Brazilian Journal of Medical and Biological Research (2007) 40: 799-806
ISSN 0100-879X

Role of *Leishmania* (*Leishmania*) *amazonensis* amastigote glycosphingolipids in macrophage infectivity

A.K. Tanaka[1], P.A.J. Gorin[2], H.K. Takahashi[1] and A.H. Straus[1]

[1]Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[2]Departamento de Bioquímica, Universidade Federal do Paraná, Curitiba, PR, Brasil

Abstract

The role of glycosphingolipids (GSLs) present in amastigote forms of *Leishmania* (*Leishmania*) *amazonensis* during infection of macrophages was analyzed, with particular emphasis on GSLs presenting the terminal Gal*p*ß1-3Gal*p*α disaccharide. Macrophage invasion by *L.* (*L.*) *amazonensis* amastigotes was reduced by 37% when the disaccharide Gal*p*ß1-3Gal*p* (1 mM) was added to the culture medium. The putative macrophage receptor/lectin for ß-Gal-globotriaosylceramide (Gal*p*ß1-3Gal*p*α1-4Gal*p*ß1-4Glc*p*ß1-1Cer) and other structurally related GSLs from *L.* (*L.*) *amazonensis* amastigotes were analyzed by micelles and parasite binding assay to peritoneal macrophage proteins fractionated by SDS-PAGE under nonreducing conditions. Micelles containing purified amastigote GSLs or a suspention of *L.* (*L.*) *amazonensis* amastigotes fixed with 2% formaldehyde were incubated with nitrocellulose membrane containing the macrophage proteins transferred by Western blotting. Binding of micelles containing purified GSLs from amastigote forms or fixed *L.* (*L.*) *amazonensis* amastigotes to nitrocellulose membrane was probed using monoclonal antibody ST-3, which recognizes the glycoepitope Gal*p*ß1-3Gal*p*α1-R present either in the micelle preparation or on the amastigote surface. Macrophage protein with molecular mass ~30 kDa bound the amastigote GSL and appeared to be a doublet on electrophoresis. The specificity of this interaction was confirmed using fixed *L.* (*L.*) *chagasi* amastigotes, which do not express GSLs such as ß-Gal*p*-globotriaosylceramides, and which do not bind to 30-kDa protein.

Correspondence
A.H. Straus
Departamento de Bioquímica
EPM, UNIFESP
Rua Botucatu, 862, Ed. J.L. Prado
04023-900 São Paulo, SP
Brasil
Fax: +55-11-5579-2509
E-mail: straus.bioq@epm.br

Research supported by FAPESP (No. 03/11274-2), CNPq (No. 477177/03), and Pronex (No. 41.96.0875.00). A.K. Tanaka was the recipient of a FAPESP grant (No. 01/13755-2).

Received July 11, 2006
Accepted March 2, 2007

Key words
- *Leishmania* (*Leishmania*) *amazonensis*
- Amastigote
- Glycosphingolipid
- Macrophage binding protein

Introduction

Species of the genus *Leishmania* (family Trypanosomatidae) are protozoans responsible for a variety of human diseases, including cutaneous, mucocutaneous, and visceral leishmaniasis, which occur throughout the world and represent a major public health problem in tropical areas. *Leishmania* is a digenetic parasite with extracellular motile promastigotes present in the alimentary tract of their insect vector, the phlebotomine sandfly, and intracellular non-motile amastigotes, which live in the mononuclear phagocytes of mammalian hosts. The etiologic agents of leishmaniasis include many com-

plex and epidemiologically diverse *Leishmania* species. *Leishmania* (*Leishmania*) *amazonensis* is responsible for most of the cases of human cutaneous leishmaniasis in the Amazon region of Brazil (1).

Leishmania phagocytosis has been studied in detail in promastigotes of various species (2). The promastigote-macrophage interaction is a multi-step phenomenon mediated mainly by the protease gp63 and lipophosphoglycan (3-6). A number of studies have identified macrophage receptors involved in the process of *Leishmania* invasion, including mannose-fucose receptor, receptors for advanced glycation end products, fibronectin, complement receptor (CR1 and CR3), and Fc receptor (2).

In contrast to abundant information available on phagocytic processes of promastigote forms, few studies have been conducted on phagocytic processes of amastigote forms, which are responsible for maintaining and disseminating infection in the vertebrate host (2,7). Previous studies on the biological roles of *Leishmania* glycosphingolipid (GSL) antigens demonstrated that monoclonal antibodies (mAbs) ST-3, ST-4 and ST-5, specifically directed at amastigote *L.* (*L.*) *amazonensis* GSLs, inhibited macrophage invasion by amastigotes by as much as 80%, suggesting that the epitope recognized by these mAbs present in surface GSLs is important in mediating binding of *L.* (*L.*) *amazonensis* amastigotes to macrophages (8,9).

To better characterize the biological role of stage-specific GSLs, in particular the role of the terminal disaccharide Gal*p*ß1-3Gal*p*, present in *L.* (*L.*) *amazonensis* amastigote GSL antigens such as Gal*p*ß1-3Gal*p*α1-4Gal*p*ß1-4Glc*p*ß1-1Cer (referred to herein as ß-Gal-globotriaosylceramide), we performed macrophage infection assays using different glycosides as inhibitors. mAb ST-3 was used to evaluate binding of amastigotes and micelles containing amastigote GSLs to specific receptors in macrophages.

Material and Methods

Parasites

Amastigote forms of *L.* (*L.*) *amazonensis* (MHOM/BR73/M2269) were maintained by footpad infection of Golden hamsters or BALB/c mice. Amastigotes (2 x 10^7) were inoculated into hamster footpads and the lesions were surgically removed after 5-6 weeks. Tissue was minced in phosphate-buffered saline (PBS) and debris were eliminated by nylon Nitex filtration (pore size, 80 µm; Tekto, Monterey Park, CA, USA). The cell suspension was centrifuged at 1800 *g* for 10 min. To lyse erythrocytes, the pellet was resuspended in ammonium chloride solution (8.29 g NH_4Cl, 1 g $KHCO_3$, 37.3 mg/L EDTA) for 10 min, and the insoluble material containing infected macrophages was disrupted in a Dounce-Potter homogenizer with about 20 strokes. The resulting suspension was centrifuged four times at 1800 *g*, and the final pellet was resuspended in RPMI 1640. The suspension was shaken for 3 h and the amastigotes were washed with PBS four times by centrifugation at 1800 *g*. The parasite yield was 5 x 10^9 amastigotes/hamster.

Amastigotes of *L.* (*L.*) *chagasi* (MHOM/BR72/LD) were maintained by intraperitoneal inoculation in Golden hamsters. Two months after infection, spleens were removed and homogenized, and amastigotes were isolated as described by Pinto et al. (10).

All experimental animal procedures were approved by the Institutional Ethics Committee.

Hybridomas and sera

Monoclonal antibodies ST-3 (mouse IgG3) directed to *L.* (*L.*) *amazonensis* GSLs (8) and CU-1 (mouse IgG3), directed to the Tn antigen (11) and used as an irrelevant antibody, were obtained from the culture supernatant. Sera of patients with visceral

leishmaniasis (1:200 dilution) were tested by indirect immunofluorescence with *L.* (*L.*) *chagasi* amastigotes before use. Amastigotes fixed with 1% formaldehyde were submitted to indirect immunofluorescence as described by Straus et al. (8).

Purification of glycosphingolipids

Amastigote GSLs were extracted and purified as described by Straus et al. (8). Amastigotes (2 x 10^{11}) were homogenized in 30 mL isopropyl alcohol/hexane/water (55: 20:25) and in 30 mL chloroform/methanol (2:1). The extracts were combined and dried in a rotary evaporator. The resulting "lipid fraction" contained sterols, phospholipids, and glycolipids. GSLs were purified from this lipid fraction by Florisil chromatography (12). Briefly, the lipid fraction was dried, treated with pyridine/acetic anhydride (2:1), and acetylated GSLs were separated from other lipids on a Florisil column. Nonpolar lipids were first eluted with 1,2-dichloroethane, and then acetylated GSLs were eluted with 1,2-dichloroethane/acetone (1:1). The GSL fraction was diacetylated with 0.5% sodium methoxide in methanol, neutralized with Dowex 50 (H^+ form), and subjected to DEAE-Sephadex ion exchange chromatography in chloroform/methanol/water 30:60:8 (13). Neutral GSLs were further subjected to HPLC on Iatrobeads column (4.6 x 300 mm, 6RS-8010; Iatron, Tokyo, Japan) and eluted with a gradient of isopropyl alcohol/hexane/water from 55:44:3 to 55:30:25 over 175 min, with a flow rate of 1 mL/min (fractions of 2 mL). GSLs were identified by HPTLC using solvent A (chloroform/methanol/aqueous $CaCl_2$ 0.02%, 60:40:9), stained with orcinol/H_2SO_4 (14). Fractions 3 to 10 (eluted from 3.7 to 5.5% water) contained monohexosylceramide (CMH), dihexosylceramide (CDH), and trihexosylceramide (CTH). Fractions 11 to 55 (eluted from 5.7 to 13.8% water) corresponded to GSLs with 4 or more carbohydrate residues, and were recognized by mAb ST-3. No contamination with lipophosphoglycan, phospholipids or peptides was detected, as described previously (8,15).

Peritoneal macrophage culture

Macrophages were harvested by washing the peritoneal cavity of BALB/c mice with PBS, and cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Approximately 5 x 10^5 macrophages were placed on sterile glass coverslips in 24-well plates, or about 5 x 10^7 cells were placed in a 150-cm^2 tissue culture flask. Non-adherent cells were removed by several washes with RPMI 1640, and the plates kept at 37ºC in a CO_2 incubator.

Alternatively, thioglycolate-elicited peritoneal macrophages of BALB/c mice were harvested 4 days after intraperitoneal thioglycolate inoculation (1.0 mL of 3% thioglycolate in PBS).

Glycoside inhibition of *Leishmania* uptake into macrophages

Fifty microliters of a 10-mM solution of the following glycosides: methyl α-D- and ß-D-galactopyranoside, ß-lactose (Gal*p*ß1-4Glc), Gal*p*ß1-3Gal*p*ß 1-OMe (all from Sigma, St. Louis, MO, USA), and Gal*p*ß1-3Gal*p* obtained by partial acid hydrolysis of native vegetal polysaccharide from *Anacardium occidentale* (16) was added to wells containing 0.45 mL RPMI 1640 and ~5 x 10^5 macrophages. After 1 h, parasites were added (2.5 x 10^5 per well) and maintained for 2 h in RPMI 1640 without FCS at 37ºC. Nonadherent parasites (less than 10% of total added amastigotes) were removed, and cultures were maintained in RPMI 1640 with 5% FCS in a CO_2 incubator for 24 h. Macrophages were fixed with methanol and stained with the Hema 3 Stain Set (Fisher, Swedesboro, NJ, USA). The percent of macrophages that had phagocyted at least one parasite was determined by examining 300 cells, as de-

scribed by Silveira et al. (17).

Western blotting

About 1 x 10^7 macrophages harvested from tissue culture flasks were homogenized with 1.0-mL sample buffer. Aliquots (20 µL) were separated by SDS-PAGE into 5 x 12 mm lanes of 8.0 or 12% polyacrylamide slab gels (80 x 60 mm) under non-reducing conditions, and transferred onto nitrocellulose (Gibco-BRL, Grand Island, NY, USA). The nitrocellulose was blocked with 5% BSA in PBS for 2 h, incubated with parasites or GSL-containing micelles as described below, and then sequentially incubated with mAb ST-3, biotin-conjugated goat anti-mouse IgG, peroxidase-conjugated avidin, and 4-chloro-naphthol (18).

Glycosphingolipid micelle and parasite binding assays

The blotted nitrocellulose membrane blocked with BSA was incubated for 45 min at room temperature with purified amastigote GSL (type I micelles), and total amastigote lipid fraction (type II micelles) prepared by ultrasonication (Branson 2200, VWR Scientific, Danbury, CT, USA) of glycolipids (450 µg carbohydrate in 3.0 mL RPMI 1640) for 45 s (19), or with a suspension of amastigotes (1 x 10^8 parasites in 3.0 mL RPMI 1640 medium) fixed with 2% formaldehyde in PBS for 10 min. Binding of parasites or GSL micelles was visualized after immunostaining with mAb ST-3.

Labeling of macrophage surface proteins with biotin

Peritoneal macrophages (1 x 10^7 cells) were washed twice with PBS and incubated with 0.1 mg/mL biotin sulfo n-hydroxysuccinimide (Sigma) in PBS for 20 min at 4ºC. The reaction was stopped by washing the cells with cold PBS. Excess biotin was blocked by incubation with PBS containing 1% BSA for 20 min at 4ºC, followed by one wash with PBS. Cells were homogenized with 0.5% Triton X-100 (Sigma) in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 1 µM leupeptin (Sigma) and centrifuged at 12,000 *g*. Triton X-100-soluble (1.0 mL) and -insoluble (resuspended in 1.0 mL of sample buffer) fractions were separated by SDS-PAGE and transferred to nitrocellulose. Biotinylated proteins were visualized after incubation of nitrocellulose with peroxidase-conjugated avidin and 4-chloronaphthol.

Isolation of macrophage soluble protein and membrane fractions

Peritoneal macrophages (1 x 10^7 cells) biotinylated or not were resuspended in PBS (1.0 mL) and lysed by three cycles of freezing and thawing in PBS containing 1 mM PMSF and 1 µM leupeptin. The lysate was centrifuged at 12,000 *g* for 10 min and the soluble proteins (supernatant) and insoluble membrane fraction (pellet) were subjected to Western blotting and to parasite binding assays, as described above.

Results

Effect of glycosides on macrophage infectivity by *L.* (*L.*) *amazonensis* amastigotes

In order to identify the biological role of specific glycan structures expressed in GSLs of amastigote forms of *L.* (*L.*) *amazonensis* in the interaction with macrophages, we pre-incubated peritoneal macrophage cultures with various glycosides and then tested them for amastigote infectivity. Among the glycosides tested the most effective infectivity inhibitor was Gal*p*ß1-3Gal*p* (Table 1). The number of infected macrophages decreased by about 37% when macrophages were pre-incubated with Gal*p*ß1-3Gal*p* (1 mM), indicating that this disaccharide, present at the

non-reducing terminus of ß-D-Gal-globotriaosylceramide, which is recognized by mAb ST-3, is involved in parasite infectivity. Gal*p*ß1-3Gal*p* inhibition was clearly dose dependent, as shown in Figure 1.

Binding of purified glycolipids and amastigotes to macrophage (glyco)proteins

Resident peritoneal mouse macrophage components were fractionated by SDS-PAGE and micelle binding to nitrocellulose membranes was probed using mAb ST-3. Binding experiments were performed using two types of micelles, type I containing purified amastigote GSLs and type II containing amastigote total lipid extract (GSLs, phospholipids and sterols), to mimic glycolipid organization in the parasite membrane. Both type I and type II micelles bound to a ~30-kDa molecule (Figure 2A, lanes 1 and 2, arrow). To rule out the possibility that GSLs with one, two, or three carbohydrate residues present in the micelle preparation were responsible for the binding, GSL fractions containing only CMH (GlcCer), CDH (Galß1-4GlcCer) and CTH (Galα1-4Galß1-4GlcCer) purified by HPLC as described in Material and Methods were incubated with a nitrocellulose membrane and, as expected, no mAb ST-3 labeling was observed (lane 3). However, when this nitrocellulose membrane was subsequently exposed to type I micelles, labeling by mAb ST-3 of the 30-kDa component was again detected (lane 4). No reactivity with the 30-kDa component was detected when an irrelevant mAb (CU-1) was used to detect binding of type I micelles to the nitrocellulose membrane (lane 5).

The macrophage 30-kDa component was also visualized in a binding assay using fixed *L.* (*L.*) *amazonensis* amastigotes instead of GSL micelles, confirming that the amastigotes also bind to the 30-kDa protein (Figure 2B, lane 1). No labeling was observed using an irrelevant antibody (lane 2). It is noteworthy that this 30-kDa protein was not recognized by *L.* (*L.*) *chagasi* amastigotes (lane 3).

Micelle binding to the macrophage membrane fraction

To further determine if the 30-kDa protein is associated with the macrophage membrane, and to analyze the possibility that galectin-3, a macrophage soluble protein, is associated with parasite binding to macrophages, resident and thioglycolate-activated peritoneal macrophages, the latter expressing large amounts of galectin-3, were isolated from the peritoneal cavity of mice and plated. After 24 h the culture medium was removed and the cells were collected from the culture plates with a minimum volume of PBS and disrupted by a freezing/thawing procedure. The resulting suspensions were centrifuged at 12,000 *g* and the supernatant (containing galectin-3 and other soluble proteins) and pellet (membrane fraction) were fractionated by SDS-PAGE. Amastigotes bound exclusively to the region of the 30-

Table 1. Inhibition by glycosides of macrophage infectivity by Leishmania (Leishmania) amazonensis amastigotes.

Glycoside (1 mM)	Macrophages infected with Leishmania (%)
Methyl ß-D-Galp	44.33 ± 2.52
Methyl α-D-Galp	37.00 ± 4.58
Galpß1-3Galp	27.50 ± 2.74*
Galpß1-3Galpß1-O-methyl	45.67 ± 1.15
Galpß1-4Glcp	36.30 ± 5.07
Control	43.67 ± 8.14

Macrophages were pre-incubated with various glycosides for 1 h and the parasites were then added and maintained for 2 h. Non-adherent parasites were removed. After 24 h, infected macrophages were fixed with methanol and stained, and the percentage of infected macrophage was determined as described in Material and Methods. Control macrophage cultures were not pre-incubated with any glycoside before parasite infection. Data are reported as means ± SD of triplicate experiments.
*$P < 0.05$ compared to control (Student t-test).

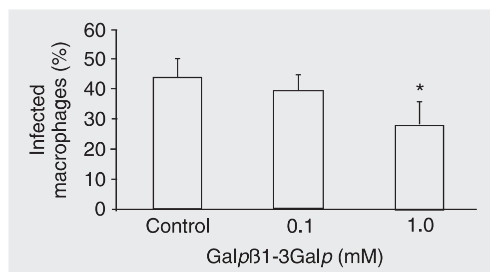

Figure 1. Macrophage infectivity by Leishmania (Leishmania) amazonensis amastigotes in the presence of various concentrations of Galpß1-3Galp glycoside. Macrophages were incubated with Galpß1-3Galp for 1 h and then infected with L. (L.) amazonensis amastigotes for 2 h at 37ºC. Non-adherent parasites were removed and infected macrophages were maintained in a CO_2 incubator for 24 h. The percent of infected macrophages was calculated as described in Material and Methods. Control macrophages were incubated without Galpß1-3Galp. Data are reported as means ± SD of triplicate experiments. *$P < 0.05$ compared to control (Student t-test).

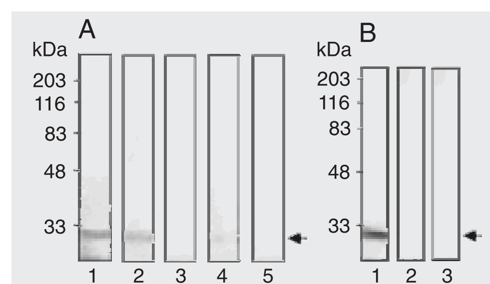

Figure 2. Binding of fixed Leishmania (Leishmania) amazonensis amastigotes and purified glycosphingolipids (GSLs) to mouse peritoneal macrophage proteins. Lysate macrophages were processed and samples containing about 10 µg protein were applied to each SDS-PAGE lane and transferred to a nitrocellulose (NC) membrane. A, Binding of micelles containing lipids and GSL antigens. Lane 1, NC incubated with type II micelles (containing total amastigote lipids); lane 2, NC incubated with type I micelles (purified amastigote GSLs); lane 3, NC incubated with micelles containing monohexosylceramide (CMH), dihexosylceramide (CDH), and trihexosylceramide (CTH), purified from amastigotes; lane 4, NC preincubated with micelles containing CMH, CDH, and CTH, followed by incubation with type I micelles; lane 5, NC incubated with type I micelles immunostained with an irrelevant antibody (mAb CU-1). Micelle binding was detected after incubation with mAb ST-3. B, Binding of formaldehyde-fixed amastigotes. Lane 1, L. (L.) amazonensis amastigotes immunostained with ST-3; lane 2, binding of L. (L.) amazonensis amastigotes immunostained with CU-1; lane 3, binding of L. (L.) chagasi amastigotes immunostained with sera positive for visceral leishmaniasis. The arrows indicate the 30-kDa component recognized by either L. (L.) amazonensis amastigotes or amastigote GSL micelles.

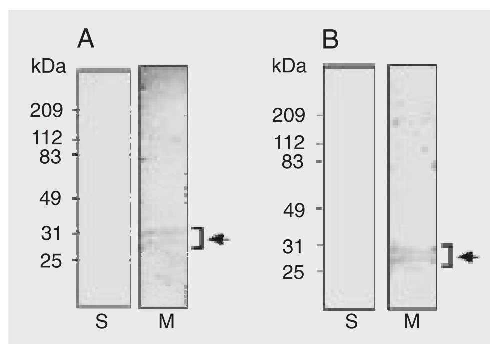

Figure 3. Binding of amastigotes to soluble proteins and to the membrane fraction of peritoneal macrophages. Resident (Panel A) and thioglycolate-elicited (Panel B) peritoneal macrophages were resuspended in PBS and subjected to a freezing/thawing procedure as described in Material and Methods. Soluble (S) proteins (about 10 µg protein/lane) and insoluble membrane (M) preparations (about 10 µg protein/lane) were fractionated by SDS-PAGE using 12% gel slabs, transferred to nitrocellulose membranes and assayed for binding of formaldehyde-fixed amastigotes immunostained with ST-3. The arrows indicate the 30-kDa doublet recognized by Leishmania (Leishmania) amazonensis amastigotes.

kDa proteins present in the pellet (corresponding to the membrane fraction), visualized as a doublet in a 12% gel slab (Figure 3, lane M). No difference in the amastigote binding pattern was observed between resident (Panel A) and thioglycolate-elicited (Panel B) peritoneal macrophages, a fact ruling out the possibility that galectin-3 is involved in the parasite-macrophage interaction modulated by GSLs.

Macrophage surface labeling

Cultured macrophages were tagged with biotin at 4ºC for surface protein labeling. Cells were removed from the plates and processed as described in Material and Methods. The fractions that were soluble (S) and insoluble (I) in 0.5% of Triton X-100 were analyzed for total protein content and stained with Coomassie blue (Figure 4A). Biotinylated proteins were identified by Western blotting revealed with peroxidase-conjugated avidin and 4-chloro-naphthol (Figure 4B). Major label proteins were present in the soluble fraction (lane S). On the other hand, the doublet of 30 kDa was the only biotinylated protein that remained in the insoluble fraction (panel B, lane I). Panel C shows that the macrophage doublet of 30 kDa recognized by amastigotes remained in the insoluble fraction of 0.5% Triton X-100 (Panel 4C, lane I).

Discussion

The numerous roles of macrophage in host defense may be reflected in the variety of lectins present in these cells. Some of them (e.g., mannose receptor, CR3) participate in microbial phagocytosis, while others are involved in cell adhesion, clearance, recruitment, or activation (20). Our laboratory recently demonstrated that glycosylinositol phospholipid-1 of *L.* (*L.*) *major* and its terminal residue, ß-D-galactofuranose, participate in the interaction of *L.* (*L.*) *major* pro-

mastigotes and amastigotes with mouse peritoneal macrophages (21), but not to interactions of other species such as *L.* (*L.*) *amazonensis*. On the other hand, in indirect experiments where Fab fragments of mAb ST-3, directed specifically to amastigote GSLs, significantly inhibited macrophage infectivity (8), we have shown that *L.* (*L.*) *amazonensis* amastigote GSLs, in addition to modulating the host cell immune response (14, 22), play a role in macrophage infectivity.

In the present study, we demonstrated that the disaccharide Gal*p*ß1-3Gal*p*, whose reducing terminus is not substituted at the anomeric carbon, presents in solution alpha (Gal*p*ß1-3Gal*p*α) and/or beta (Gal*p*ß1-3Gal*p*ß) isomers and inhibits invasion of macrophage cells by *L.* (*L.*) *amazonensis* amastigotes by ~37%. In contrast, the disaccharide Gal*p*ß1-3Gal*p*ß1-O-methyl, which has the subterminal galactose locked in a beta configuration, did not inhibit macrophage infectivity. These results clearly indicate the key role of the anomeric configuration of the subterminal galactose unit in the parasite binding to macrophages. Thus, the inhibition of the macrophage infectivity by amastigote forms observed with Gal*p*ß1-3Gal*p* is related to the presence of the Gal*p*ß1-3Gal*p* isomer, whereas the Gal*p*ß1-3Gal*p*ß isomer does not bind the 30-kDa protein/receptor.

Using mAb ST-3, which recognizes ß-Gal*p*-globotriaosylceramide and related amastigote GSLs, we analyzed the putative receptor/lectin of macrophages for these GSLs. By Western blotting of a macrophage preparation, we identified a single band with a molecular mass of 30 kDa using micelles prepared with highly purified amastigote GSLs. No inhibition was observed when nitrocellulose was pre-incubated with CMH, CDH, and CTH purified from *Leishmania* preparations. The same protein was identified when the nitrocellulose sheet was incubated with fixed *L.* (*L.*) *amazonensis* amastigotes. However, when the nitrocellulose sheet was incubated with fixed *L.* (*L.*) *chagasi* amastigotes, which do not express GSLs such as ß-Gal*p*-globotriaosylceramides, no labeling of the 30-kDa protein was detected.

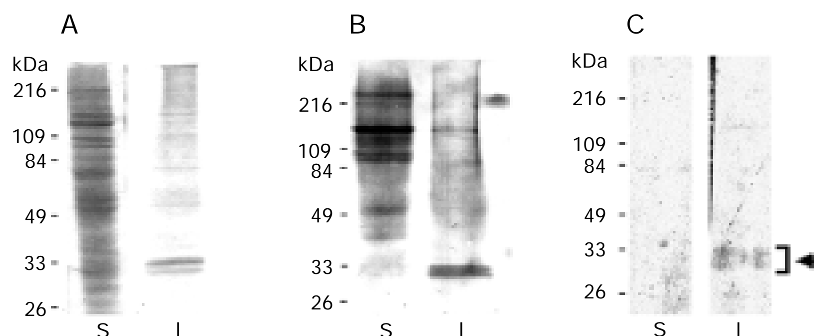

Figure 4. Macrophage surface protein labeling with biotin. Macrophage cultures were labeled with biotin sulfo n-hydroxysuccinimide at 4ºC as described in Material and Methods. The labeled cells were incubated with 0.5% Triton X-100 and the soluble (S) and insoluble (I) fractions obtained after centrifugation were separated by SDS-PAGE using 12% gel slab (S, 10 µg protein/lane; I, 1.2 µg protein/lane). Panel A, SDS-PAGE of biotinylated macrophage proteins stained with Coomassie blue. Panel B, Western blotting of biotinylated macrophage proteins visualized after nitrocellulose incubation with peroxidase-conjugated avidin, and 4-chloro-naphthol. Panel C, Binding of formaldehyde-fixed amastigotes to nitrocellulose containing 0.5% Triton X-100 soluble and insoluble fractions. The arrows indicate the 30-kDa doublet recognized by Leishmania (Leishmania) amazonensis amastigotes.

The 30 kDa-protein detected by PAGE-SDS using an 8% acrylamide gel slab is resolved as a doublet in a 12% acrylamide gel slab and is recognized by *L.* (*L.*) *amazonensis* amastigotes in SDS-PAGE carried out under reducing or not-reducing condition (data not shown). The 30-kDa doublet protein recognized by *L.* (*L.*) *amazonensis* amastigotes and its GSLs i) is present on the macrophage surface as demonstrated by macrophage biotin labeling, ii) is insoluble in 0.5% Triton X-100, and iii) is recognized by concanavalin A (data not shown), indicating that this receptor is a glycoprotein. These data, taken together with the fact that the 30-kDa putative receptor is not detected in the culture medium and its expression is not altered by macrophage activation with thioglycolate, rule out the possibility that this molecule could correspond to galectin-3. The amino acid sequence of the 30-kDa receptor is currently under investigation.

References

1. Lainson R, Shaw JJ. New World Leishmaniasis - The Neotropical Leishmania species. In: Cox FEG, Kreier JP, Wakelin D (Editors), Topley & Wilson's Microbiology and Microbial Infections. London: F.E.G. Cox; 1998. p 241-266.
2. Handman E. Cell biology of Leishmania. Adv Parasitol 1999; 44: 1-39.
3. Chang KP, Chaudhuri M. Molecular determinats of Leishmania virulence. Annu Rev Microbiol 1990; 44: 499-529.
4. Turco SJ, Descoteaux A. The lipophosphoglycan of Leishmania parasites. Annu Rev Microbiol 1992; 46: 65-94.
5. Kelleher M, Moody SF, Mirabile P, Osborn AH, Bacic A, Handman E. Lipophosphoglycan blocks attachment of Leishmania major amastigotes to macrophages. Infect Immun 1995; 63: 43-50.
6. Brittingham A, Chen G, McGwire BS, Chang KP, Mosser DM. Interaction of Leishmania gp63 with cellular receptors for fibronectin. Infect Immun 1999; 67: 4477-4484.
7. Guy RA, Belosevic M. Comparison of receptors required for entry of Leishmania major amastigotes into macrophages. Infect Immun 1993; 61: 1553-1558.
8. Straus AH, Levery SB, Jasiulionis MG, Salyan ME, Steele SJ, Travassos LR, et al. Stage-specific glycosphingolipids from amastigote forms of Leishmania (L.) amazonensis. Immunogenicity and role in parasite binding and invasion of macrophages. J Biol Chem 1993; 268: 13723-13730.
9. Straus AH, Valero VB, Takizawa CM, Levery SB, Toledo MS, Suzuki E, et al. Glycosphingolipid antigens from Leishmania (L.) amazonensis amastigotes. Binding of anti-glycosphingolipid monoclonal antibodies in vitro and in vivo. Braz J Med Biol Res 1997; 30: 395-399.
10. Pinto AR, Beyrodt CG, Lopes RA, Barbieri CL. Identification of a 30 kDa antigen from Leishmania (L.) chagasi amastigotes implicated in protective cellular reponses in a murine model. Int J Parasitol 2000; 30: 599-607.
11. Takahashi HK, Metoki R, Hakomori S. Immunoglobulin G3 monoclonal antibody directed to Tn antigen (tumor-associated alpha-N-acetylgalactosaminyl epitope) that does not cross-react with blood group A antigen. Cancer Res 1988; 48: 4361-4367.
12. Saito T, Hakomori SI. Quantitative isolation of total glycosphingolipids from animal cells. J Lipid Res 1971; 12: 257-259.
13. Toledo MS, Suzuki E, Straus AH, Takahashi HK. Glycolipids from Paracoccidioides brasiliensis. Isolation of a galactofuranose-containing glycolipid reactive with sera of patients with paracoccidioidomycosis. J Med Vet Mycol 1995; 33: 247-251.
14. Suzuki E, Mortara RA, Takahashi HK, Straus AH. Reactivity of MEST-1 (antigalactofuranose) with Trypanosoma cruzi glycosylinositol phosphorylceramides (GIPCs): immunolocalization of GIPCs in acidic vesicles of epimastigotes. Clin Diagn Lab Immunol 2001; 8: 1031-1035.
15. Giorgio S, Jasiulionis MG, Straus AH, Takahashi HK, Barbieri CL. Inhibition of mouse lymphocyte proliferative response by glycosphingolipids from Leishmania (L.) amazonensis. Exp Parasitol 1992; 75: 119-125.
16. Menestrina JM, Iacomini M, Jones C, Gorin PA. Similarity of monosaccharide, oligosaccharide and polysaccharide structures in gum exudate of Anacardium occidentale. Phytochemistry 1998; 47: 715-721.
17. Silveira TG, Suzuki E, Takahashi HK, Straus AH. Inhibition of macrophage invasion by monoclonal antibodies specific to Leishmania (Viannia) braziliensis promastigotes and characterisation of their antigens. Int J Parasitol 2001; 31: 1451-1458.
18. Silveira TG, Takahashi HK, Straus AH. Immunolocalization of Leishmania (Viannia) braziliensis membrane antigens recognized by mAbs SST-2, SST-3, and SST-4. Parasitology 2003; 127: 449-456.
19. Kotani M, Tajima Y, Shimoda Y, Irie A, Kubo H, Tai T. Ganglioside GT1b in rat brain binds to p58, a brain-specific sodium-dependent inorganic phosphate cotransporter: expression cloning with a specific monoclonal antibody to ganglioside GT1b-binding protein. J Biochem 2000; 127: 13-22.
20. Linehan SA, Martinez-Pomares L, Gordon S. Macrophage lectins in host defence. Microbes Infect 2000; 2: 279-288.
21. Suzuki E, Tanaka AK, Toledo MS, Takahashi HK, Straus AH. Role of beta-D-galactofuranose in Leishmania major macrophage invasion. Infect Immun 2002; 70: 6592-6596.
22. Giorgio S, Santos MR, Straus AH, Takahashi HK, Barbieri CL. Effect of glycosphingolipids purified from Leishmania (Leishmania) amazonensis amastigotes on human peripheral lymphocytes. Clin Diagn Lab Immunol 2003; 10: 469-472.