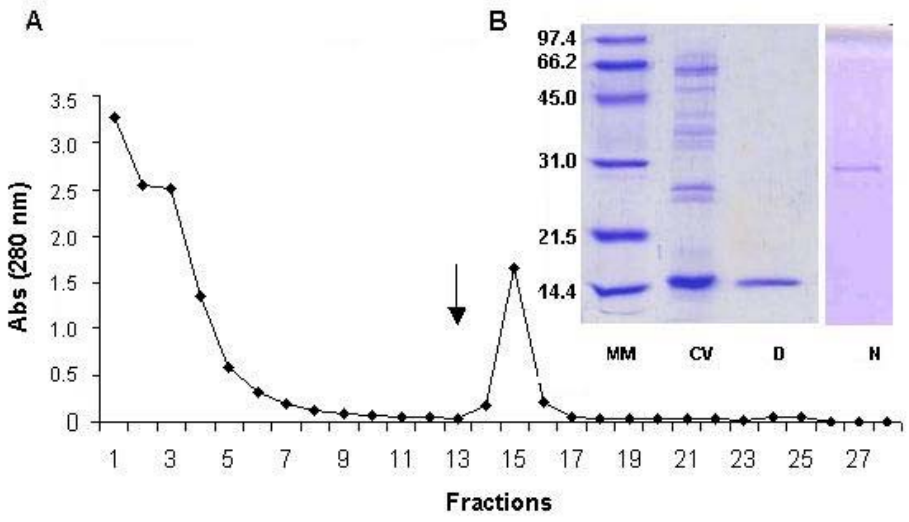
The purification procedure for BjcuL was monitored using agglutination assays with human erythrocytes (type A) and SDS-PAGE analyses. Total isolation was achieved

with a single chromatoghaphic step, using a column of agarose-D-galactose affinity gel, eluted with 100mM lactose in CTBS (Figure 1A). As shown in Table 1, in a typical purification procedure from 100mg crude venom, ~5mg of purified lectin was obtained with increased hemagglutinating activity. It migrated on Coomassie-stained SDS-PAGE gels as a single band of ~15kDa or ~30kDa in the presence or absence of 2-mercaptoethanol, respectively (Figure 1B). This is consistent with mass spectrometry results, which showed a major ion of 32028Da and minor ones of ~16000 and 64000Da (Figure 1C), suggesting that the lectin is mainly a dimer composed of two identical subunits linked by cystine bonds, but other aggregate levels are possible.

Table 1. Properties of the fractions obtained during lectin purification (from 100mg of crude venom).

Fraction	Volume (ml)	Protein concentration ($mg.ml^{-1}$)	Total protein (mg)	Agglutination titer[a]	Total activity (AU)[b]	Specific activity (AU/mg)[c]
Crude venom	5	20	100	4	20	0.2
Purified fraction	28	0.18	5.2	16	448	86

[a] Titer is recorded as the reciprocal highest dilution showing a +4 agglutination degree.
[b] Volume X agglutination titer
[c] AU/total protein



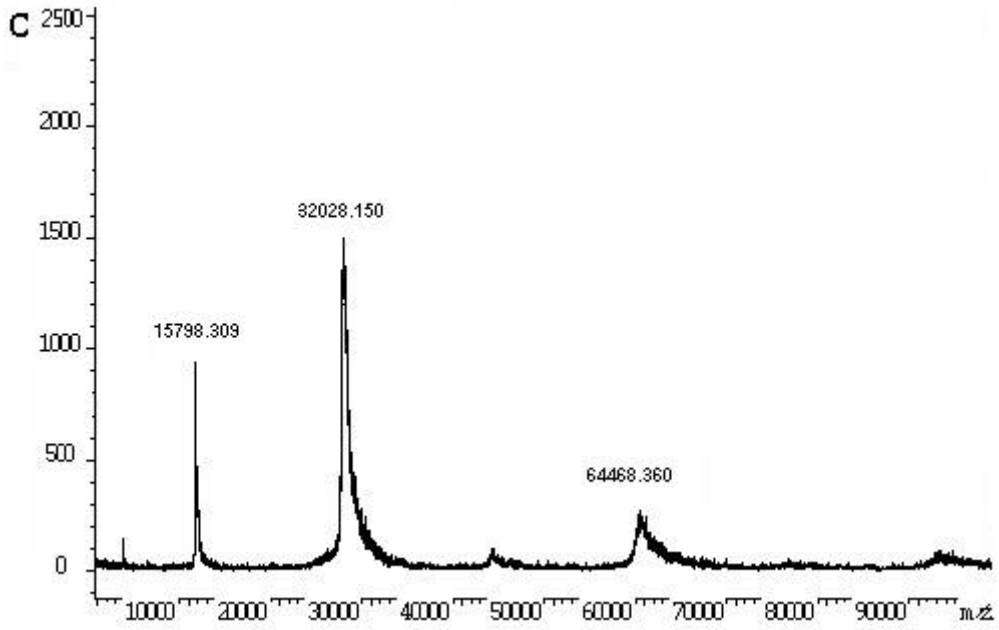


Figure 1. Lectin purification. (A) Elution profile of crude venom (100mg) on agarose-D-galactose column (5ml), collected in 2ml fractions. Total proteins were eluted with CTBS, pH 7.5, and adsorbed fraction with 100mM lactose solution in the same buffer (indicated by the arrow). (B) SDS-PAGE of the purified fraction. MM: molecular mass marker proteins (kDa); CV: *Bothrops jararacussu* crude venom; D: denaturant conditions in the presence of 2-mercaptoethanol; N: natural conditions. (C) MALDI-TOF mass spectrometry of *B. jararacussu* lectin. The sample was applied using the dry droplet technique. The spectrum was acquired in linear mode.

Hemagglutination Characterization

BjcuL agglutinated all types of human erythrocytes, but showed a high preference for type B over type A (data not shown), which indicates blood-group specificity, depending on the cell-surface carbohydrate moieties. Divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} were required for BjcuL activity, as their complete absence reduced hemagglutination (Figure 2). The best results were obtained with Ca^{2+} and Mg^{2+} at 40 and 50mM, respectively. The lectin was more effective at a neutral pH range, showing a gradual increase in activity from pH 5.0 to 7.0, 7.5 then decreasing up to pH 8.5. It showed total loss of activity when assayed at pH values below 4.0 and above 9.0. Agglutinating activity remained stable up to 60min at 25°C, but increased when the lectin was left at 35°C for at least 15min. Its activity also remained stable from 40 to 50°C up to 30min, then drastically reduced. At 70°C, the lectin was completely inactivated (Figure 2).

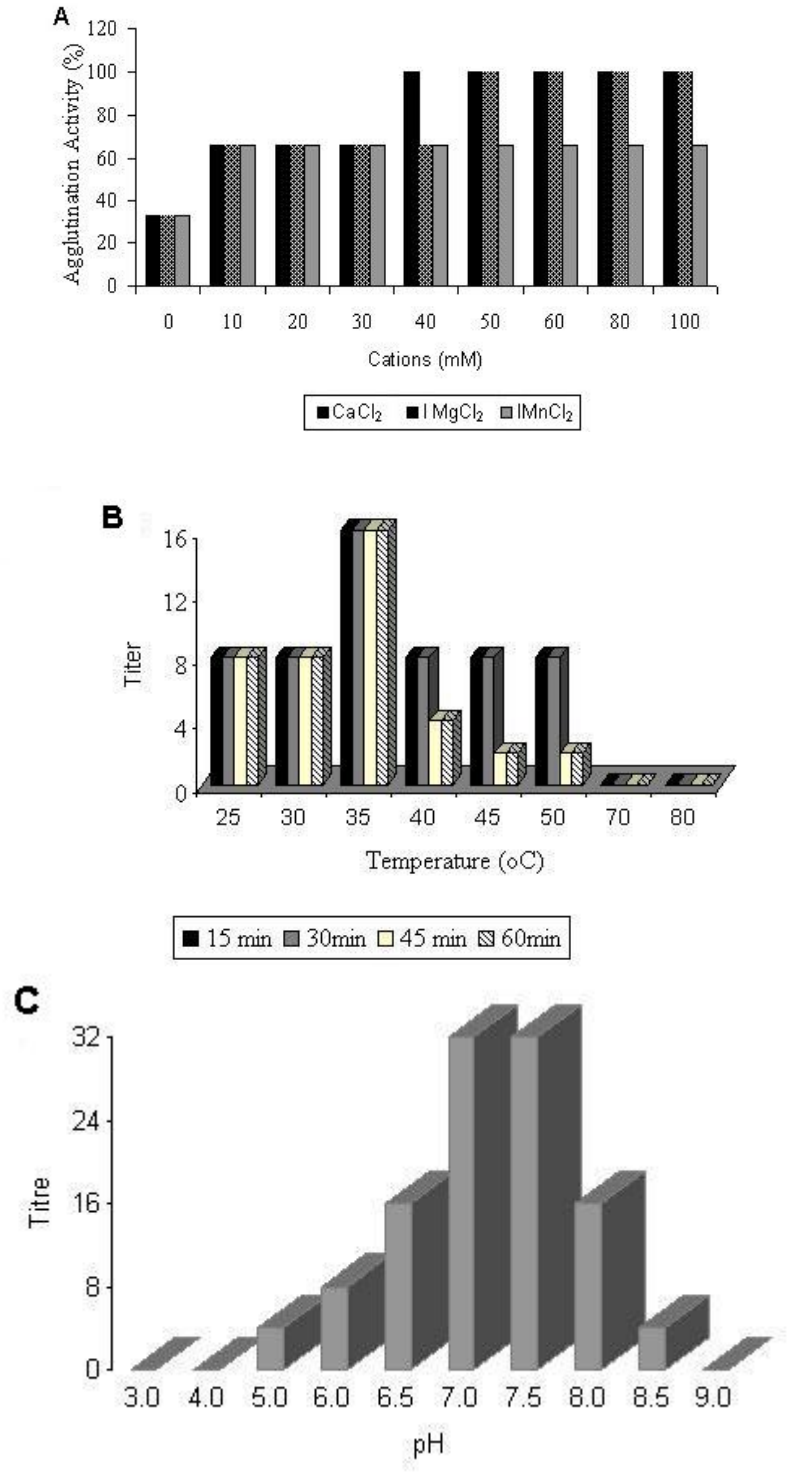


Figure 2. Effect of divalent cations (A), temperature (B) and pH (C) on Bothrops jararacussu lectin hemagglutinating activity. Thermal stability was verified by maintaining the lectin solution at different temperatures for increasing exposure times. The effect of pH was measured by incubating the lectin solution with different pH-buffered solutions for 1h at 25°C. All procedures were followed by activity assays under standard conditions.

Adhesion Assays

Adhesion assays to ECM glycoproteins showed that the biotinylated lectin (0.039–5.0µg/100µl) was capable of binding, in a dose-dependent manner, to fibronectin and vitronectin (Figure 3A). Binding was partially inhibited in the presence of D-galactose. Mannose, up to a concentration of 100mM, did not inhibit BjcuL binding to the glycoproteins (Figure 3B).

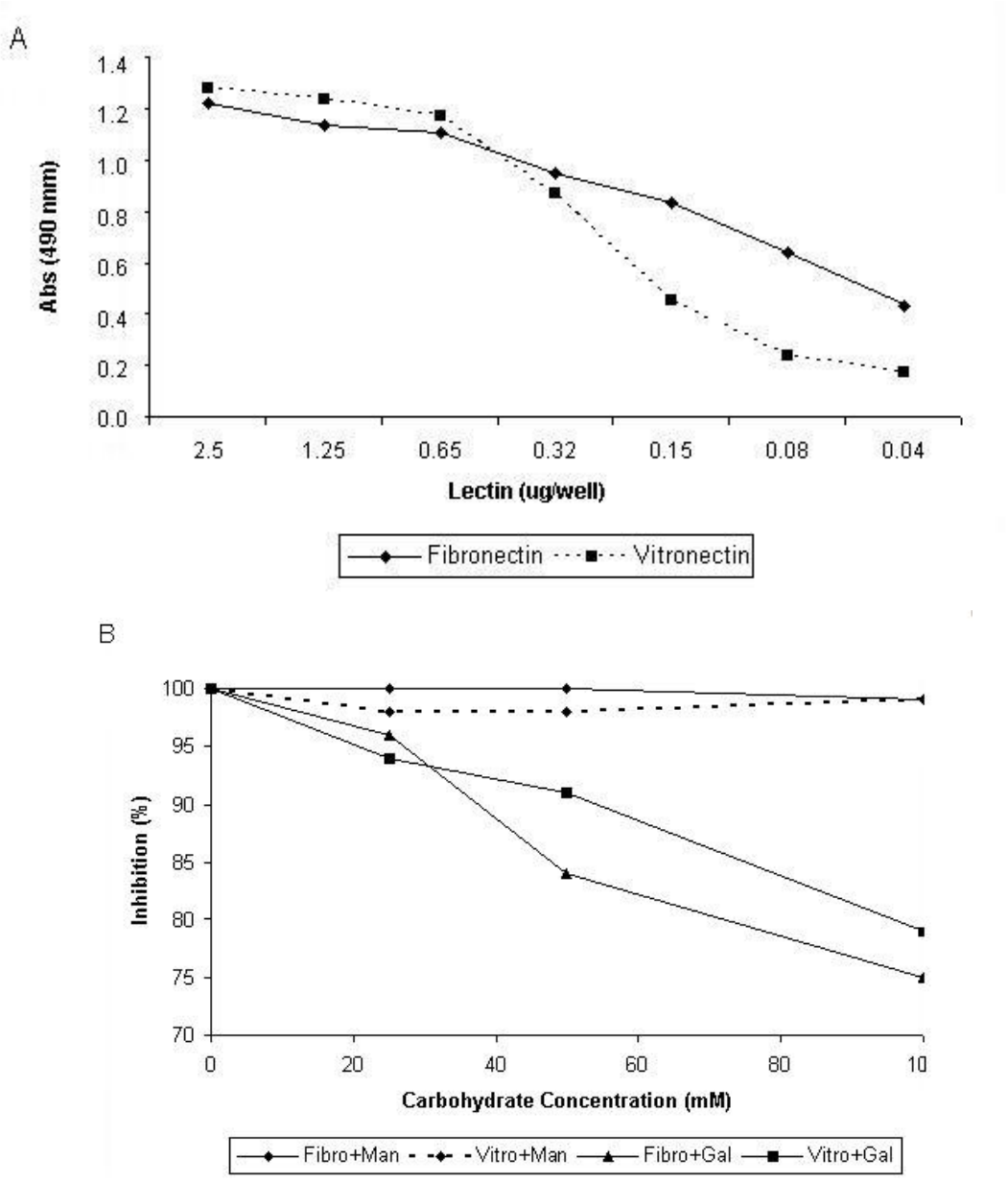
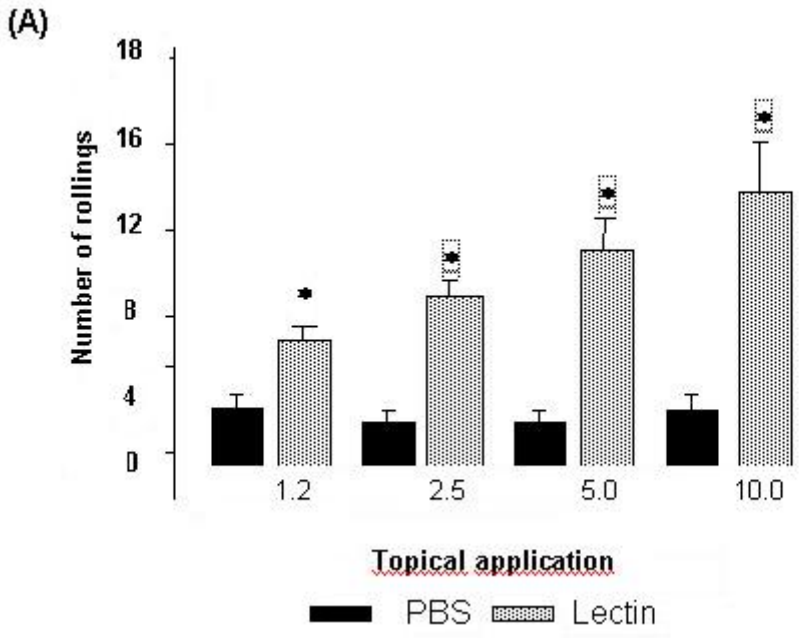


Figure 3. Adhesion of *Bothrops jararacussu* venom lectin to extracellular matrix proteins, fibronectin and vitronectin (A), and the interference of galactose (Gal) and mannose (Man) at different concentrations (B). Negative control in the absence of lectin.

Intravital Microscopy

To investigate BjcuL potential for leukocyte rolling and adhesion to endothelial cells under the conditions prevailing in living microvessels, the cremaster muscle of mice was exposed for topical application of lectin (1.25, 2.5, 5, and 10μg/30μl) and evaluated after 30min. A few rolling leukocytes (velocity>30μm/s), but essentially no firmly adherent cells, were observed in the postcapillary venules of control mice (black bars, Figure 4). Numerous leukocytes interacted with the endothelium in the cremaster of BjcuL-treated mice (Figure 4A), and the vast majority of these cells adhered firmly to the vessel walls after lectin application. The increase in rolling and adherent leukocytes, induced by lectin, was dose-responsive (Figure 4B). The average number of rolling and adherent leukocytes was higher in the lectin group than in the control group until 30min (Figure 5). Analysis of the recorded videotapes did not show any evidence of accumulated platelets in postcapillary venules of cremaster muscle of BjcuL or PBS-treated mice (data not shown).



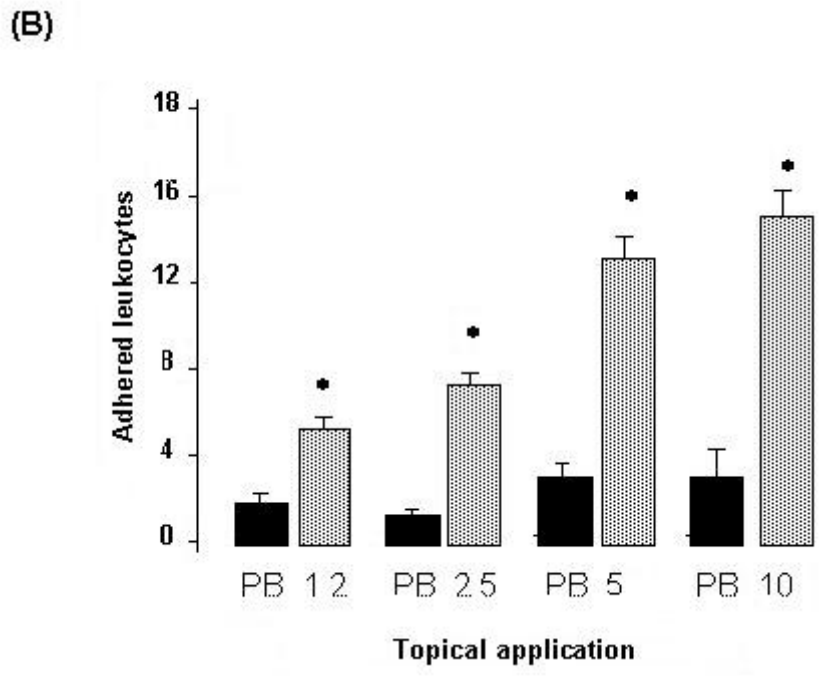
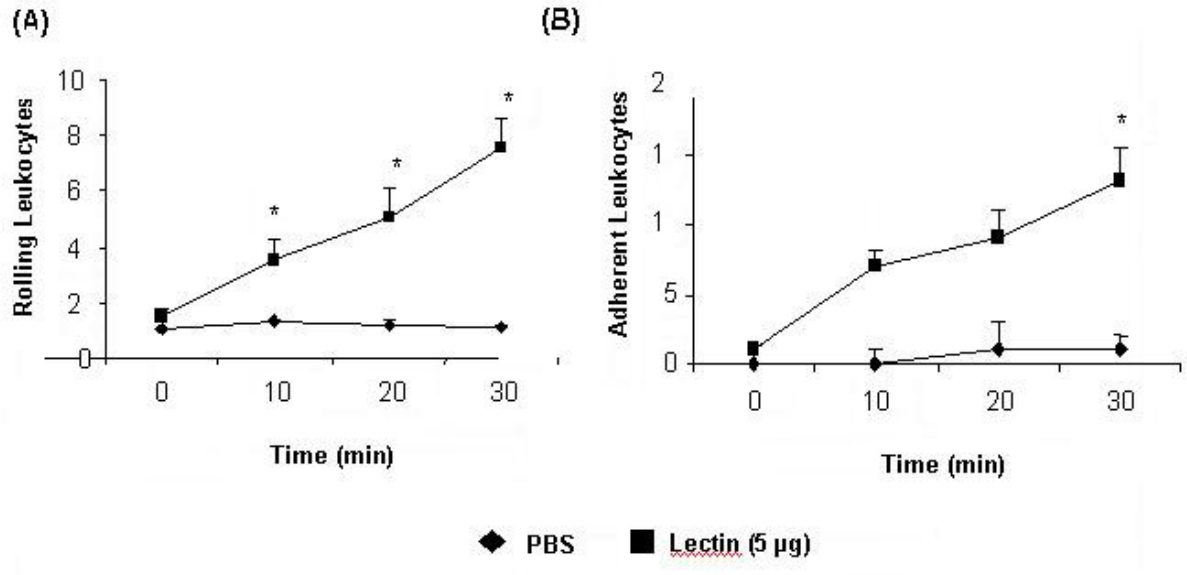


Figure 4. Evaluation of rolling (A) and adherent (B) leukocytes induced by C-type lectin from *Bothrops jararacussu* venom. Samples (30μl) containing different doses of lectin were topically applied on the cremaster muscle of anesthetized mice. The aspect of pre and postcapillary venules was observed after 30min. * $p<0.05$, compared with control group.



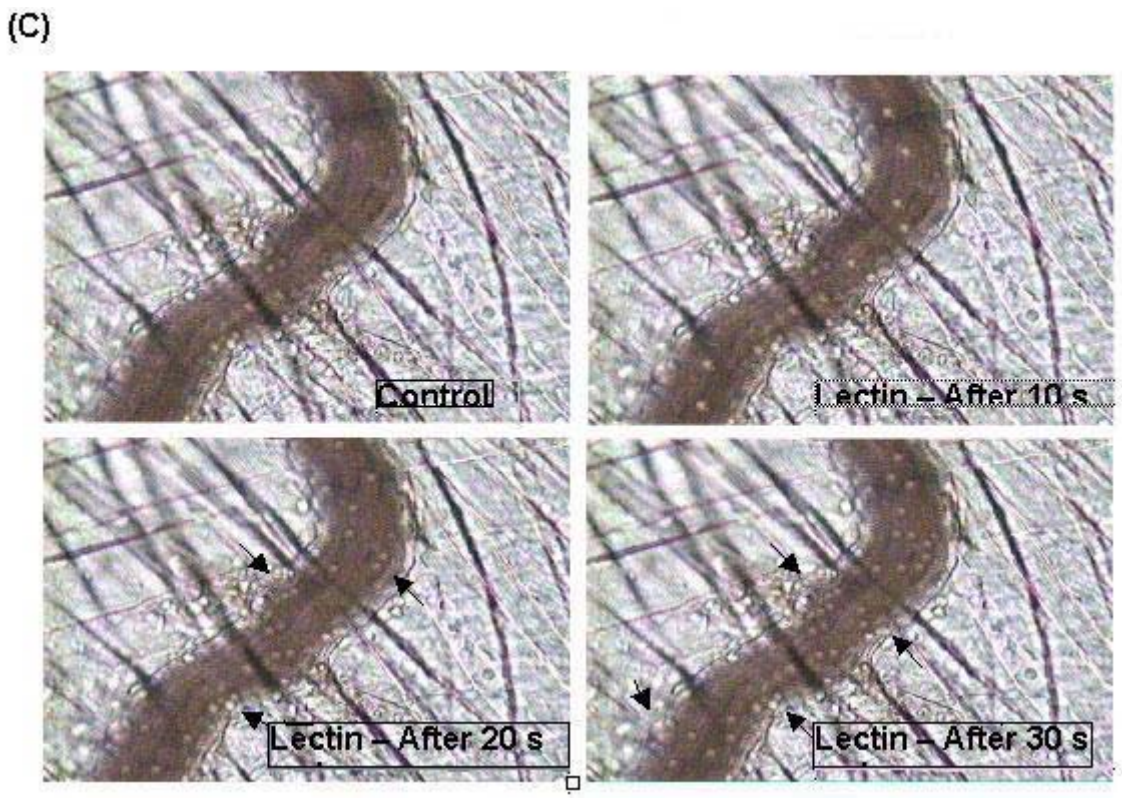


Figure 5. Evaluation of the kinectics of rolling and adherent leukocytes. Samples (30μl) containing 5μg lectin were topically applied on the cremaster muscle of anesthetized mice. The aspect of pre and postcapillary venules was observed every 10min during 30min and rolling (A) and adhesion (B) were recorded during 1min. The photos represent an intravital micrograph of cremaster muscle after 0, 10, 20 and 30s of topical application of lectin (C). Arrowheads indicate adhered leukocytes. * $p<0.05$, compared with control group.

DISCUSSION

Thrombolectin was the first lectin isolated from snake venom. It was discovered in the venom of *Bothrops atrox* and identified as a β-galactoside-binding dimeric lectin of *M*r 28000, similar in certain aspects to other Ca^{2+}-dependent (C-type lectins; CTL) β-galactoside-binding lectins of vertebrates (12). Soon after, hemagglutinins from different snake venoms were discovered and characterized as a large family of structurally homologous proteins. These are dimers of ~30kDa, composed of similar disulfide-linked monomers with the CTL motif presenting saccharide-binding activity (14).

BjcuL is a typical component of this group of molecules. Its purification was first described by Carvalho *et al*. (4) using D-galactose affinity chromatography with 0.9%

yield. Panunto *et al*. (29) isolated the lectin using a non-derivatized Sepharose 4-B column and obtained a similar result. Our modification of the former method consisted in a 4h agitation period at 4°C prior to elution, which allowed an ~5% yield. Apparently, it permitted a better interaction between the venom-pool lectin molecules and the gel matrix. The result of MALDI-TOF confirmed the purity of the carbohydrate-free fraction: carbohydrate might have been present because of an inefficient dialysis step or as column debris. Furthermore, this result confirms the expected molecular weight of BjcuL monomers of ~16kDa (3). The amount of recovered lectin is consistent with the analysis of *B. jararacussu* venom gland transcriptome, which showed transcripts related to C-type lectin proteins to an order of 5% (17).

BjcuL can adhere, although weakly, to human metastatic breast cancer and human ovarian carcinoma cell lines, suppressing the viability of these tumor cells (5). However, considering its action in envenomation, few speculations were presented. BjcuL has been shown to be capable of producing edema and briefly increasing vascular permeability in mouse hind paws (29), which are signs of inflammatory processes. Extravasation of immune cells from peripheral blood through the vascular endothelium into the ECM is also a common event in inflammatory manifestations. We evaluated the cellular infiltration induced by lectin and our results, using intravital microscopy, show that the lectin acts directly on endothelial cells of postcapillary venules, creating an adhesive surface for the rolling of a great number of leukocytes.

Neutrophil influx induced by ip injection of *B. jararaca* venom into mice has been shown to be related to the expression of adhesion molecules, responsible for rolling, firm adhesion, and transmigration events associated with neutrophil migration (33). Molecules in venom responsible for these effects have not been described yet, but it is possible to anticipate that lectins are involved, as lectin-carbohydrate interactions in leukocyte recruitment into inflammatory sites have become more evident (1). Galectin-3 is chemotactic in humans, promoting adhesion of neutrophils as well as other cell types to ECM proteins in an integrin-dependent manner (19,23). This bridging of neutrophils to ECM due to galectin-3 appears to involve activation of the cells via Ca^{2+} and Mg^{2+}-dependent processes. Ganiko and collaborators (10,11) suggested that the interaction of a D-mannose-binding lectin (KM+), also known as

artocarpin, with laminin, fibronectin and heparin makes possible the establishment and maintenance of a KM+ molecular gradient, a necessary phenomenon to induce the directed movement of neutrophils.

Here, we showed the interaction of BjcuL with ECM components and evaluated the dependence of the carbohydrate recognition domain (CRD) in the interaction of BjcuL with fibronectin and vitronectin. Snake venom lectins are known to bind mostly to galactose and also to mannose (34). However, our results demonstrated that lectin pre-incubated with 100mM D-galactose had its interaction capacity reduced by about 20%, whereas its incubation with 100mM D-mannose did not give rise to any alteration. These results reinforce the hypothesis that CRDs are involved in the inductive activity of migration (30) as well as the galactoside-binding preference of BjcuL.

Hemagglutination assays were carried out in order to analyze the integrity of the purified BjcuL as well as to establish the optimum conditions for biological studies. Our results indicated the requirement for divalent cations other than Ca^{2+}, such as Mg^{2+}, besides physiological conditions of pH and temperature for a better binding activity, which was expected, as the venom must act on mammalian preys.

In conclusion, we demonstrated a further characterization of BjcuL with respect to its hemaglutinating activity, which can be increased at neutral pH and temperature around 37°C, in the presence of Mg^{2+}, as a replacement for Ca^{2+}. We showed that BjcuL can adhere to fibronocetin and vitronectin and act directly on endothelial cells of postcapillary venules, creating an adhesive surface for the rolling of a great number of leukocytes. However, to consider its participation in inflammation events, further investigation on its effect over leukocytes is needed.

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