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Insights into anti-parasitism induced by a C-type lectin from *Bothrops pauloensis* venom on *Toxoplasma gondii*

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ABSTRACT

Here we evaluate the effects of BpLec, a C-type lectin isolated from *Bothrops pauloensis* snake venom, on *Toxoplasma gondii* parasitism. BpLec (0.195–12.5 μg/mL) did not interfere with HeLa (host cell) viability by MTT assay, whereas higher doses decreased viability and changed HeLa morphology. In addition, the host cell treatment before infection did not influence adhesion and proliferation indexes. BpLec did not alter *T. gondii* tachyzoite viability, as carried out by trypan blue exclusion, but decreased both adhesion and parasite replication, when tachyzoites were treated before infection. Galactose (0.4 M) inhibited the BpLec effect on adhesion assays, suggesting that BpLec probably recognize some glycoconjugate from *T. gondii* membrane. Additionally, we performed cytokine measurements from supernatants collected from HeLa cells infected with *T. gondii* tachyzoites previously treated with RPMI or BpLec. MIF and IL-6 productions by HeLa cells were increased by BpLec treatment. Also, TGF-β1 secretion was diminished post-infection, although this effect was not dependent on BpLec treatment. Taken together, our results show that BpLec is capable of reducing *T. gondii* parasitism after tachyzoite treatment and may represent an interesting tool in the search for parasite antigens involved in these processes.

1. Introduction

Toxoplasmosis presents a high infection rate of 1/3 of the seropositive population worldwide, associated with death in many cases [1]. In Brazil, seroprevalences up to 84% have been reported in children, while pregnant women (between 36% and 92%) may be seropositive as well, depending on the Brazilian region studied. These values indicate Brazil as one of the countries with the highest seroconversion rates for toxoplasmosis [2]. It has also been stated that the pathogenesis is more severe in Brazilian children than in Europeans [2,3]. Other findings demonstrate a high infection rate in human and animal populations from Minas Gerais State, in Southeast Brazil, emphasizing the importance of investigating this disease in the country [4].

The development of new drugs for toxoplasmosis treatment is fundamental considering that its most common therapy, a combination of sulfadiazine and pyrimethamine, displays many side effects [5]. In this way, new alternatives for toxoplasmosis treatment should be of keen interest. Many studies have demonstrated the use of snake venom toxins in biotechnological applications such as disease therapy, more specifically for hypertension, thrombosis, cancer and antiparasitism, among others [6–9]. Furthermore, snake venom toxins have been designated as structural models for targeting commercial drugs [10,11].

Lectins are non-enzymatic proteins known for recognizing and specifically binding carbohydrates in a non-covalent, but reversible manner [12]. They are present in different organisms besides animals, from microorganisms to plants [13,14]. In snake venoms, these proteins are divided into C-type lectins and C-type lectin-like [15]. C-type lectins are carbohydrate-binding homodimers, usually galactose-binding, which present a calcium-ligand loop that is crucial for their biological activity [16]. As a consequence of its property of binding to cell membrane carbohydrates, lectins from different

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organisms can be useful as glycoconjugate markers in many cell types [17–19].

Toxoplasma gondii virulence is influenced by many glycoproteins from its micronemes, rhoptries and dense granules [20]. Despite glycosylation being thought to be rare in *T. gondii*, glycoproteins are relatively abundant in RH strain tachyzoites and may participate in disease progression [18]. Many of these glycoproteins are involved in parasite attachment to its host cell, as well as in the invasion process, and were identified by techniques employing the association with lectins, such as Concanavalin A (ConA), wheat germ agglutinin (WGA) and jacalin. Proteins from tachyzoite glideosome were also recognized by ConA, suggesting that treatment with the isolated lectin would interfere with parasite functions [17,18]. Interestingly, a lectin from *Dolichos biflorus* (DBA) specific for N-acetylgalactosamine has been used to identify *T. gondii* bradyzoite differentiation and for quantifying cysts even at low concentrations [5,21,22].

In addition, proteomic analyses with lectin association are also employed not only for understanding the mechanisms of parasite infection, but also for detecting new drug targets in the parasite membrane [23]. Several studies use lectin labeling to detect glycoproteins existent in *T. gondii* tachyzoites and bradyzoites, but experiments demonstrating these lectins' effects on parasite functions remain to be unraveled [17,18].

In this regard, we have investigated the antiparasite effects of BpLec, a C-type lectin isolated from *Bothrops pauloensis* snake venom [24]. This protein is capable of inhibiting Gram-positive bacteria growth and agglutinating cat and dog erythrocytes and *Leishmania* (*Leishmania*) *amazonensis* promastigotes as well. Given its previously demonstrated biological activities, in the present work we demonstrate the effects of BpLec upon *T. gondii* tachyzoite adhesion and proliferation.

2. Materials and methods

2.1. BpLec purification

The C-type lectin named BpLec was purified from *B. pauloensis* snake venom, as described by [24]. Briefly, crude dried venom from *B. pauloensis* was dissolved in Tris saline buffer containing calcium chloride (CTBS: 150 mM NaC1, 20 mM Tris, 5 mM $CaC1_2$, pH 7.4) and centrifuged twice at 2450 × *g* during 10 min each, at 4 °C, for excluding insoluble material. The sample was applied to an affinity agarose column immobilized with D-galactose (0.5 cm × 4 cm, Pierce, USA) and incubated for approximately 1 h. The column, which was previously equilibrated with CTBS, was initially eluted with the same buffer, and later 0.4 M D-galactose was added to the solution for lectin obtention. Subsequently, in order to removing the carbohydrate, BpLec was subjected to an exclusion chromatography on two coupled desalting G-25 columns (1.5 × 2.5 each, GE HealthCare, Sweden), equilibrated and eluted with 0.1 ammonium bicarbonate, at a flow rate of 0.5 mL/min, collecting 500 μL in each tube.

2.2. Cell culture

The HeLa cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in 75 cm^2 flasks with RPMI-1640 medium (Gibco, Paisley, UK), supplemented with 25 mM HEPES, 23 mM sodium carbonate, penicillin (100 U/mL), streptomycin (100 μg/mL) (Sigma Chemical Co., St Louis, MO, USA) and 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil) in a humidified incubator at 37 °C and 5% CO_2 [25]. Tachyzoites of the *T. gondii* RH strain were initially obtained from peritoneal exudates of previously infected Swiss mice [26] and maintained by serial passages in HeLa cells cultured in the same medium with 2% heat-inactivated fetal bovine serum, in order to obtain *in vitro* parasites [25]. In this regard, parasites were infected in Hela, where they replicated and evade to the medium. The medium containing evaded tachyzoites was centrifuged and parasites were obtained from the pellet for further experiments.

2.3. HeLa viability

The cytotoxic effects of BpLec upon HeLa were assayed based on mitochondrial oxidation of the MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (Sigma), according to [27], with some modifications. HeLa (3×10^4 cells/mL) was plated on a 96-well culture plate and BpLec (0.195–100 μg/mL) was incubated with the cells in serial twofold dilutions in the next day. After 24 h of incubation in a humidified incubator (37 °C and 5% CO_2), the supernatants were discarded and 10 μL of MTT reagent (0.5 mg/mL) were added to each well and maintained in the humidified incubator during 3 h. Afterward, formazan crystals were solubilized by 10% SDS in 50% dimethylformamide and the absorbance measured at 570 nm after 30 min. This assay was performed in triplicate.

2.4. RH strain viability

T. gondii (4×10^6 tachyzoites/mL) was treated with BpLec (2.5 μg/mL, 5.0 μg/mL and 10 μg/mL) during 30 min and dyed by trypan blue. Viable cells, which present a clear cytoplasm and exclude trypan blue staining, were counted in an optical microscope [28]. RPMI-treated tachyzoites were considered as a control. The assay was performed in triplicate.

2.5. Adhesion

In this assay, two treatment approaches were carried out separately: BpLec (3.125 μg/mL, 6.25 μg/mL and 10.0 μg/mL) was incubated with HeLa during either 1 or 24 h and non-treated parasites were added later. The other approach was based on incubating tachyzoites with BpLec (2.5 μg/mL, 5.0 μg/mL and 10 μg/mL) during 30 min before infection, without treating HeLa cells. Each assay was implemented using one of these treatments exclusively. Also, for testifying BpLec specificity, all BpLec doses from tachyzoite treatment were inhibited with 0.4 M galactose during 30 min. As a control, tachyzoites or HeLa were also treated with only RPMI medium. Briefly, as suggested by [29], HeLa (2×10^4 cells/mL) was plated in a 24-well culture plate containing 13 mm cover slips in each well. In the following day, cells were fixed with 8% paraformaldehyde/4% PBS during 30 min and, after extensive washing in PBS for removing this solution, *T. gondii* tachyzoites (1×10^5 cells/mL) were added to each well. After 3 h of interaction, the parasites were also fixed overnight, after an extensively washing to discard the excess of *T. gondii* that did not adhere to HeLa. In the next day, the cover slips were stained with toluidine blue. On each slip, 200 cells were counted in the light microscope and the following parameters were analyzed: the number of cells with adhered parasites and the total number of parasites adhered to those cells. Three independent experiments in triplicate were accomplished for each treatment approach.

2.6. Proliferation

Both parasite and HeLa treatments were also performed separately, as described above. The assay was based on the β-galactosidase reaction, according to [30], with some modifications. HeLa (2×10^4 cells/mL) was plated on a 96-well culture plate and incubated or not with BpLec (3.125 μg/mL, 6.25 μg/mL and