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Effect of BjcuL, a lectin isolated from *Bothrops jararacussu*, on human peripheral blood mononuclear cells

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ABSTRACT

BjcuL is a C-type lectin with specificity for the binding of β-D-galactose units isolated from *Bothrops jararacussu* venom. It triggers cellular infiltration in post capillary venules, increases edema and vascular permeability in murine models, contributes to *in vitro* neutrophil activation and modulates macrophage functional activation towards an M1 state. The purpose of this study was to investigate the effect of BjcuL on human peripheral blood mononuclear cells (PBMCs) activation with a focus on PBMCs proliferation and inflammatory mediators release. Results showed that BjcuL is not toxic to PBMCs, that BjcuL inhibits PBMCs proliferation and that it stimulates PBMCs to produce superoxide anion and hydrogen peroxide, primarily via lymphocyte stimulation, but does not stimulate the production of nitric oxide and PGE_2. These results demonstrate that BjcuL has an immunomodulatory effect on PBMCs. Further studies are needed to confirm the immunomodulatory effect of BjcuL, to elucidate the molecular mechanisms of action responsible for its effects and to determine its potential application as an immunopharmacological and biotechnological tool.

1. Introduction

Bothrops jararacussu is one of Brazil's most dreaded snakes. This important pit-viper may grow to a length of 2.2 m and its venom glands can deliver up to 1000 mg (dry weight) of highly-lethal venom in a single bite. It is responsible for 0.8% to 10% of serious snakebites in São Paulo State, Brazil (Milani Júnior et al., 1997). *Bothrops jararacussu* venom is composed primarily of enzymatic proteins such as phospholipases A_2 (PLA_2), serine and metalloproteases, and non-enzymatic lectin proteins.

Snake venom lectins comprise a class of toxins that bind, non-covalently and with high specificity, to terminal galactoside residues of glycans. The recognition of specific glycans by lectins is an event that plays a key role in a variety of biological phenomena (Kishore et al., 1997). The lectin BjcuL was isolated from *Bothrops jararacussu* venom. This lectin is a C-type lectin with specificity for the binding of β-D-galactose units (de Carvalho et al., 2002).

BjcuL has been shown to bind to lactose moieties and induce agglutination of erythrocytes. The literature shows that BjcuL weakly adheres to MDAMB-435, a human metastatic breast cancer cell line, and to OVCAR-5, a human ovarian carcinoma cell line (de Carvalho et al., 2001). Moreover, BjcuL inhibits growth in several cancer cell lines (Damasio et al., 2014; de Carvalho et al., 2001; Nolte et al., 2012; Pereira-Bittencourt et al., 1999). However, BjcuL does not inhibit adhesion of these cancer cells to the extracellular matrix proteins fibronectin, laminin and type I collagen (de Carvalho et al., 2001).

BjcuL triggers cellular infiltration in post capillary venules (Elifio-Esposito et al., 2007), increases edema and vascular permeability in murine models (Panunto et al., 2006) and contributes to in vitro neutrophil activation (Elifio-Esposito et al., 2011). The literature on the inflammatory effects of BjcuL indicates that BjcuL modulates macrophage functional activation towards an M1 state (Dias-Netipanvi et al., 2016); the aim of this study was to investigate the effect of BjcuL on human PBMCs activation with a focus on PBMCs proliferation and inflammatory mediators release.

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2. Material and methods

2.1. Chemicals and reagents

MTT, RPMI-1640, L-glutamine, gentamicin, lipopolysaccharide (LPS), concanavalin A (Con-A), phorbol myristate acetate (PMA), Histopaque 1077, DMSO, OPD (o-1,2-phenylenediamine dihydrochloride), horseradish peroxidase, nitroblue tetrazolium (NBT), carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and anti-iNOS antibody were purchased from Sigma (MO, USA). Cell Viability Kit, PE anti-human CD14, PE anti-human CD4, PE anti-human CD8 and PerCP anti-human CD3 were purchased from BD Pharmingen (CA, USA). 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Molecular Probes (OR, USA). PGE_2 specific enzymatic immunoassay (EIA), anti-COX-1 and anti-COX-2 antibodies were purchased from Cayman Chemicals (MI, USA). Fetal bovine serum (FBS) was obtained from Cultilab (São Paulo, Brazil). All salts and reagents were obtained from Merck (Darmstadt, Germany).

2.2. Ethical statement

This study was approved by the Brazilian Institutional Review Board of the Centre for Research in Tropical Medicine (CEPEM-RO), under protocol numbered 067/08. All subjects were adult volunteers and all signed a Statement of Informed Consent.

2.3. Venom

The *Bothrops jararacussu* snake venom was provided by Serpentário Proteínas Bioativas (Batatais – SP) and maintained refrigerated (8 °C) in the Bank of Amazon Venoms at the Center of Biomolecular Studies Applied to Health, CEBIO-UNIR-FIOCRUZ-RO, (authorization: CGEN/CNPq 010627/2011-1 and IBAMA 27131-1).

2.4. Affinity column preparation

Initially, 20 mL of Sepharose 6B resin was transferred to a Buchner funnel and washed with 30 mL of distilled water. After the complete elimination of water, the resin was suspended in 15 mL of NaOH 1 M containing 30 mg of sodium borohydrate. The contents were transferred to a plastic beaker and 5 mL of bisepoxi was added. The activation was processed for 5 h at room temperature under gentle agitation. The activated resin was transferred to a Buchner funnel and washed with 300 mL of distilled water. Lactose coupling proceeded for 24 h at room temperature in bicarbonate buffer 0.5 M (pH 10.0), using an equal volume of ligand solution and activated resin. After lactose coupling, the remaining epoxi groups were blocked with β-mercaptoethanol for 2 h at room temperature. The coupled resin was washed with 200 mL of sodium bicarbonate 0.5 M, 50 mL of sodium acetate 0.2 M (pH 4.0), and 50 mL of saline, respectively. The coupled resin (Sepharose 6B-CL-lactose) was maintained at 4 °C until use.

2.5. Purification of a lectin from Bothrops jararacussu venom

Bothrops jararacussu venom (100 mg) was dissolved in CaTBS buffer (0.02 M Tris, 0.15 M NaCl and 0.01 $CaCl_2$) (pH 7.4), and clarified by ultracentrifugation at 3500 ×*g* for 5 min. The supernatant was applied and fractionated on a column (3 cm × 1 cm) of Sepharose 6B-CL-lactose which was pre-equilibrated with the same buffer used to dissolve the venom. The resin was washed until the optical density measured 280 nm returned to basal levels. The retained material was eluted using 0.1 M lactose in CaTBS. The resin fraction was filtered using Sephadex G-25 to remove the lactose. The purity of the eluted protein was evaluated with RP-HPLC using a C18 column (Discovery 0.45 cm × 25 cm; Supelco – USA) under a linear gradient 0–60% of acetonitrile in 0.1% trifluoroacetic acid (*v*/v). All chromatographic steps were done using an Akta Purifier 10 system (GE Lifescience Healthcare – USA). SDS-PAGE 12.5%, performed as described by Laemmli (1970), confirmed the apparent weight and molecular homogeneity of the resin fraction. The protein concentration was estimated using Bradford reagent with a solution of BSA as the protein standard.

2.6. Molecular mass determination

Molecular mass was determined by mass spectrometry performed with MALDI equipment (*matrix-assisted laser desorption ionization*) using TOF analyzers (AXIMA TOF^2 Shimadzu Biotech) and a saturated solution of sinapinic acid as the ionization matrix. The sample was co-crystallized with the ionization matrix (1:3 ratio, respectively) and analyzed in linear mode.

2.7. Hemagglutinanting activity

Hemagglutinanting activity was performed using a microagglut ination technique to determine the presence of BjcuL. This technique used human erythrocytes previously collected with heparin (50 U/mL), and microtiter V-well plates. Each well contained 50 μL of 3% erythrocytes suspension and CaTBS with 50 μL samples in the same buffer. The negative control contained 50 μL of cell suspension and 50 μL of CaTBS. Following the addition of erythrocytes, the plates were shaken briefly and incubated at room temperature. The agglutination was monitored visually after 2 h.

2.8. Isolation of human peripheral blood mononuclear cells (PBMC)

Blood was donated by healthy volunteers who had not used medication in the last 48 h. Blood was collected in vacuum tubes containing heparin, and diluted in phosphate buffered saline (PBS - 14 mM NaCl, 2 mM $NaH_2PO_4 \cdot H_2O$, 7 mM $Na_2HPO_4 \cdot 12H_2O$) (pH 7.4). PBMCs were isolated by density gradient method—according to Lacouth-Silva et al. (2015)—using Histopaque 10,771 (Sigma; MO, USA), as per the manufacturer's instructions. Briefly, the blood diluted in PBS was layered on Histopaque at a 1:1 ratio and subjected to centrifugation at 400 ×*g* for 30 min. The white layer representing PBMCs was gently aspirated out and aseptically transferred into sterile centrifuge tubes. After centrifugation, the cell suspension containing the PBMCs was washed 3 times with PBS and cultured in sterile RPMI assay medium [RPMI-1640 medium supplemented with 100 μg/mL of gentamicin, 2 mM of L-glutamine and 10% of fetal bovine serum (FBS)]. Aliquots of the isolated PBMCs were used to determine the total number of cells in a Neubauer's chamber following cell staining (1:20, v/v) with Turk solution (0.2% violet crystal in 30% acetic acid). The purity of the isolated cell populations was determined by Panotic staining of cytospin preparations and by flow cytometry analysis (FACscan). The mean purity was 99% for PBMC preparation. The number of cells was adjusted to 10^5 or 10^6, relative to the amount of cells necessary for each experiment.

2.9. Cell viability assay by MTT reduction

The cytotoxicity of BjcuL on PBMCs was determined by an MTT assay (Reilly et al., 1998; Lacouth-Silva et al., 2015), which made it possible to analyze the capacity of living cells to reduce the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma; MO, USA) to a purple formazan product. Cells were plated in 96-well plates (2×10^5/well) and maintained in RPMI assay medium. PBMCs were incubated with RPMI (negative control) or BjcuL (5 and 10 μg/mL diluted in RPMI; experimental group) for 96 h at 37 °C, under an atmosphere of 5% CO_2. After incubation, the plate was centrifuged at 400 ×*g* for 5 min and the supernatant was replaced by fresh RPMI containing MTT (0.5 μg/mL). Four hours later, the plates were washed three times with PBS. The formazan crystals were dissolved in 100 μL DMSO over 90 min and were evaluated by spectrophotometer at 540 nm (Bio-Tek