Sialoglycoconjugates in Herpetomonas megaseliae: role in the adhesion to insect host epithelial cells

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Abstract
Herpetomonas megaseliae is a monoxenic trypanosomatid isolated from the phorid fly Megaselia scalaris. In the present report, the expression of cell surface sialoglycoconjugates in this parasite was analyzed by Western blotting, flow cytometry and fluorescence microscopy analyses using lectins that specifically recognize sialic acid residues. A strong reaction was detected when parasites were treated with Limax flavus, Maackia amurensis and Sambucus nigra lectins. Analysis of crude protein extracts by Western blotting revealed that bands with molecular masses ranging from 19 to 80 kDa were reactive to these lectins, which showed a sugar-inhibited recognition with the parasite extract. These results indicated that molecules containing α2,3- and α2,6-sialylgalactosyl sequences are present in this protozoan. The role of the surface sialomolecules in the interaction with explanted guts from Aedes aegypti was assessed. The interaction of H. megaseliae with the insect gut was strongly inhibited in the presence of mucin (71%), fetuin (68%) and sialyllactose (68%). Collectively, our results suggest a possible involvement of sialomolecules in the interaction between this insect trypanosomatid and the invertebrate host.

Introduction
Sialic acids consist of a family of acidic nine-carbon sugars that are typically located at the terminal positions of a variety of glycoconjugates. Naturally occurring sialic acids show an immense diversity of structure, and this reflects their involvement in a variety of physiologically and pathologically important processes, including microorganism binding that leads to infections, regulation of the immune response and the progression and spread of human malignancies. Cellular recognition in microorganism binding involves the direct participation of sialic acids in recognition events through specific interactions with complementary protein or glycoprotein receptors (Lehmann et al., 2006; Varki & Varki, 2007). Several agglutinins of plant origin have been used in these studies, such as elderberry bark lectin (Sambucus nigra, SNA), seeds of the leguminous plant Maackia amurensis (MAA) and the lectin isolated from the slug Limax flavus (LFA) (reviewed by Schauer & Kamerling, 1997).

The protozoan cell surface is heavily glycosylated (De Souza, 1995). In trypanosomatids, cumulative evidence indicates that glycoconjugates are relevant in several phases of host–parasite interactions such as cellular recognition, adhesion, penetration, survival within host cells, escape from the immune system and cell antigenicity (De Souza, 1995; Colli & Alves, 1999; Descoteaux & Turco, 1999; Chava et al., 2004). The Trypanosomatidae family is comprised of at least 11 distinct genera and includes parasites of plants, insects and vertebrates. However, only two genera, Leishmania and Trypanosoma, are usually found in humans and are etiologic agents of important illnesses such as leishmaniasis, Chagas’ disease and African trypanosomiasis (McGhee & Cosgrove, 1980). Consequently, they have been the focus of extensive research. In addition to these pathogenic parasites, several genera, including Herpetomonas,
Crithidia, Blastocrithidia and Leptomonas, are composed wholly of single host (monoxenic) parasites of the midgut and associated organs of a wide range of insects (McGhee & Cosgrove, 1980). The monoxenic trypanosomatids have been used as models for comparative studies in order to understand the physiology, biochemistry, ultrastructure and molecular biology of the pathogenic species, especially because they are easily cultured under axenic conditions and possess homologues of virulence factors from pathogenic trypanosomatids (reviewed by Santos et al., 2006, 2007). Also, trypanosomatids that are not normally infectious to humans were isolated from immunosuppressed patients, mainly in HIV-positive individuals, in whom the parasites caused either visceral or cutaneous lesions (reviewed by Chiarro & Alvar, 2003). Collectively, these studies emphasize the need for further investigation in the biochemical machinery of the insect trypanosomatids.

Few data are available dealing with the cell surface carbohydrate structures in Herpetomonas megaseliae, a monoxenic trypanosomatid isolated from the phorid fly Megasesia scalaris (Dagget et al., 1972). In this work, we evaluated the surface distribution of sialoglycoconjugates in H. megaseliae by flow cytometry and fluorescence microscopy using fluorescein isothiocyanate (FITC)-labeled lectins and we also determined the sialoglycoprotein profile by Western blotting assay with peroxidase-labeled lectins (LFA, SNA and MAA). In an effort to reveal a functional role for the cell surface glycoconjugates, we analyzed the adhesion process of H. megaseliae to explanted guts from adult Aedes aegypti female mosquitoes.

Materials and methods

Chemicals

Media constituents, reagents used in electrophoresis, Western blotting and buffer components were purchased from Amersham Life Science (Little Chalfont, UK). Low-molecular-weight standards were acquired from Gibco BRL (Grand Island, NY). The lectins LFA, SNA and MAA were obtained from EY Lab (San Mateo, CA). Bovine serum albumin (BSA), mucin, fetuin, sialyllactose and lactose were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were either of analytical grade or superior.

Microorganism and cultivation

Herpetomonas megaseliae (ATCC 30029) was provided by Dr Erney Camargo (Universidade de Sao Paulo, SP, Brazil). The trypanosomatid was maintained by weekly transfers at 26 °C in brain–heart infusion (BHI) medium supplemented with 1% heat-inactivated fetal bovine serum.

Flagellate extracts

Parasites (1.0 × 10⁸ cells) were harvested at the log phase (48 h) by centrifugation at 1500 g for 5 min at 4 °C and washed three times with cold phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2). Cells were then resuspended in 100 μL of PBS and disrupted by sonication lysis at 4 °C following centrifugation at 5000 g for 10 min at 4 °C. The supernatant obtained after centrifugation corresponds to the parasite cellular extract (Santos et al., 2002a,b).

Western blotting analysis

Samples containing the equivalent to 5.0 × 10⁶ cells were added to 10 μL of sample buffer [125 mM Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.002% bromophenol blue] and mixed with 10% (v/v) β-mercaptoethanol, followed by heating at 100 °C for 5 min. Proteins were analyzed in 12% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Electrophoresis was carried out at 4 °C, at 100 V, for 2 h. Subsequently, the polypeptides were electrophoretically transferred at 4 °C at 100 V/300 mA for 2 h to nitrocellulose membranes. The membranes were placed in a blocking solution (150 mM NaCl, 10 mM Tris, pH 7.5, 10% Tween 20, and 5% BSA) for 2 h at room temperature. The membranes were incubated in blocking solution for 1 h with the following peroxidase-labeled lectins (50 μg mL⁻¹): LFA, SNA and MAA. The specificity of the binding was checked by incubating the membrane strips in the presence of each lectin and its specific sugar inhibitor(s) (2 μg mL⁻¹ fetuin plus 2 μg mL⁻¹ sialyllactose, 2 μg mL⁻¹ sialyllactose and 2 μg mL⁻¹ fetuin, respectively). For the visualization of reactive molecules, the membranes were washed five times in the blocking solution and developed by chemiluminescence. The relative molecular masses of the reactive polypeptides were calculated by comparison with the mobility of Gibco BRL SDS-PAGE standards.

Fluorescence microscopy for cell surface sialoglycoconjugates

For fluorescence microscopy analysis, the parasites (1.0 × 10⁷ cells) were fixed for 1 h at room temperature in 0.4% paraformaldehyde diluted in PBS, followed by extensive washing in the same buffer. The fixed cells maintained their morphological integrity, as verified by microscopic observation. The cells were then washed three times in PBS and incubated in the presence or absence of the FITC-labeled lectins (LFA, SNA and MAA) at 50 μg mL⁻¹ for 1 h at room temperature. The cells were washed three times in PBS and observed under a Zeiss epifluorescence microscope (Axioskop 2). The specificity of the binding was checked by the incubation of the parasites in the presence of each lectin...
(LFA, SNA and MAA) and its specific sugar inhibitor(s) (2 μg mL⁻¹ fetuin plus 2 μg mL⁻¹ sialyllactose, 2 μg mL⁻¹ sialyllactose and 2 μg mL⁻¹ fetuin, respectively). The images were digitally recorded using a cooled CCD camera (Color View XS, Analysis GmBH, DE), and analyzed using ANALYSIS system software (AnalySIS, DE).

**Flow cytometry analysis**

For flow cytometry analysis, 1.0 × 10⁷ cells were fixed in 0.4% paraformaldehyde in PBS for 1 h at room temperature, washed three times with PBS and incubated sequentially for 30 min in the same buffer containing 150 mM NH₄Cl and then in 1% BSA in PBS for 1 h. The fixed cells retained their morphological integrity, as verified by microscopic observation. The cells were then washed three times in PBS and incubated in the presence of the FITC-labeled lectins at 50 μg mL⁻¹ for 1 h at room temperature. The specificity of the binding was checked by incubating the parasites in the presence of each lectin (LFA, SNA and MAA) and its specific sugar inhibitor(s) (2 μg mL⁻¹ fetuin plus 2 μg mL⁻¹ sialyllactose, 2 g mL⁻¹ sialyllactose and 2 μg mL⁻¹ fetuin, respectively). After incubation, the parasite-associated fluorescence was excited at 488 nm and quantified on a fluorescence-activated cell sorter FACSCalibur (BD Bioscience). At least 50,000 cells were analyzed per sample with four repeats per experiment. Control cells were first analyzed to determine their auto fluorescence (Santos et al., 2002a, b; d’Avila-Levy et al., 2005). Windows Multiple Document Interface Flow Cytometry Application (WINMDI) was used to generate the histogram.

**Protozoa–insect gut interaction**

Adult female mosquitoes (A. aegypti) were reared and maintained at Laboratório de Fisiologia e Controle de Vetores, Departamento de Entomologia (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil). The binding of protozoa to insect guts was performed as described previously (Pimenta et al., 1992). The dissected guts were treated for 15 min with the following glycoconjugates used as inhibitors: fetuin, mucin, sialyllactose and lactose at 250 μg mL⁻¹. The trypansomatids (2.0 × 10⁶ cells in 200 μL) were then added to 10 dissected guts per group and incubated for 1 h at room temperature in PBS. The interaction of the trypansomatids with untreated insect guts was used as a control. The guts were then extensively washed with PBS, individually transferred to microcentrifuge tubes containing 40 μL of PBS and homogenized. The released trypansomatids were counted in a Neubauer chamber. The dissected guts are preserved under the experimental conditions used (Fampa et al., 2003). Results are shown as the mean ± SE of the mean of two independent experiments. The data were analyzed statistically by means of Student’s t-test using EPI-INFO 6.04 (Database and Statistics Program for Public Health) computer software.

**Results**

In the present work, the sialoglycoprotein composition of *H. megaseliae* was examined using lectins with monosaccharide specificity for sialic acid-α2,3-galactose (SAα2,3Gal) (MAA), SAα2,6-Gal (SNA) and N-acetylneuraminic acid (LFA). The flagellate contains glycoconjugates that bind to the three lectins (Figs 1–3). Generally, all applied lectins showed a sugar-inhibited reaction with parasite extracts. The molecular masses of the glycoproteins identified by lectin blots were very broad, ranging from 19 to 80 kDa. The LFA agglutinin recognizes all sialic acid linkages, with a major predilection for N-acetylneuraminic acid rather than N-glycolyneuraminic acid residues. This lectin revealed the most complex pattern of sialoglycoconjugates, comprised of at least eight major bands migrating approximately at 19, 22, 25, 30, 40, 46, 60 and 80 kDa. The lectin binding was specifically inhibited in the presence of 2 μg mL⁻¹ fetuin plus 2 μg mL⁻¹ sialyllactose (Fig. 1).

SNA and MAA recognize sialic acids in α2,6 and α2,3 linkages, respectively. For SNA binding, galactose or N-acetyl-d-galactosamine is the required sugar unit, whereas for MAA, the necessary underlying sequence is Galβ1-4-N-acetyl-d-glucosamine. When we used the SNA agglutinin, only the 25 and 30 kDa bands were not detected. The binding was completely inhibited by 2 μg mL⁻¹ sialyllactose. The MAA agglutinin generated a pattern composed of 19, 40, 46, 60 and 80 kDa bands. The binding was totally inhibited by 2 μg mL⁻¹ fetuin (Fig. 1).

![Fig. 1](image-url)

Western blotting showing the sialoglycoproteins detected in *Herpetomonas megaseliae*. The gel strips containing the extract from 5 × 10⁶ cells were revealed with peroxidase-labeled LFA, SNA or MAA. The lanes (i) represent nitrocellulose membrane strips preincubated with each specific sugar inhibitor at 2 μg mL⁻¹ fetuin plus sialyllactose, sialyllactose or fetuin, respectively. The numbers on the left indicate the relative molecular mass markers expressed in kilodaltons.
The flow cytometry and fluorescence microscopy assays using FITC-labeled lectins (Figs 2 and 3) showed that *H. megaseliae* intact cells are recognized by LFA, SNA and MAA. The results from microscopy revealed that LFA binding was spread throughout the cytoplasm. The SNA lectin was detected at the parasite surface, concentrated in spots, while MAA appears to be expressed on the surface, but also in the cytoplasm. The specificity of the binding was checked by the incubation of the parasites in the presence of each FITC–lectin; fetuin + MAA, cells previously incubated with the sugar inhibitor and then incubated with FITC–MAA. Scale bar = 10 μm.

The flow cytometry analysis revealed that after incubation of the parasites with the three lectins (Fig. 3), two distinct populations with different affinities for the lectins were identified in *H. megaseliae* cells, indicating that sialic acids are not equally expressed by this parasite.

Aiming to assess a possible role of *H. megaseliae* cell surface saccharides in the parasite adhesion to insect guts, we have performed binding assays with *A. aegypti* explanted guts. Our data showed that the trypanosomatid adhesion was significantly inhibited in the presence of fetuin (68%) and mucin (71%), which are sialic acid-rich glycoconjugates (Fig. 4). These molecules could either block specific receptors for the protozoa binding, or may be masking the

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**Fig. 2.** Binding of FITC–lectins to the cell surface of *Herpetomonas megaseliae*. Experimental systems were analyzed under differential interferential contrast images and fluorescence. Control, untreated cells were analyzed for autofluorescence. LFA, SNA and MAA, cells incubated with each FITC–lectin; fetuin + MAA, cells previously incubated with the sugar inhibitor and then incubated with FITC–MAA. Scale bar = 10 μm.

**Fig. 3.** Flow cytometric analysis of FITC–lectin binding to *Herpetomonas megaseliae* surface. Analysis of 50,000 cells is shown. Analysis of unstained cells (a), cells incubated with FITC–LFA (b), FITC–SNA (c) and FITC–MAA (d).

**Fig. 4.** *In vitro* binding of *Herpetomonas megaseliae* to guts of *Aedes aegypti*. Untreated insect guts (control), and insect guts previously incubated with the following molecules: fetuin, mucin, sialyllactose or lactose were analyzed. The values represent the mean of two independent experiments, which were performed with 10 explanted guts per analysis. The bars represent the SE of the mean. Values statistically different from control (*P < 0.001*) are marked with an asterisk in the figure.
receptor by binding at high density on adjacent sugars, due to their size. This prompted us to perform binding assays in the presence of sialyllactose, which also caused a significant reduction in the protozoa binding (68%). In order to assess whether lactose, rather than the sialyl groups, was responsible for this reduction, we have shown that this disaccharide did not cause a significant reduction in the binding of *H. megaseliae* to the insect gut (Fig. 4).

**Discussion**

The majority of carbohydrates exposed on the plasma membrane of eukaryotic cells are associated with glycoproteins or glycolipids. Valuable information on the structure and function of cell surface saccharides is derived from studies with lectins. Several techniques using lectins have been performed in order to analyze the distribution of sialic acids in trypanosomatids (reviewed by Souto-Padrón, 2002; Chava *et al*., 2004). A combination of Western blotting, flow cytometry and fluorescence microscopy analysis has been used only recently in these microorganisms to identify and characterize sialoglycoconjugates in parasites (Santos *et al*., 2002b; Romeiro *et al*., 2003; d’Avila-Levy *et al*., 2005).

Sialic acids are a family of carboxylated nine-carbon sugars found in glycoproteins and glycolipids at the non-reducing end of oligosaccharides, linked to galactose and *N*-acetyl-β-galactosamine (Schauer & Kamerling, 1997). Sialoglycoproteins can contain both SA2,6Gal and SA2,3Gal sequences, and the type of linkage depends on individual glycoproteins. The present study demonstrates for the first time by Western blotting analysis that the *H. megaseliae* sialoglycoconjugates show a slight prevalence of SA2,6Gal over SA2,3Gal linkages. This has already been observed in the *C. falciparum* (Mattia *et al*., 1999; d’Avila-Levy *et al*., 2004, 2005), *Phytomonas* (Santos *et al*., 2002a) and *Blastocrihtidia* (d’Avila-Levy *et al*., 2005) genera, and also in *Herpetomonas samuelepessai* (Santos *et al*., 2002b). Binding to the same component by lectins MAA and SNA probably indicates a carbohydrate moiety composed of α2,3- and α2,6-linked sialic acid residues, respectively. The majority of the sialoglycoconjugates identified in the present study possess these two moieties (19, 40, 46, 60 and 80 kDa), while the 22-kDa band contained just SA2,6Gal sequences.

Sialic acids are the most abundantly available monosaccharides present as the terminal residue of cell surface sugar chains. Its strategic terminal position provides accessibility to it, reflected in its regulation of a multitude of cellular and molecular interactions (Kelm & Schauer, 1997). The sialoglycoconjugates identified at the cellular surface of *H. megaseliae* could be directly involved in some parasite-host cell (insect) interaction, such as preferential host site colonization and the mode of attachment of the flagellates to the host tissue. In addition, the presence of sialic acids in proteins may induce conformational changes in the glycoconjugates (Schauer & Kamerling, 1997), which may protect proteins from enzymatic hydrolysis (Fukuda, 1991), or, as they do in other trypanosomatids, modulate infectivity, parasite survival, interaction with host cells or protect parasites against immunological destruction (Chava *et al*., 2004). The presence of such protective structures may be important for parasite growth and differentiation in the insect vector, survival against the action of digestive enzymes and escaping from the insect’s immune response. Finally, with regard to conformation, sialic acids may contribute to the specific structure of a given glycoconjugate molecule and may thus influence its biological activity, for example, by masking its receptors or promoting the interaction with specific receptors on the cell surface of the insect host. It should be pointed out that the sialoglycoconjugates detected in the present report are not exclusively restricted to the protozoa cell surface.

In this context, we assessed a possible role of *H. megaseliae* cell surface saccharides using explanted *A. aegypti* guts previously incubated with different glycoconjugates. Our results indicated that mucin-, fetuin- and sialyllactose-treated guts showed a significant reduction in trypanosomatid binding. These observations suggest a probable involvement of sialomolecules in the adhesion process of this insect trypanosomatid to *A. aegypti* guts, an insect vector experimental model (reviewed by Santos *et al*., 2006). Previous studies from our group revealed that other insect trypanosomatids, *Blastocrihtidia culicis* and *C. falciparum*, also possess sialoglycoconjugates at the cellular surface that are involved in insect gut recognition and adhesion (d’Avila-Levy *et al*., 2005).

Glycoconjugates have been implicated in trypanosomatid interaction with the insect vector. *Leishmania* glycoconjugates, for instance, include molecules that share a common phosphoglycan repeat unit. These phosphoglycans consist of glycoproteins, such as cell surface and released proteophosphoglycans, and glycolipids such as lipophosphoglycan. Glycoconjugates also include non-phosphoglycan-containing molecules, such as the major surface glycoprotein, the metalloprotease gp63 (Kamhawi, 2006). Among these, lipophosphoglycans is the largest and most abundant surface glycoconjugate of promastigotes. Genetic and biochemical studies have implicated lipophosphoglycan in the attachment of leishmanial nectomonads to the sand fly midgut, preventing their loss with the excreted blood meal (Sacks *et al*., 2000; Ilg, 2001; Sacks & Kamhawi, 2001; Soares *et al*., 2002). The interaction between *A. aegypti* and monoxenic trypanosomatids is an excellent model to help elucidate their biological interplay. It has been shown that *Herpetomonas* sp. is found repeatedly in this insect (Weinman & Cheong, 1978), which has been used as an experimental model to study the interaction between monoxenic...
trypanosomatids and insect hosts (Fampa et al., 2003; d’Avila-Levy et al., 2005, 2006, 2008; Nogueira de Melo et al., 2006; Santos et al., 2006; Pereira et al., 2008). Intriguingly, experimental infection of A. aegypti by B. culcis, a monoxenic trypanosomatid, shows that this parasite remains in the digestive tract of the insect for 38 days after feeding (Corrêa-da-Silva et al., 2006), indicating that trypanosomatids can survive and colonize A. aegypti. Finally, we have previously shown that H. megaseliae adheres more readily to A. aegypti midgut than to the gut of its natural host, M. scalaris (Nogueira de Melo et al., 2006). Also, the adhesion process in these two insects seemed to be at least partially mediated by gp63-like molecules (Nogueira de Melo et al., 2006). The role of gp63 in the life cycle of Leishmania during the insect colonization is poorly explored. Nevertheless, it is known that gp63 is predominantly expressed on the surface of promastigotes residing in the midgut of the phlebotomine sandfly vector (Grimm et al., 1987; Davies et al., 1990). However, data obtained from gp63 knockouts in Leishmania major do not support a major role for this metallopeptidase in parasite survival in the insect (Joshi et al., 2002). Conversely, in Leishmania amazonensis the downregulation of gp63 adversely affected its early development in the neotropical Lutzomyia longipalpis sand fly (Hajmová et al., 2004).

The Western blotting, flow cytometry and fluorescence microscopy analyses were performed with parasites obtained from an axenic culture, and consequently one cannot rule out the possibility that the carbohydrate expression in the invertebrate host may be directly influenced by the protozoa–insect interaction. Accordingly, Romeiro et al. (2003) showed a variable lectin-binding pattern in Leptomonas wallacei depending on the source of the parasite. For instance, an analysis with parasites isolated directly from its natural host (Oncopeltus fasciatus) showed that during the passage from the midgut to the hindgut, the surface of the parasites evolves such that glycoconjugates containing galactose and N-acetyl-d-galactosamine are newly expressed. Furthermore, cultured forms present a carbohydrate profile similar to the hindgut-isolated parasite. Therefore, further studies using direct isolates from the insect host may be necessary to define whether there other sialic acid-rich molecules are also involved in parasite adhesion to the invertebrate host gut.

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References


