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Leishmania chagasi heparin-binding protein: Cell localization and participation in *L. chagasi* infection

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ABSTRACT

Visceral leishmaniasis is a fatal human disease caused by the intracellular protozoan parasite *Leishmania chagasi* that is captured by host cells in a process involving classics receptors mediated phagocytosis. The search for molecules involved in this process is important to design strategies to disease control. In this work, we verified the presence of heparin-binding protein (HBP) in *L. chagasi* promastigotes forms. HBP is a lectin of the group of ubiquitous proteins, whose main characteristic is to bind to carbohydrates present in glycoproteins or glycolipids, which is poorly studied in *Leishmania* species. *L. chagasi* HBP (HBP*Lc*) was purified by affinity chromatography using heparin–agarose column in FPLC automated system. Its localization in the parasite was assessed by immunolabeling and electronic transmission microscopy tests using anti-HBP*Lc* polyclonal antibodies, which showed HBP spread over the parasite outer surface and internally next to the kynetoplast. In addition, we verified that HBP*Lc* participates in the process of parasite infection, since its blocking with heparin generated a partial reduction in the internalization of *Leishmania* by RAW macrophages "in vitro". According to these results, it is believed that, in further "in vivo" studies, interference on this parasitic protein may provide us prophylactic and therapeutic alternatives against visceral leishmaniasis.

1. Introduction

Neglected tropical diseases refer to infectious diseases that occur in tropical regions of the world, affecting the world's poorest people [1]. Among these diseases is leishmaniasis, an infectious disease caused by the protozoa *Leishmania* sp., which is an obligate intracellular parasite that infects humans and other mammalian species. This disease can cause a wide spectrum of clinical presentations [2] and is responsible worldwide for the second highest number of deaths from parasitic infections [3]. Visceral leishmaniasis (VL) or kala-azar is the most severe clinical form of leishmaniasis, and is responsible for the higher incidence of deaths associated with the disease, hence representing a serious public health problem. It is clinically characterized by chronic low-grade fever, weight loss, hepatosplenomegaly, and laboratory findings which included pancytopenia, low albumin levels, and hypergammaglobulinemia; these symptoms render it fatal if not properly treated [4,5]. VL ranks second in mortality and fourth in morbidity among tropical diseases, with a mortality rate of approx. 20,000–40,000 deaths per year—reviewed in [6]. The treatment of VL is generally limited to the use of pentavalent antimonials as first-line drugs and pentamidine or amphotericin B as second-line drugs. However, an increased resistance to the first-line drugs as well as several side effects and high toxicity of the second-line drugs have been detected [7,8]. Thus, there is a need for the development of new drugs and alternative strategies to block, control, and prevent the disease [9]. Therapeutics that can disrupt certain mechanisms important for the virulence of the microorganism also becomes a potential target, aiming to control the established infection. In leishmaniasis, the characterization of parasite-specific molecules that contribute to their virulence mechanisms (called virulence factors), such as antigenic potential, is the subject of several research groups. These molecules include the *Leishmania* homolog of receptors for activated C-kinase (LACK) [10,11], the lipophosphoglycan (LPG), the

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gp63 glycoprotein [12–15], the cistein-peptidases [16], enzymes that participate in the catabolism of extracellular ATP [17], and proteinases, enzymes that hydrolyze peptide bonds and thus have the potential to degrade proteins and peptides that participate in a broad range of biological functions, including the infection process [18].

During the blood meal, the insect vector deposits metacyclic promastigotes in the skin of the mammalian host. These promastigotes are infective forms of *Leishmania* that interact with macrophages and dendritic cells, in a process mediated by classic receptors that initiate phagocytosis [18]. The intervention in the adhesion and penetration processes is important to prevent the infection. Some surface molecules of both the parasites and macrophages are responsible for this interaction: complement receptors (CR) such as CR 1 and CR3 (Mac-1); the fibronectin receptors in promastigotes; and the fucose–mannose receptors (FMRs) on the surface of macrophages play important roles in the binding of promastigotes to the host cells; binding to multiple receptors is required for parasite uptake or to trigger a protective immune response [19,20]. Other parasitic molecules potentially involved in infectious diseases are the lectins, a group of ubiquitous proteins whose main feature is to bind to soluble carbohydrates present in glycolipids or glycoproteins. This feature has aroused interest on the possible role of lectins in the access of pathogenic microorganisms to mammalian cells. Previous several studies demonstrate the involvement of host cell lectins in the recognition of carbohydrates present on the surface of different pathogenic species [21,22], such as parasites of the genus *Leishmania* [23,24]. The results of these studies prove that the modulation of the host immune response is due to the activation of signaling pathways which is triggered by the stimulation of surface lectins of the immune system cells by the carbohydrates present in pathogens. Interestingly, in some cases, lectins are involved in therapeutic purposes, including inhibition of HIV infection [25]. Parasitic lectins are also involved in infectious diseases caused by protozoan parasites that cause important diseases in humans: heparin-binding proteins (HBP) present on the surface of infectious forms of *Trypanosoma cruzi* are involved in the adhesion of amastigotes and epimastigotes forms to the host cells and intestinal epithelium of triatomines, respectively [26–28]; galactose and *N*-acetyl-galactosamines-binding lectins present in the surface of *Entamoeba histolytica* are important virulence factors for this protozoan parasite, which is responsible for the third highest number of deaths caused by parasitic diseases in the world, after malaria and schistosomiasis [29–31]. Several aforementioned studies on vertebrate hosts, plants or microorganisms have been conducted, which consider the biological effects of lectins on infectious agents [25,32,33]. However, the number of studies that involve profiling of the expression of surface lectins in *Leishmania* parasites which mainly consider the participation of these molecules in cellular adhesion or their influence over the mammalian host immunological response is less. Nevertheless, certain studies based on the investigation of the presence of HBP in *Leishmania braziliensis* promastigotes provide evidences of the expression of these molecules, thus, suggesting the participation of surface lectins in the interaction of the parasite with intestinal cells in *Lutzomyia* species as well as in the life cycle of the parasite [34–36].

In this study, we aimed to detect, purify, and determinate the localization of HBP in *Leishmania chagasi* promastigotes (HBP*Lc*), describing by the first time the molecular mass profile of this protein. Additionally, we investigated the participation of this lectin in the adhesion and internalization of *L. chagasi* promastigotes in mammalian cells. Once HBP*Lc* is identified and characterized, the next step may be directed to the study of the effects of blocking of protein on the development of infectious diseases, giving us perspectives to provide new strategies to control infection by *L. chagasi*.

2. Materials and methods

2.1. Ethics statement

C57BL/6 mice (4–8 weeks old) were obtained from the University's animal facility (Biotério Central—UFV, Viçosa, Brazil). Animals were given water and food "ad libidum". Animal experimentation was done respecting ethical principles of the Code of Professional Veterinarian, according to the opinion of the Ethics Committee for Animal Use (approved by CEUA/UFV – Research project – process number: 22/2011), based in the actual Brazilian Legislation (Law no. 11.794, October 08, 2008), in the Normative Resolutions edited by CONCEA/MCTI, as in the "Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos" (DBCA), following the orientations to the practices of euthanasia commended by CONCEA/MCTI.

2.2. Parasites

L. chagasi promastigotes, M2682 strain (MHOM/BR/75/M2682), were cultured in Grace's insect medium (GIBCO BRL, Grand Island, N.Y., USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; LGC Biotecnologia, Cotia, SP, Brazil), 2 mM L-glutamine (GIBCO BRL) and 100 U/ml penicillin G potassium (USB Corporation, Cleveland, OH, USA), pH 6.5, at 26 °C. The cultures were started with cell density of 10^5 cells/ml. On the fifth day of culture, the material was centrifuged at 1540 × *g*/4 °C/10 min, the parasites were washed two times with phosphate buffered saline (PBS), pH7.2, and the pellet was stored frozen at −20 °C.

2.3. L. chagasi *promastigotes protein extract preparation*

Parasitic pellets were thawed and pooled. Amount corresponding to 1.5×10^{11} parasites were resuspended in 10 ml of 50 mM sodium phosphate/150 mM sodium chloride buffer, pH 7.0, and disrupted by sonication (ultrasonic Q) at a frequency of 6 KHz, performing six cycles of 15 s with 1 min intervals in ice cold. All resulting material was centrifuged at 7000 × *g*/4 °C/20 min and the supernatant of soluble protein was recovered, filtered through a 0.45 μM pore diameter membrane, and stored at 4 °C.

2.4. Purification of HBPLc

The soluble protein extract of *L. chagasi* was subjected to affinity chromatography on heparin column, using 50 mM sodium phosphate/150 mM sodium chloride buffer, pH 7.0 to equilibrate the column and to elute non-adsorbed fraction. The adsorbed fraction was eluted with the same buffer plus NaCl 2 M. Purification was performed on automated Fast Protein Liquid Chromatography (FPLC) system (Akta Purified, GE®) at a flow rate of 1 ml/min and monitored by absorbance reading at 280 nm. The fractions of interest were collected, pooled and subjected to size exclusion chromatography technique in a *D-salting* column (1 ml bed volume). The buffer used for elution was 50 mM sodium phosphate/150 mM sodium chloride, pH 7.0. The new samples were collected and submitted to protein dosage. Samples were aliquoted to be stored at −20 °C, subjected to freeze drying or submitted directly to the immunolocalization tests.

2.5. Electrophoretic analysis of HBPLc

Electrophoretic analysis was carried out on polyacrylamide gel under dissociating conditions (SDS-PAGE) performed in "Mini Ver-

tical 10.8 V-Gel Electrophoresis System" (GIBCO BRL). The amount of 30 μg of protein samples was suspended in sample buffer (2.5% SDS and 50% glycerol in Tris-HCl 0.5 M, pH 6.5) and applied to 12% polyacrylamide gel. The electrophoretic run was performed in 50 min at 80–120 mA and 190 V. Proteins in the gels were detected using silver staining.

2.6. Production of polyclonal anti-HBPLc antibodies

The amount of 200 μg of purified protein was diluted in PBS buffer (500 μL final volume), and was added to 500 μL of 15 mg/ml alum adjuvant. Four C57BL/6 mice were immunized intraperitoneally with 40 μg of purified protein plus 7.5 mg of alum/animal. The animals were submitted to booster immunizations at 15 and 30 days after the first inoculation, consisted of 20 μg of purified protein/animal plus the same amount of alum adjuvant. One week after the last booster, the animals were euthanized by cervical dislocation for collection of blood by cardiac puncture. After clot formation, collected blood was centrifuged at 520 × g/25 °C/10 min in order to separate immune serum. A pool of sera of the animals was obtained, incubated in water bath at 57 °C for 30 min and subsequently stored frozen at −20 °C. Mice that were not immunized (PBS treated) were used as experimental control. The same protocol for blood collection and serum obtainment was used as described for the immunized animals.

2.7. IgG purification

IgG from serum samples were purified by affinity chromatography using G Protein Sepharose column (3 ml bed volume), which was previously equilibrated with 20 mM sodium phosphate buffer, pH 7.0. Non-adsorbed material was removed with the same buffer, while adsorbed material was eluted with 100 mM glycine-HCl buffer, pH 2.7, which was collected in 1 ml aliquots in micro centrifuge tubes containing 70 μL of 1 M Tris–HCl buffer, pH 9.0. All fractions were submitted to protein dosage using Bradford assay kit (Bio Agency—#500-0006N). Samples of interest were gathered in pool.

2.8. Immunoblotting

Standardized samples (30 μg of purified protein) were submitted to SDS-PAGE as described above. Proteins in the gel were transferred to a nitrocellulose membrane. The blotting run was performed at 200 mA/2 h. Then, the nitrocellulose membrane was soaked in a blocking solution (PBS with 5% milk powder) and left under stirring for 30 min. After this period, the solution was removed and the membrane was submitted to 3 washes with PBS and it was incubated with primary anti-HBP*Lc* IgG antibody (48 μg/ml) for 1 h. The membrane was washed 3× with PBS and incubated with secondary rabbit anti-mouse IgG antibody conjugated with alkaline phosphatase (SIGMA, St. Louis, MO, USA) for 30 min according to the manufacturer's recommended dilution (1:5000). The membrane was washed with PBS and stained with developer BCIP®/NBT—Liquid Substrate System (SIGMA).

2.9. Fluorescence microscopy

Parasites were cultivated as described above and quantified, washed twice in PBS and settled onto glass slides containing 0.1% poly-L-lysine. After one wash with PBS, they were directly fixed for 10 min at room temperature with PBS containing 4% paraformaldehyde and then blocked in PBS plus 2% BSA. The experiment was designed showing a group that was submitted to plasmatic membrane permeabilization treatment and another group without this treatment. The permeabilization was made by addition of 200 μl of 0.3% triton X in PBS by 10 min, followed by the washing of the wells twice with cold PBS. The samples were incubated with a purified polyclonal antibody against PLH*Lc* at concentration of 240 μg/ml, diluted 1:10 in PBS plus 2% BSA, for 1 h at room temperature. The slides were washed in blocking solution and subsequently incubated for 30 min at 37 °C with FITC labeled rabbit anti-mouse Fc IgG secondary antibody (SIGMA) at a dilution of 1:400. The cells were further incubated with 0.6 μg/ml 4,6-diamidino-2-phenylindole (DAPI), for nuclei and kinetoplast staining. All the samples were washed twice with PBS, mounted on glass slides with 0.1 M *N*-propyl gallate to reduce fading, and analysed by fluorescence microscope Olympus BX60.

2.10. Electronic transmission microscopy

For transmission electron microscopy analysis, promastigotes were fixed in 4% paraformaldehyde, 1% glutaraldehyde, 5 mM calcium chloride, and 3.7% sucrose in a 100 mM sodium cacodylate buffer (pH 7.2). The samples were gradually dehydrated in alcohol at low temperatures, infiltrated, and finally embedded in LR White resin at 60 °C. Ultrathin sections were collected on nickel grids of 300 mesh and incubated for 20 min at room temperature in 50 mM ammonium chloride in PBS at pH 7.2. Next, the sections were incubated in PBS at pH 8.0 containing 1.5% albumin and 0.01% Tween 20 for 20 min at room temperature and then overnight in the presence of purified anti-HBP*Lc* antibodies (1:100). Control test was performed using purified antibodies from the serum of non-immunized animals. The grids were washed with PBS and finally incubated with a 1:30 dilution of a secondary 10 nm gold-conjugated goat anti-rabbit IgG for 60 min. The ultrathin sections were contrasted with solutions of 3% uranyl acetate and 0.2% lead citrate. All of the samples were observed and photographed in a transmission electron microscope (Zeiss EM 109) at the Núcleo de Microscopia e Microanálise at Universidade Federal de Viçosa, Minas Gerais, Brazil.

2.11. Adhesion and internalization assays

RAW 264.7 macrophages was acquired from LIMA—Laboratory of Animal Molecular Infectology/UFV and maintained in RPMI-1640 culture medium (HiMedia Laboratories, Swastik Disha Business Park, Mumbai, India), plus 1% penicillin and 10% FBS at 37 °C/5% CO_2. For adhesion and penetration assays of *Leishmania* into macrophages, 500 μL of a suspension containing 2.5×10^5 RAW macrophages in RPMI (HiMedia Laboratories), 10% FBS, pH 7.2, were added to each well of a 24-well tissue culture plate covered with circular glass coverslips (Ø 13 mm). The plate was incubated overnight at 37 °C/5% CO_2 for adhesion of macrophages to the coverslips. After this period, the supernatant was removed and the wells were washed twice with PBS pH 7.2 to remove non-adherent macrophages. The test of parasitic HBP*Lc* treatment with anti-HBP*Lc* antibodies was performed by macrophage infection with 5×10^6 *L. chagasi* promastigotes forms in 1 ml of RPMI (HiMedia Laboratories), 10% FBS, pH 7.2. Parasites were pretreated with anti-HBP*Lc* antibodies (30 min at 25 °C) in different concentrations (5 μg/ml, 10 μg/ml or 50 μg/ml) or inespecific IgG antibodies (non-immune serum IgG—50 μg/ml). As control group, the same amount of non-treated parasites was used. For adhesion or penetration assays, macrophages were incubated in the presence of parasites by 30 min or 3 h, respectively, at 37 °C/5% CO_2. After incubation, coverslips were washed with PBS pH 7.2 and removed. Adhered macrophages were fixed in methanol and stained using Panoptic staining kit (Renylab, Barbacena, MG, Brazil) following the manufacturer's protocol. In parallel, a blockage test was made using heparin pretreated parasites prior to the macrophages infection. Heparin was used at concentrations of 0.2 UI/ml, 1 UI/ml or 2 UI/ml

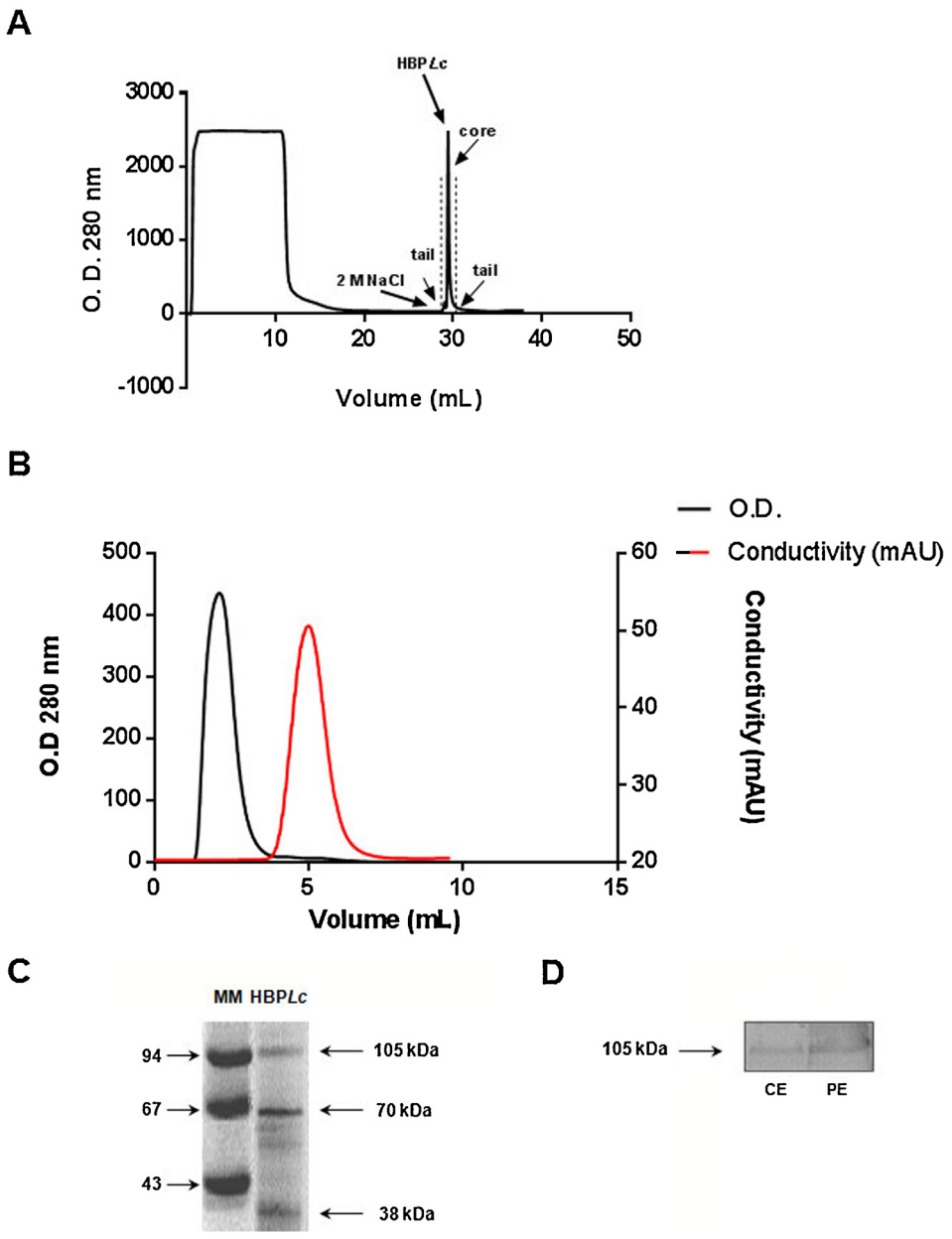

Fig. 1. Purification of HBP*Lc*. (A) Affinity chromatography on heparin column. Crude extract of *L. chagasi* promastigotes was subjected to FPLC as described in Section 2. (B) Size exclusion chromatography in D-salting column. Fractions corresponding to the tails an core from FPLC were pooled and eluted in constant flux 50 mM sodium phosphate/150 mM sodium chloride, pH 7.0 as equilibration buffer. O.D. at 280 nm and conductivity and were measured and plotted. (C) SDS-PAGE of purified HBP*Lc* in non-reducing conditions. 30 μg of HBP*Lc* was submitted to SDS-PAGE, which was subjected to silver staining, as described in the methodology. The arrows indicate the position of the protein of interest. MM: Molecular Mass standard. (D) Western blotting (WB) of protein extract of *L. chagasi* promastigotes. Crude extract of *L. chagasi* promastigotes submitted to SDS-PAGE was submitted to immunoblotting using anti-HBP*Lc*, as described in the methodology. Crude extract (CE); Purified Extract (PE).

and the protocols of pretreatment and infection were the same employed in anti-HBP*Lc* treatment assay, using untreated parasites in control group. Both tests were performed in duplicate. The results were represented as the mean of the relative index between each group and the data from the control group with untreated parasites. The index was calculated from data related to the percentage of macrophages with attached or internalized parasites or to the number of *Leishmania* per macrophage with attached or internalized parasites.

2.12. Statistical analysis

Statistical analysis was performed by column statistics analysis, considering $p < 0.05$ as statistically significant. All comparisons were performed using the GraphPad Prism 5.01® statistical software (GraphPad Software, Inc., San Diego, CA, USA). Bars and symbols represent mean + 1 SEM from two or three separate experiments.

3. Results

Proteins with lectin activity were purified from *L. chagasi* by HPLC using a heparin column, as described in the methodology. Three distinct fractions were collected: the "core" of the diluted sample (having the highest concentration) and the other two, called the "tails" of the sample. The pool of these eluates was termed HBP*Lc* (Fig. 1A).

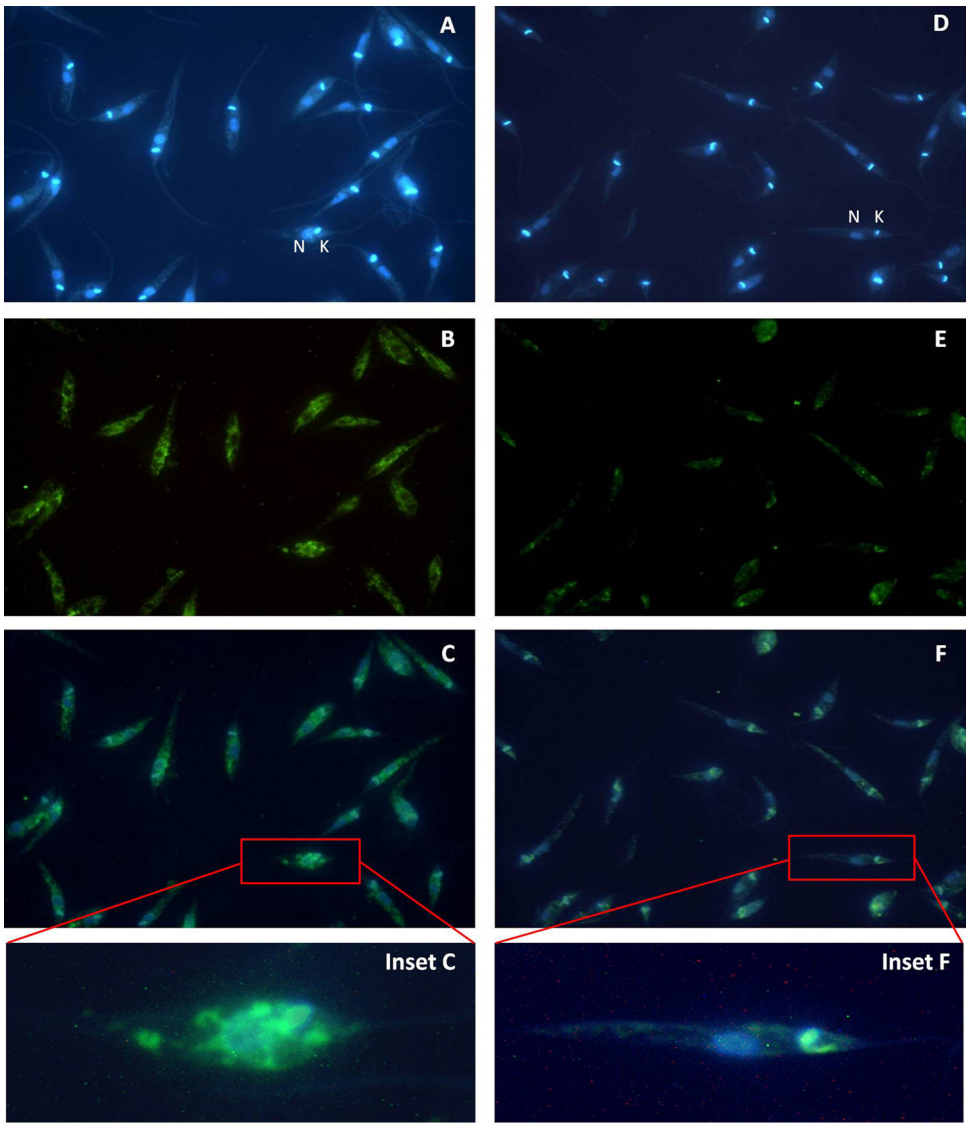

Fig. 2. Immunolabeling of HBP*Lc*. *L. chagasi* promastigotes were labeled with FITC and DAPI (0.6 μg/ml), as described in the methodology. Left column: unpermeabilized parasites; Right column: permeabilized parasites. (A and D) DAPI stained DNA. (B and E) FITC stained HBP*Lc* (48 μg/ml). (C and F) DAPI and FITC stained parasites – overlapped Images – X 1000 (insets C and F, respectively) Parasites detached and amplified from figures C and F. N = nucleus; K = kinetoplast.

To remove the NaCl used in the elution process, the samples were subjected to size exclusion chromatography in a desalting column. The differentiation of two peaks corresponding to optical density/OD (protein) and conductivity (salt) indicates desalinization of the amount (Fig. 1B). To verify the purity of HBP*Lc* and estimate its molecular mass, 30 μg of purified protein was submitted to SDS-PAGE under non-reducing conditions. After silver staining, we observed the presence of three bands corresponding to molecular masses of approximately 105, 70, and 38 kDa (Fig. 1C). The same result was observed when the fractions (core and tails) were analyzed separately (data not shown).

Total IgG anti-HBP*Lc* antibodies were produced in mice and purified using G protein Sepharose column, as described in the methodology. To check the purity of the samples, the product collected after column purification was applied to SDS-PAGE under reducing and non-reducing conditions. Following the electrophoresis and staining with Coomassie blue, the results revealed bands corresponding to the antibody and, when compared with the control unpurified serum, a large decrease in the intensity of the band corresponding to albumin, indicating satisfactory purification of the samples (data not shown).

Purified polyclonal IgG anti-HBP*Lc* antibodies were submitted to Western blotting to verify their specificity to recognize HBP*Lc*. The result as seen in Fig. 1D shows that the purified antibodies react with the 105-kDa protein present in the purified and crude (total) extracts. Importantly, a single band was revealed for both extracts, showing the high specificity of this antibody to recognize HBP*Lc*.

The localization of HBP*Lc* in promastigote forms of the parasite was evaluated by an immunolabeling assay. We observed a wide distribution of this protein in the parasite plasma membrane, except in the flagellum (Fig. 2C and inset C). When the parasites were permeabilized, we detected the presence of this protein in the cytoplasm, being more evident in regions closer to the kinetoplast (Fig. 2F and inset F).

To confirm these results, an electronic transmission analysis was carried out which showed the presence of the protein on the cell surface, next to the nucleus, on the flagellum where it intrudes into the flagellar pocket, and in the flagellar pocket, the last two being undetected by the previous immunolabeling assay. Additionally, the gold particles stained the kinetoplast as well as the mitochondrial and internal vesicles (Fig. 3).

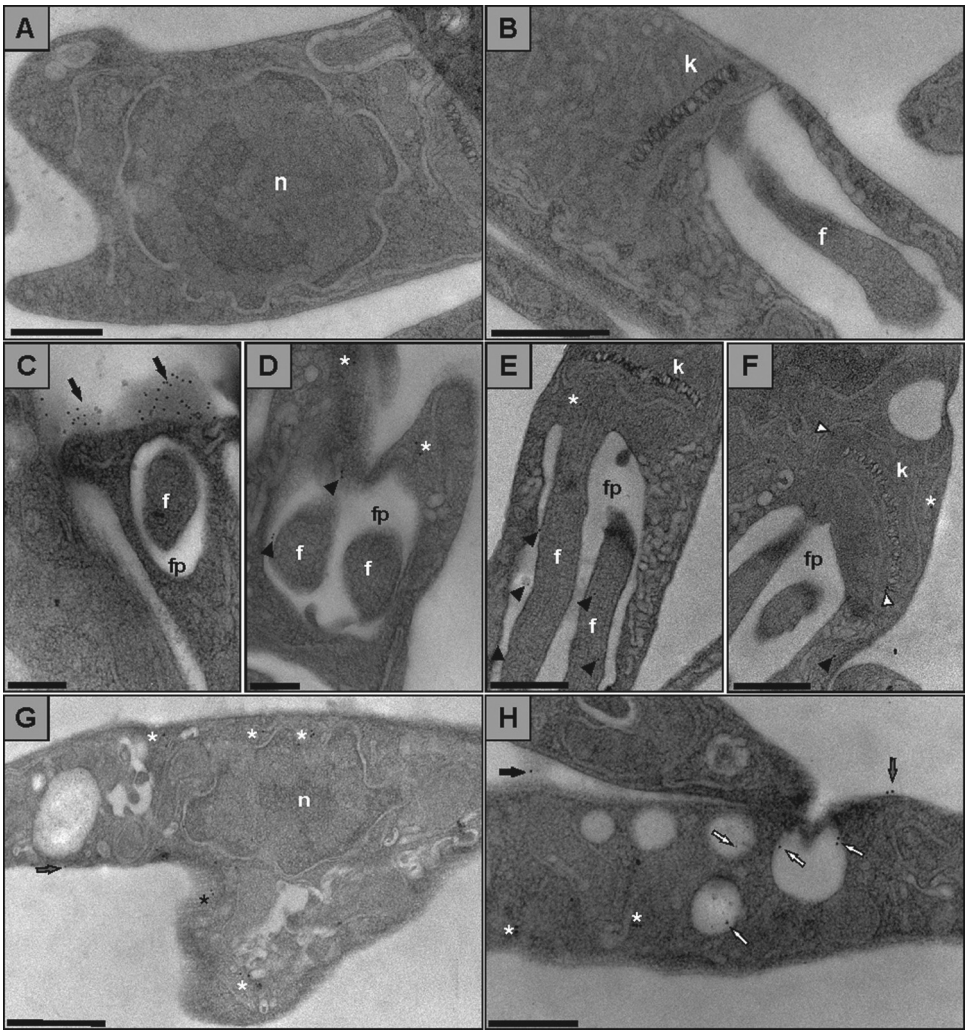

Fig. 3. Sub-cellular localization of HBP*Lc* in *L. infantum chagasi* promastigotes. (A and B) Controls. (C–H) Electron micrographs using polyclonal antibodies anti-HBP*Lc* (2.4 μg/ml) and anti-IgG conjugated to 10 nm colloidal gold. Letters and symbols indicate different localizations: next to kinetoplast (k) (white arrow head), flagellum (f) (black arrow head), vesicles (white arrow), cell surface (grey arrow), cytoplasm and nucleus (n) (white asterisk), secreted (black arrow), cytoskeleton (black asterisk). Bars: C and D = 0.2 μM; A, B, G and H = 0.5 μM; E and F = 5 μM. fp: flagellar pocket.

To determine the role of HBP*Lc* in the infection of macrophages by *L. chagasi* promastigotes, the parasites were previously treated with anti-HBP*Lc* antibodies or heparin. We observed that the pre-treatment of the parasites with anti-HBP*Lc* antibodies does not inhibit the process of parasitic adhesion; on the contrary, a trend of an increase in the adhesion index when the parasites were pre-treated with 5, 10, or 50 μg/ml anti-HBP*Lc* antibody (Fig. 4A and B).

Analogously, when the parasites were pretreated with anti-HBP*Lc* antibodies and subjected to infection assays, the antibodies were unable to prevent the internalization of the parasite in macrophages; on the contrary, an increase in the number of macrophages with internalized parasites was observed when the parasites were pretreated with 5 or 50 μg/ml of anti-HBP*Lc* antibody, compared to the control (untreated parasites) (Fig. 4C). The same profile was observed considering the number of parasites per macrophages with internalized *Leishmania*, when the parasites were pretreated with 10 μg/ml anti-HBP*Lc* antibody (Fig. 4D).

In addition to the use of anti-HBP*Lc* antibodies which react with the parasite lectin, heparin sodium was used as HBP*Lc* blocking agent. When the parasites were pretreated with 0.2, 1, and 2 IU/ml sodium heparin, there was no important reduction either in the percentage of macrophages with parasites attached or in the average number of parasites attached to each macrophage submitted to parasitic adhesion (Fig. 5A and B, respectively).

Otherwise, the treatment with heparin was able to prevent internalization of the parasites in macrophages, as evidenced by the reduction of the internalization index considering the percentage of macrophages with internalized parasites in the groups treated with 1 or 2 IU/ml heparin compared to that of those parasites in the control group (Fig. 5C).

4. Discussion

Intracellular pathogen–host cell interactions are dynamic and complex processes, where the recognition of molecules between the host cell and pathogen surface is an important step in the invasion process [26,37]. In this context, a class of HBPs, the glycosaminoglycans (GAGs), has been a focus of investigation. It has been demonstrated that GAGs, specifically reacting with their counterpart lectins present in the parasite, may influence the life cycle of the *Leishmania* parasite in both invertebrate and vertebrate hosts [34].

Structures that bind to host cell GAGs have been identified in many pathogens [34,38,39]; however, the investigation of the pres-

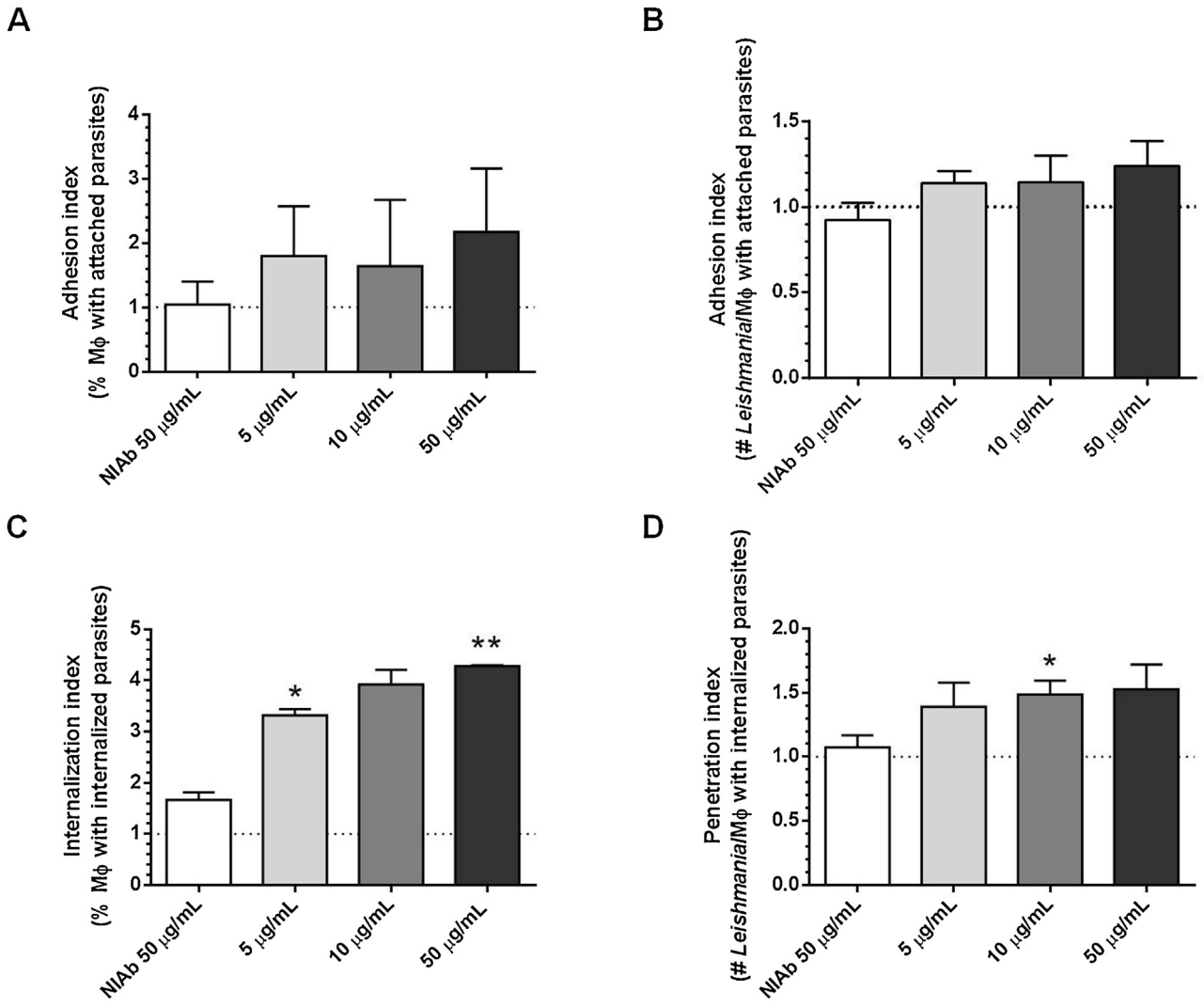

Fig. 4. Adhesion and internalization of *L. chagasi* pretreated with anti-HBP*Lc* antibodies in macrophages. Promastigote forms of *L. chagasi* were treated with anti-HBP*Lc* antibodies and used to infect RAW 264.7 macrophages by 30 min or 3 h to adhesion or internalization tests, respectively, as described in Section 2. (A) Index of macrophages with attached *Leishmania*. (B) Index of average of adhered parasites by macrophages submitted to adhesion. (C) Index of macrophages with internalized *Leishmania*. (D) Index of average of internalized parasites per infected macrophage. NIAb 50 μg/ml: infection with *L. chagasi* pretreated with antibodies from not immunized mice. 5, 10 and 50 μg/ml: Pretreatment of parasites with 5, 10 or 50 mg/ml of polyclonal anti-HBP*Lc*. Bars and symbols represent mean + 1 SEM from two or three separate experiments. Asterisk indicates statistical difference between antibody treated and control (antibody untreated) groups ($p < 0.05$).

ence of HBPs in *L. chagasi* promastigotes had not yet been described and characterized.

In this article, we report and characterize the presence of HBPs in *L. chagasi* promastigotes, which in respect to their behavior as adhesion molecules, may be important to the processes of recognition, adherence, and/or internalization of the parasite in mammalian host macrophages.

L. chagasi metacyclic promastigotes and amastigotes are infective forms that play important roles in the maintenance of the parasite life cycle, respectively being transmitted to the mammalian host by the insect vector or spreading the infection from infected macrophages. HBPs predominate in promastigotes infective forms of *Leishmania donovani* and have also been described in others *Leishmania* spp., as *Leishmania amazonensis* and *L. major*. However, contrary to what has been observed for *L. donovani*, in these species those proteins appear to be predominant in amastigotes [38]. These facts justify the experimental purpose of this manuscript regarding the use of promastigote forms.

Azevedo-Pereira et al. [34]. demonstrated HBPs with molecular masses of 54.5 and 65 kDa in *L. (Viannia) braziliesis*, and suggested that these proteins may have the form of a complex structural organization with more than one subunit. Following SDS-PAGE of purified HBP*Lc*, we detected three protein bands of molecular masses 105, 70, and 38 kDa, approximately (Fig. 1C). However, Western blotting performed with purified polyclonal anti-HBP*Lc* antibodies revealed the recognition of only one band (approx. 105 kDa). These data lead us to suggest that HBP*Lc* is either a protein complex or has three different proteins in its composition. In the first case, the polyclonal antibody used, which was obtained from the immunization of mouse by the native protein, may have recognized only the suggested protein complex (approx. 105 kDa) in amounts sufficient to be detected by Western blotting. This would have been disfavored when each suggested unit (approx. 70 or 38 kDa) reacted. In the second case, each of the three bands represented independent proteins, and only one (approx. 105 kDa) was sufficiently immunogenic to induce antibodies that allow for protein detection by Western blotting.

Within the class of HBPs are certain commonly encountered proteins with different molecular masses according to the species from which they are extracted. For example, *Anadara granosa* displays a

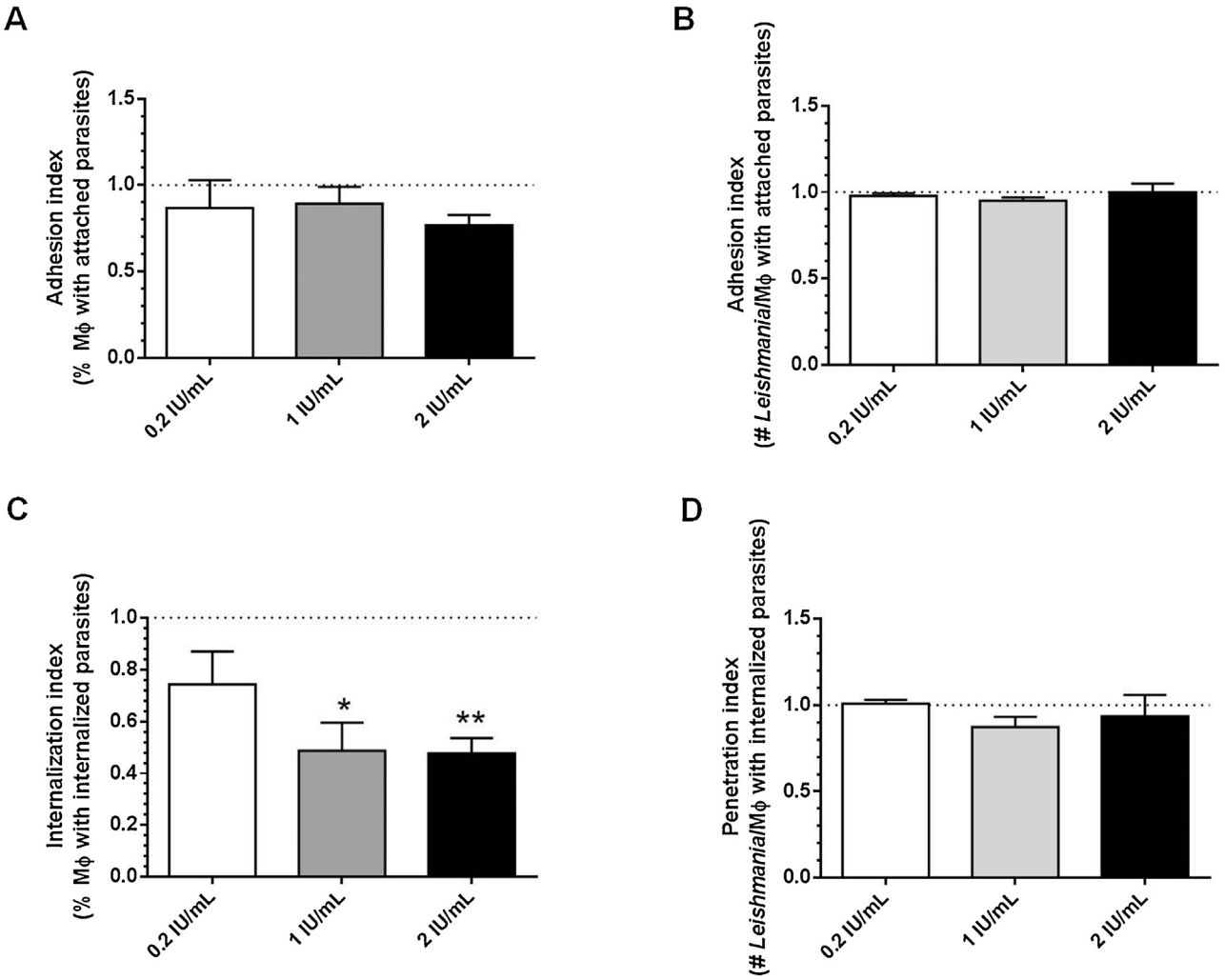

Fig. 5. Adhesion and internalization of *L. chagasi* pretreated with heparin in raw macrophages. Promastigote forms of *L. chagasi* were treated with heparin and used to infect RAW 264.7 macrophages by 30 min or 3 h to adhesion or internalization tests, respectively, as described in Section 2. (A) Index of macrophages with attached *Leishmania*. (B) Index of average of adhered parasites by macrophages submitted to adhesion. (C) Index of macrophages with internalized *Leishmania*. (D) Index of the average of internalized parasites per infected macrophage. 0,2, 1 and 2 UI/ml: Pretreatment of parasites with 0,2, 1 and 2 UI/ml of heparin. Bars and symbols represent mean + 1 SEM from two or three separate experiments. Asterisk indicates statistical difference between heparin treated and control (heparin untreated) groups ($p < 0.05$).

protein with a native molecular mass of 300 kDa and which is composed of identical subunits of 60 kDa [40]. De Castro-Cortes et al. [35]. investigated the presence of HBPs in two sub-cellular fractions from *L. (V.) braziliensis,* flagellar and membrane HBPs. An analysis by SDS-PAGE revealed two main protein bands (55 and 65 kDa) for both fractions. Also, a study related to the isolation of this protein class from *T. cruzi* epimastigotes revealed molecular masses of 59 and 65.8 kDa [28]. These structural differences between the HBPs from both trypanosomatids may be related to the involvement of these proteins in distinct physiological processes in their life cycles [34].

In our work, the polyclonal antibodies produced against HBP*Lc* showed specific binding to it. This enables us to assess the protein localization in promastigote forms of the parasite by fluorescence microscopy. We visualized a homogeneous distribution of lectin on the surface of the parasite (Fig. 2: inset C), in the extension of the flagellum, though not completely (Fig. 2), in the flagellar pocket, in the insertion of the flagellum into it (Fig. 3), and near the kinetoplast of the parasite (Fig. 2: inset F and Fig. 3E and F), suggesting the involvement of this protein with this structure.

The fact that host cell surface heparan sulfate proteoglycans can activate signaling cascades involved in cytoskeleton remodeling [41] lead us to assume that sulfated proteoglycans can also mediate pathogen invasion. This assumption was tested in the adhesion and infection assays, which demonstrated that the blocking of HBP*Lc* by heparin partially prevented the internalization of the parasites (Fig. 5C). A similar result was found in the evaluation of the ability of heparin to prevent invasion by *T. cruzi* cardiomyocytes in culture; a reduction of 82% in infection when the parasites were pretreated with heparin was observed [26]. A reduction of 60% of *L. amazonensis* amastigote binding in murine peritoneal macrophages was also seen when the parasites were pretreated with heparin [38]. This result is analogous to that obtained by us, as observed in Fig. 5C. This suggests an interaction between parasitic HBP and host heparin besides additional recognition mechanisms of *L. amazonensis* amastigotes by mammalian host cells.

Interestingly, when Love et al. [38]. and Bambino-Medeiros et al. [26]. used other GAGs such as chondroitin sulphate, dermatan sulphate, and keratan sulfate to prevent adhesion or internalization of parasites, no inhibition results were obtained, thus, showing that the recognition and infection processes is highly related to the presence of heparin. Is also noteworthy that heparin/HBP interaction may be used to elucidate the nature and function of parasitic proteins and enzymes that play some role in the cell biology of

host–*Leishmania* interaction, since heparin binding to *L. donovani* HBP is able to control the degree and the pattern of parasite protein phosphorylation as a result of the inhibition of the parasitic protein kinase C [42].

When we treated *L. chagasi* promastigotes with anti-HBP*Lc* antibodies, a trend of increase in the adhesion index and higher number of macrophages with internalized parasites compared to that in non-treated controls was observed, thus, indicating that anti-HBP*Lc* antibodies do not prevent the infection, but rather exacerbate it.

It has been argued that for leishmaniasis, a protective immune response is associated with an efficient cellular Th1 response with production of cytokines such as IL-12, IFN-γ, and TNF-α. Moreover, susceptibility to disease is associated with reduced Th1 response, associated with the development of a type Th2 immune response with cytokines IL-4 and IL-10 [43]. When there is a development of a Th2 response associated with a humoral response, there is production of antibodies. The antibodies may recognize the pathogens, opsonize them, and bind to Fc receptors present on macrophages. This mechanism facilitates phagocytosis of the parasite, since these antibodies are not blockers. Considering the behavior of *Leishmania* under the effects of antibodies, it is known that the infective metacyclic forms of *L. donovani* are resistant to lysis by human serum [44]. Beside this resistance to antibodies, opsonization may facilitate the process of macrophage infection by the parasite. In fact, IgG antibodies *in vivo* not only fail to provide protection but even contribute to the progression of leishmaniasis [45]. This process of recognition of opsonized parasites and their uptake by macrophages may explain the results obtained from our tests using anti-HBP*Lc* antibodies. Opsonized parasites may be recognized by macrophages that internalize them, resulting in the favoring of the infection, since these parasites have the ability to proliferate into the parasitophorous vacuole.

HBP*Lc*, widely distributed over the parasite plasma membrane (Fig. 2C and inset C), can, hence, be recognized by the host cell membrane heparan sulfate and trigger the process of infection, as seen in the *T. cruzi* infection process [26]. Moreover, polyclonal anti-HBP*Lc* antibodies act in favor of the uptake of the parasites by the macrophages. These evidences were obtained from our *in vitro* experiments indicating that HBP*Lc* may act as an additional virulence factor that contributes to the recognition and entry of the parasite into the macrophage, either by its direct binding to heparan sulfate or by the binding of lectin-opsonized antibodies to Fc receptors present in the cell membrane of the phagocytes. To ascertain this last possibility, further studies should be carried out with Fc-fragment or immune immunoglobulin, both produced in experimental infection by *L. chagasi* derivatives and sub fragments of heparin.

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Ethical and regulatory guidelines

Animal experimentation was done respecting ethical principles of the Code of Professional Veterinarian, according to the opinion of the Ethics Committee for Animal Use (approved by CEUA/UFV – Research project – process number: 22/2011), based in the actual Brazilian Legislation (Law no. 11.794, October 08, 2008), in the Normative Resolutions edited by CONCEA/MCTI, as in the "Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos" (DBCA), following the orientations to the practices of euthanasia commended by CONCEA/MCTI.

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