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Identification of sialic acids on *Leishmania donovani* amastigotes

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Abstract

The presence of Neu5Ac on promastigotes of *Leishmania donovani*, the causative organism of Indian visceral leishmaniasis, has been reported recently. Here we report the occurrence of Neu5Ac as a major component on amastigotes, as well as Neu5Gc, Neu5,9Ac2 and Neu9Ac5Gc as indicated by fluorimetric high performance liquid chromatography and gas liquid chromatography/electron impact mass spectrometry. Furthermore, binding studies with *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), and various Siglecs, showed the presence of both ($\alpha2\rightarrow6$)- and ($\alpha2\rightarrow3$)-linked sialic acids; their binding was reduced after sialidase pretreatment. Western blotting of amastigote membrane glycoproteins with SNA demonstrated the presence of two sialoglycoconjugates of M_r values of 164 000 and 150 000. Similarly, binding of MAA demonstrated the presence of five distinct sialoglycans corresponding to molecular masses of 188, 162, 136, 137 and 124 kDa. Achatinin-H, a lectin that preferentially identifies 9-*O*-acetylated sialic acid ($\alpha2\rightarrow6$)-linked to GalNAc, demonstrated the occurrence of two 9-*O*-acetylated sialoglycans with M_r 158 000 and 150 000, and was corroborated by flow cytometry; this binding was abolished by recombinant 9-*O*-acetylesterase pretreatment. Our results indicate that Neu5Ac [($\alpha2\rightarrow6$)- and ($\alpha2\rightarrow3$)-linked], as well as Neu5Gc and their 9-*O*-acetyl derivatives, constitute components of the amastigote cell surface of *L. donovani*.

Keywords: Achatinin-H; amastigotes; *Leishmania donovani*; *O*-acetylated sialic acid; visceral leishmaniasis.

Introduction

Protozoan parasites of the genus *Leishmania* are obligate intracellular parasites that reside in mononuclear phagocytes. They cause a wide spectrum of clinical manifestations resulting in substantial morbidity and mortality, and 10% of the world's population is at risk of infection. Depending on the causative species and immunological state of the host, the major clinical presentations range from a simple cutaneous lesion to the disfiguring mucocutaneous leishmaniasis and the visceralized form (kala-azar) that could be fatal if left untreated (Guerin et al., 2002).

Cell membrane-bound carbohydrates play a key role in parasite survival and proliferation. Most of these specialized molecules are members of a family of phosphoglycans, while others are members of a family of glycoinositol phospholipids (Ferguson, 1999). Studies have shown the presence of *N*-acetyl- and *N*-glycolylneuraminic acid on *Trypanosoma* species (Schauer et al., 1983). However, the topography of *Leishmania* parasites with regard to their sialoglycan profile remains a poorly investigated area and it was only recently that the status of sialoglycans on *Leishmania donovani* promastigotes was reported (Chatterjee et al., 2003).

Sialic acids are typically present as terminal residues on glycoproteins, and glycolipids are known to play a significant role in the mediation of many biological phenomena involving cell-cell and cell-matrix interactions by either reacting with specific surface receptors or masking other carbohydrate recognition sites (Kelm and Schauer, 1997; Schauer, 2000; Angata and Varki, 2002). Among over 40 diverse structural modifications of the parent molecule, the most common are *O*-acetyl substitutions at C-4, C-7 and/or C-9 (Schauer and Kamerling, 1997). Taking into account that *O*-acetyl esters at the C-7 position are known to migrate to the C-9 position (Kamerling et al., 1987; Vandamme-Feldhaus and Schauer, 1998), 9-*O*-acetylated sialic acids (9-*O*AcSA) usually predominate, generating a family of 9-*O*AcSGs (9-*O*-acetylated sialoglycoconjugates) (Varki, 1992; Schauer, 2000).

Currently available data of the cell surface architecture and biochemistry of several microorganisms like fungi, bacteria and protozoa indicate that sialic acid and its principle derivatives are major components of their glycocalyx. They assist the cells in interactions with their external environment through functions ranging from survival, infectivity and host-cell recognition such as anti-recognition molecules, thereby aiding the pathogen to evade host defense mechanisms (Vimr and Lichtensteiger, 2002). Thus it may be envisaged that sialic acids and their derivatives could play an important role in pathophysiological conditions in visceral leishmaniasis and, accordingly, merit further investigation. We have recently reported the presence of sialic acids and their predomi-

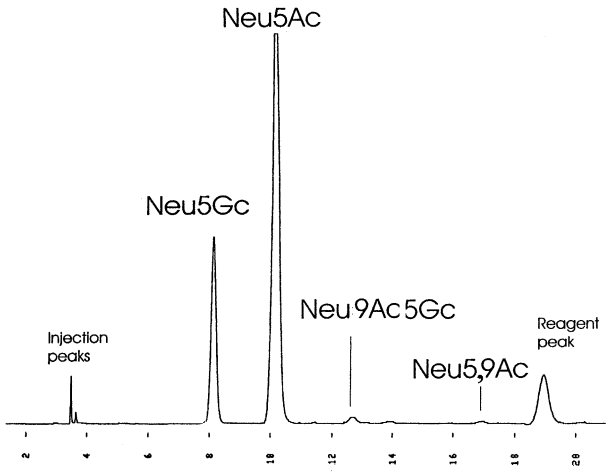

Figure 1 Fluorimetric HPLC analysis of sialic acids on *Leishmania donovani* amastigotes.
Glycosidically-bound sialic acids were released with 2 M acetic acid (3 h, 80°C), derivatized with 1,2,-diamino-4,5-methylenedioxybenzene, and analyzed as described in section materials and methods.

nant derivative 9-OAcSA on *Leishmania donovani* promastigotes (Chatterjee et al., 2003). Considering the biological relevance of sialic acids in protozoal diseases, in the present study we investigated the status of sialic acids in amastigotes, the biologically relevant form of the parasite. We detected both ($\alpha2\rightarrow6$)- and ($\alpha2\rightarrow3$)-linked *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), as well as their 9-*O*-acetylated derivatives Neu5,9Ac_2 and Neu9Ac5Gc, on the parasite surface. We envisage that identification of these sialylated determinants on amastigotes will open up new avenues of research.

Results

Achatinin-H preferentially binds to 9-*O*-acetylated sialoglycoconjugates

In earlier studies it was shown that Achatinin-H preferentially binds to bovine submandibular mucin (BSM), which has 9-*O*AcSA as a terminal constituent of *O*-linked glycans, ($\alpha2\rightarrow6$)-linked to GalNAc (Reuter et al., 1983). The strong inhibition of binding with purified 9-*O*AcSA and the absence of inhibition with 4-*O*-acetylated sialic acid pointed toward its specificity for 9-*O*AcSA (Mandal and Basu, 1987; Mandal et al., 1989; Mandal and Mandal, 1990; Sen and Mandal, 1995). No inhibition occurred with de-*O*-acetylated BSM and asialo-BSM, reconfirming the lectin specificity. Sheep submaxillary mucin having a terminal sialic acid in either $\alpha2\rightarrow6$ or $\alpha2\rightarrow3$ linkage did not show any inhibition. These observations clearly indicate that the *O*-acetyl substituent at C-9 is crucial for lectin binding (Sen and Mandal, 1995).

Identification of sialoglycans on *L. donovani* amastigotes

The presence of sialic acids on *L. donovani* amastigotes was investigated by fluorimetric high performance liquid chromatography (HPLC) following the release of sialic acids with 2 M acetic acid and labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB). In Figure 1, a representative profile of this analysis is shown for amastigotes. The chromatogram exhibited two well-resolved, intense peaks, which coincide with that of Neu5Gc and Neu5Ac, and two small peaks co-migrating with Neu9Ac5Gc and Neu5,9Ac_2. The quantitative data for the four sialic acids is shown in Table 1 and was determined by adding a measured amount of DMB-Neu5Ac. The total amount of sialic acids was quantified to be 658 ng/1.6×10^9 cells, of which the majority is Neu5Ac (385 ng). To exclude that sialic acids were not loosely adhered to the cell surface, the parasites were extensively washed three times (50 ml per wash) with phosphate-buffered saline (PBS) and the washes were similarly examined for their sialic acid content; the final wash contained only 2 ng of Neu5Ac (Table 1). The number of copies of sialic acid residues per cell was estimated to be 12.8×10^5.

Confirmation of the assigned (9-*O*-acetylated) sialic acids was obtained by gas liquid chromatography/electron impact mass spectrometry (GC/EI-MS) analysis of their trimethylsilyl ether, methyl ester derivatives (Kamerling and Vliegenthart, 1982). Typical fragment ion values (structural reporter peaks) for each sialic acid are presented in Table 2.

Cell surface localization and linkage specificity of sialic acids on *L. donovani* amastigotes

The surface density of sialoglycoconjugates present on *L. donovani* amastigotes was examined by flow cytometric analysis using two sialic acid binding plant lectins, *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), which recognize Neu5Ac ($\alpha2\rightarrow6$) Gal/GalNAc and Neu5Ac ($\alpha2\rightarrow3$) Gal, respectively (Shi-

Table 1 Quantitative analysis of sialic acids on *Leishmania donovani* amastigotes.

Derivative	Amastigotes ng/1.6×10^9 cells[b]	Wash 1[a]	Wash 2[a]	Wash 3[a]
Neu5Gc	175	33	23	14
Neu5Ac	385	4	2	2
Neu9Ac5Gc	12	2	1	1
Neu5,9Ac_2	4	–		
	576 (87.5%)	39 (5.9%)	26 (4.0%)	17 (2.4%)

[a] Cells were extensively washed three times with PBS (50 ml per wash) and each aliquot was similarly analyzed for estimation of sialic acids.
[b] Sialic acids were determined by HPLC analysis as described in section materials and methods. The % distribution of total sialic acids (658 ng) in each aliquot is indicated in parentheses.

Table 2 Qualitative analysis of sialic acids on amastigotes by GC/EI-MS.

Sialic acid	Retention	R_{Neu5Ac} time (min)	Mass fragment ions A-F[a] (*m/z*)
Neu5Ac	13.6	1.00	668, 624, 478, 400, 317, 298
$Neu5,9Ac_2$	15.2	1.12	638, 594, 478, 400, 317, 298
Neu5Gc	23.6	1.74	756, 712, 566, 488, 386, 317
Neu9Ac5Gc	27.0	1.98	726, 682, 566, 488, 386, 317

[a] For an explanation of the mass fragment ions A-F, see Kamerling and Vliegenthart (1982).

buya et al., 1987; Wang and Cummings, 1988). As demonstrated in Figure 2 and Table 3, the parasites had a predominance of ($\alpha 2 \rightarrow 6$)-linked sialic acids as the binding of SNA was three-fold higher than MAA, $87.7 \pm 6.5\%$ vs. $27.7 \pm 3.4\%$, respectively. Its specificity toward Neu5Ac was confirmed by the diminished binding after sialidase treatment. Following sialidase treatment the binding of SNA and MAA was reduced to $30.6 \pm 8.5\%$ and $4.0 \pm 5.6\%$, respectively, representing decreases of 65% and 84% (Figure 2). We observed that the *O*-acetyl moiety inhibited sialidase activity, because treatment with *O*-acetylesterase followed by sialidase decreased the binding of SNA further to 7.7%; the binding of MAA remained unaffected.

Binding of Siglecs indicates the presence of both ($\alpha 2 \rightarrow 3$)- and ($\alpha 2 \rightarrow 6$)-linked sialic acids

Corroborative evidence for the presence of ($\alpha 2 \rightarrow 3$)- and ($\alpha 2 \rightarrow 6$)-linked sialic acid-containing glycans on *L. donovani* amastigotes was provided by the binding of various recombinant sialic acid-binding lectins (Siglecs). Although the binding pattern showed wide variation, ranging from 40–65%, most Siglecs tested showed a certain degree of binding (Table 3). Siglecs exhibit widely differing preferences for the linkages between sialic acid and subterminal monosaccharides. For example, CD22/Siglec-2 binds only to ($\alpha 2 \rightarrow 6$)-linked sialic acids, whereas sialoadhesin/Siglec-1 prefers ($\alpha 2 \rightarrow 3$)-linked sialic acids and Siglec-5 binds epitopes with both linkages. Taken together, our results indicate that both ($\alpha 2 \rightarrow 3$)- and ($\alpha 2 \rightarrow 6$)-linked sialic acids are present on *Leishmania donovani* amastigotes.

Molecular characterization of sialoglycans present on the cell surface of *L. donovani* amastigotes

In order to define the molecular determinants on the parasite surface that are sialylated, the reactivity of SNA and MAA was examined on Western blots of amastigote

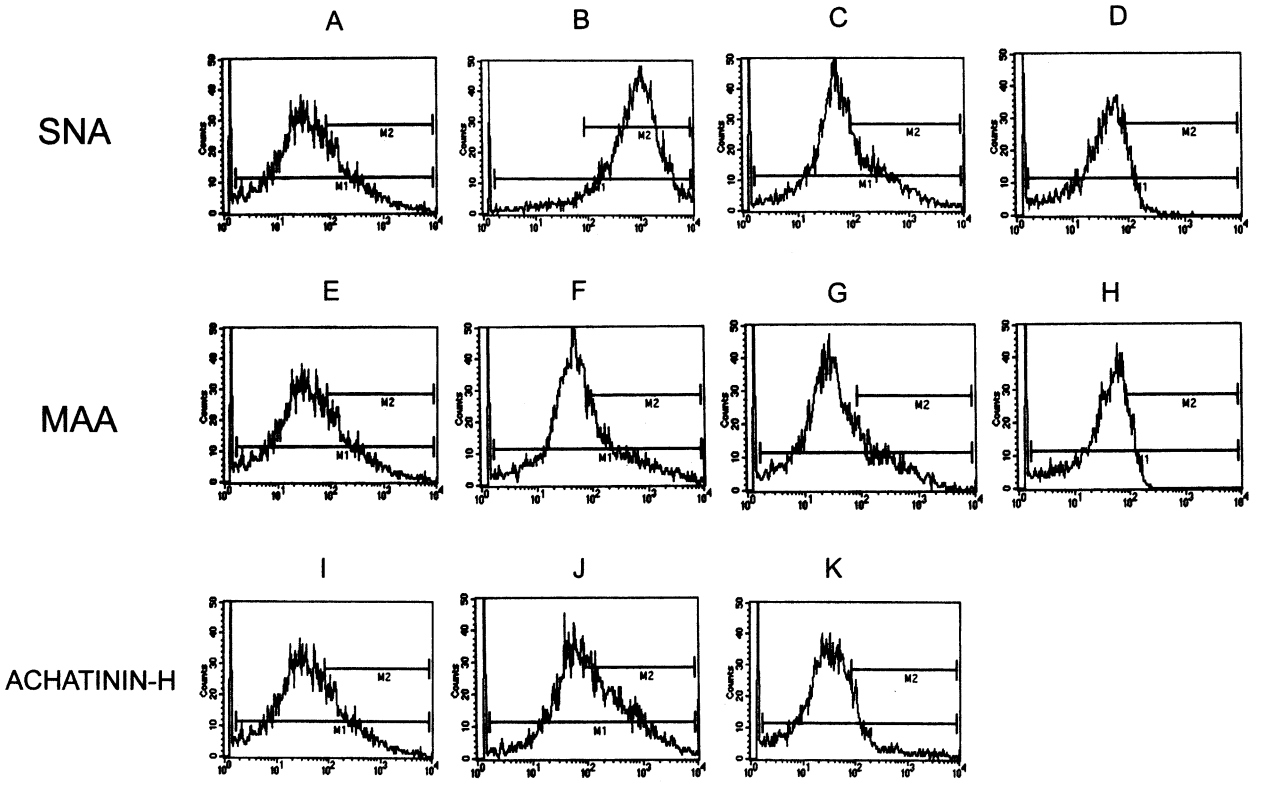

Figure 2 Flow cytometric analysis of cell surface sialoglycans on *Leishmania donovani* amastigotes using SNA (A-D), MAA (E-H) and achatinin-H (I-K).
Parasites were incubated in the absence (A, E and I) and presence of SNA (B), MAA (F) and Achatinin-H (J) and binding was measured as described in section materials and methods. The binding specificity was demonstrated by pre-incubating the cells with either sialidase (C, G) or sialidase and *O*-acetylesterase (D, H) or *O*-acetylesterase (K) as described in section materials and methods. The bar (M2) represents the cell population positive for the lectin staining.

Table 3 Lectin binding patterns in *Leishmania donovani.*

Probe	Linkage specificity	Occurrence	Binding[a] (%)	
			Promastigotes[b]	Amastigotes
SNA	$\alpha2\rightarrow6$	*Sambucus nigra agglutinin*	72.0±14.3	87.7±6.5
MAA	$\alpha2\rightarrow3$	*Maackia amurensis agglutinin*	41.0±6.3	27.7±3.4
Achatinin-H	9-OAcSA$\alpha2\rightarrow6$ GalNAc	*Achatina fulica* snail	44.3±3.4	49.3±4.5
Siglec-1	$\alpha2\rightarrow3>\alpha2\rightarrow6$	Macrophages	17.3±3.7	48.0±2.7
Siglec-2	$\alpha2\rightarrow6\gg\alpha2\rightarrow3$	B cells	50.5±11.4	54.0±3.5
Siglec-5	$\alpha2\rightarrow3=\alpha2\rightarrow6$	Neutrophils, myeloid cells	31.4±3.7	60.0±2.6
Siglec-7	$\alpha2\rightarrow6>\alpha2\rightarrow3$	NK cells, monocytes	19.0±4.3	61.0±3.1
Siglec-8	$\alpha2\rightarrow3$	Eosinophils	28.6±4.2	65.0±3.7
Siglec-10	$\alpha2\rightarrow3=\alpha2\rightarrow6$	Myeloid cells	32.5±1.5	40.0±2.2

[a] Mean±SD of three independent experiments of percent positive cells as determined by FACS analysis using sialic acid binding probes as described in section materials and methods.
[b] Chatterjee et al., 2003.

membranes (Figure 3). Using SNA, two sialoglycoproteins with molecular masses of 164 and 150 kDa were identified on the parasite membranes (Figure 3, lane 2). In the case of MAA, five distinct sialoglycans were identified with molecular masses of 188, 162, 136, 137 and 124 kDa (Figure 3, lane 1). The binding specificity of SNA and MAA was confirmed by the absence of binding following sialidase treatment.

Abolition of binding of Achatinin-H by esterase treatment reconfirmed that parasite membranes have glycoconjugates with terminal 9-*O*-acetylated sialic acid

The strong binding of Achatinin-H (49.0±4.5%) to *L. donovani* amastigotes, as demonstrated by flow cytometry (Figure 2), indicated the presence of cell surface 9-*O*AcSA. To confirm that this binding is directed toward the 9-*O*-acetyl moiety, cells were incubated with a recombinant *O*-acetylesterase; the resultant de-*O*-acetylation caused a 4.8-fold reduction in lectin binding, from 49.3±4.5% to 10.2±3.7%, thus supporting the presence of 9-*O*-acetylated glycotopes on amastigotes.

Molecular determinants recognized by Achatinin-H

To further characterize the surface 9-*O*AcSGs on *L. donovani* amastigotes, Western blotting was performed using Achatinin-H. We observed that Achatinin-H bound to two 9-*O*AcSGs with molecular masses of 158 and 150 kDa.

Discussion

Recently a growing interest in the pathophysiological role of microbial sialoglycoconjugates has arisen following the identification of sialic acids and their 9-*O*-acetylated derivatives on the cell surface of viruses, bacteria, fungi and protozoan species (Crocker and Varki, 2001a). Leishmanial parasites have adapted not only to survive, but also to proliferate, largely due to protection conferred by unique glycoconjugates that include a family of phosphoglycans and glycoinositol phospholipids whose principal features are the presence of Gal ($\beta1\rightarrow4$) Man ($\alpha1\rightarrow PO_4$) repeating units (Ferguson, 1999; Turco et al., 2001). We have recently demonstrated the presence of sialic acid derivatives on the surface of promastigotes of *Leishmania donovani* parasites, but the status of these glycotopes in amastigotes remained undetermined (Chatterjee et al., 2003). Accordingly, in this study we have analyzed the status of sialic acid derivatives on the cell surface of *L. donovani* amastigotes.

To investigate the linkage specificity of these sialoglycans, two sialic acid binding lectins, e.g. SNA and MAA, were used. The binding of SNA was distinctly higher, indicating predominance of the Neu5Ac ($\alpha2\rightarrow6$) Gal/GalNAc glycotope. Binding specificities of SNA and MAA toward Neu5Ac were verified by sialidase pretreatment that resulted in a reduction in binding of 65% and 85%, respectively. The more complete removal of $\alpha2\rightarrow3$ linked sialic acids may be attributed to the faster hydrolysis of $\alpha2\rightarrow3$ linked sialic acids than those with $\alpha2\rightarrow6$ linkages (Corfield et al., 1983, 1986). Interestingly, we have observed that the *O*-acetyl moiety inhibited sialidase activity, as treatment with *O*-acetylesterase followed by sialidase decreased the binding of SNA further to 7.7%.

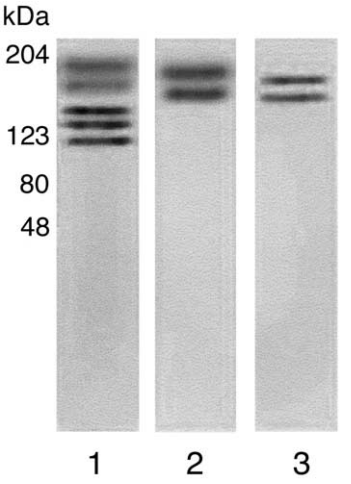

Figure 3 Western blot analysis of sialoglycoproteins present on *Leishmania donovani* amastigotes.
Membrane proteins (20 μg/lane) from *L. donovani* amastigotes were electrophoresed (10% SDS PAGE) and transferred onto nitrocellulose. The membranes were incubated with biotinylated MAA (lane 1) or biotinylated SNA (lane 2) or Achatinin-H (lane 3). Binding was detected using peroxidase-conjugated streptavidin as described in section materials and methods.

Overall, the proportion of binding of SNA and MAA to amastigotes was similar to that observed with promastigotes.

Siglecs, members of the immunoglobulin superfamily, bind to sialic acids and are mainly expressed by cells of the hematopoietic system (Powell and Varki, 1994; Crocker and Varki, 2001b). In general, the binding of Siglecs was two- to three-fold higher in amastigotes than in promastigotes. Like promastigotes, the interaction of Siglec-1, -2, -5, -7, -8 and -10 with amastigotes exhibited a complex pattern of binding specificities (see Table 3) that may be due to the subterminal linkages as well as penultimate sugars present. Accordingly, the binding of Siglecs is difficult to correlate with the total sialic acid and the binding data of MAA and SNA. A notable difference was the three-fold higher binding of amastigotes, as compared to promastigotes, with Siglec-1, which is abundantly present on macrophages. It remains to be investigated whether these sialoglycans are important for the infectivity and intracellular survival of the parasite within the macrophage.

To assess the status of 9-*O*AcSAs, we used a lectin, Achatinin-H, which preferentially binds to sialoglycans containing the 9-*OAcSA* ($\alpha 2 \rightarrow 6$) GalNAc glycotope (Sen and Mandal, 1995). Flow cytometric analysis demonstrated binding of Achatinin-H to amastigotes ($49.0 \pm 4.5\%$); its specificity toward *O*-acetylated determinants was confirmed by an 80% reduction in binding following 9-*O*-acetylesterase treatment. Under the given experimental conditions, we were unable to achieve near complete removal of the sialic acids and the *O*-acetyl moiety. There are two likely possibilities; first, we could have achieved this by increasing the enzyme concentration and/or incubation time. Alternatively, the existence of an additional epitope that is resistant to enzymatic cleavage cannot be ruled out. Although we were unable to demonstrate the presence of the *O*-acetylated sialoglycans on promastigotes by mass spectrometry, the presence of such derivatives was proven by GC/EI-MS in amastigotes, possibly indicating the presence of higher amounts of 9-*OAc*SA. Another notable difference is the presence of Neu5Gc, not observed on promastigotes by a similar analysis (Chatterjee et al., 2003). Although Neu5Gc is a major sialic acid derivative in most mammals (including our closest evolutionary relatives, the great apes; Muchmore et al., 1998), it is thought to be absent in healthy humans (Schauer, 1982). Considering the wide differences in Neu5Gc expression in certain parasitic diseases (Karlsson et al., 2000), it is important to check the status of this sugar and its functional relevance in *Leishmania donovani*; such studies are ongoing. HPLC analysis also demonstrated that amastigotes have a 2-fold higher copy number of Neu5Ac than promastigotes (Chatterjee et al., 2003).

Western blotting of amastigote membranes with SNA and MAA clearly showed discrete sialoglycoprotein bands that were different from those obtained with promastigotes (Chatterjee et al., 2003). This suggests that during transformation to the amastigote form, parasites acquire a new array of sialoglycans on their surface. The obvious question that arises is: what is/are the source(s) of these sialoglycans? It is possible that sialic acids are either adsorbed from the host cells or are 'borrowing' sialoglycans from intracellular pools. We have previously shown that *L. donovani* promastigotes acquire Neu5Ac from the growth medium by direct incorporation of serum components (Chatterjee et al., 2003). In contrast, *Trypanosoma cruzi* acquires sialic acid by transglycosylation (Pereira-Chiocolla et al., 2000).

Another unanswered question is the source for the presence of the *O*-acetylated derivative on amastigotes. The metabolism of *O*-AcSA is under the control of two groups of enzymes, *O*-acetyltransferases and 9-*O*acetylesterases (Klein and Roussel, 1998). *O*-acetyltransferases are difficult to purify, and attempts to clone their genes have failed in isolating the true 9-*O*-acetyltransferase cDNA, accounting for the limited information regarding expression of *O*-AcSA (Klein and Roussel, 1998). The capsular polysaccharide of *Escherichia coli* K1 is a linear polymer of *N*eu5Ac in $\alpha 2 \rightarrow 8$ linkage. Certain substrains of *E. coli* K1 (designated *O*Ac+) modify the polysaccharide by *O*-acetylation of sialic acids. This modification occurs by an acetyl-coenzyme A:polysialosyl *O*-acetyltransferase, and was found only in *E. coli* K1 *O*Ac+ substrains. Thus, expression of this enzyme can account for the *O*Ac+ phenotype and the variation in forms between *O*Ac+ and *O*Ac− substrains (Higa and Varki, 1988). In this context it is worthwhile to consider whether such a system exists in *L. donovani* amastigotes; such studies are underway. Parasitologic research is limited by the lack of availability of genomic data on protozoa and therefore the search for genes possibly involved in the biosynthesis, activation or transfer of sialic acids in protozoa has to be continued in the future (Angata and Varki, 2002).

Materials and methods

Parasites

Promastigotes of an Indian *Leishmania donovani* strain MHOM/IN/83/AG83 (Chatterjee et al., 2003) were grown at 22–25°C in M199 medium containing HEPES buffer (20 mM, pH 7.5) supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). Amastigotes were obtained from golden hamsters that were infected with stationary phase promastigotes (1×10^6 cells/100 μl). After 4–6 weeks, hamsters were sacrificed and spleen tissue was collected in sterile PBS.

Amastigote suspensions were prepared by homogenization of excised spleen lesions in sterile PBS containing 6% glucose, disrupted by four passages through 22-gauge needles, and centrifugation at 250 *g* for 10 min. The resultant supernatant was centrifuged at 1400 *g* for 10 min, and the pellet was resuspended in serum-free RPMI 1640 medium. The suspension was agitated for 4 h at 25°C and centrifuged at 250 *g* for 10 min. The final pellet contained purified amastigotes free of cellular contamination (Barbieri et al., 1993).

Purification of Achatinin-H, a 9-*O*-acetylated sialic acid binding lectin

Achatinin-H, a 9-*O*AcSA binding lectin, was affinity purified from the hemolymph of the giant African land snail, *Achatina*

fulica, using bovine submaxillary mucin (BSM) coupled to Sepharose-4B (Pharmacia, USA) as the affinity matrix, based on the long-standing evidence that BSM contains a high percentage of *O*AcSA (Reuter et al., 1983). The presence of 9(8)-*O*-acetylated sialic acid derivatives on BSM was estimated fluorimetrically and found to be 22.5% (Shukla and Schauer, 1982; Sharma et al., 1998, 2000). This was further reconfirmed by fluorimetric HPLC (Chatterjee et al., 2003). Furthermore, the specificity of Achatinin-H toward 9-OAcSA was confirmed using several approaches (Sen and Mandal, 1995).

Fluorimetric HPLC of sialic acids

Amastigotes (1.6×10^9 cells) of *L. donovani* were extensively washed in PBS (0.02 M, pH 7.2) and the cell pellet was lyophilized. In order to release sialic acids, glycoconjugates in amastigote pellets and the three washes were subjected to mild acid hydrolysis with 200 μl of 2 M acetic acid for 3 h at 80°C. Samples were cooled on ice for 10 min, and then used for HPLC and GC/EI-MS analyses.

Aliquots of the free sialic acid-containing samples (from the amastigotes and three washes) were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) for fluorimetric HPLC analysis (Hara et al., 1989). In brief, 200 μl of a 7 mM DMB solution, containing 1.4 M acetic acid, 0.75 M β-mercaptoethanol and 18 mM sodium dithionite, were added to 150 μl of each sample, then heated for 2.5 h at 50°C in the dark. After cooling on ice, a fixed volume (2–20 μl) was used for analysis by HPLC.

The fluorescently labeled sialic acids were analyzed on a Cosmosil 5C18-AR-II column (4.6×250 mm; Waters, Germany) using acetonitrile/methanol/water (9:7:84, v/v/v) as the solvent system at a flow rate of 1.0 ml/min. Authentic sialic acids (Sigma, USA) were used as reference compounds. Fluorescence detection was performed using an excitation wavelength of 373 nm and an emission wavelength of 448 nm. For quantification, the DMB derivative of Neu5Ac was used as an internal standard.

Gas chromatography/mass spectrometric analysis of sialic acids as trimethylsialylated methyl ester derivatives

Aliquots of the sialic acid-containing samples (from amastigotes and three washes; 50 μl) were diluted with 100 μl of methanol, then evaporated to dryness with a nitrogen flow and re-dissolved in dry methanol (0.5 ml). Dowex H^+ in methanol (80 μl) was added, the mixture was filtered over cotton wool, and diazomethane in ether was added until a faint yellow color remained for 5 min at 25°C. The solution was evaporated using a stream of nitrogen and dried over P_2O_5. The residue was dissolved in 6 μl TMS reagent (pyridine/hexamethyldisilazane/trimethylchlorosilane 5:1:1, v/v/v). After 2 h at room temperature, samples (3 μl) were analyzed by GC/EI-MS (Kamerling and Vliegenthart, 1982).

The following equipment and parameters were used for GC/EI-MS analysis: a Fisons Instruments GC 8060/MD800 system (Interscience, Breda, The Netherlands); an AT-1 column (30×0.25 mm, Alltech, Breda, The Netherlands); temperature program, 220°C for 25 min, 6°C/min to 300°C, 6 min constant; injector temperature, 230°C.

Flow cytometric analysis

To establish the presence of sialic acids on parasite membranes, binding of two sialic acid binding lectins, SNA and MAA (Vector Labs, Burlingame, USA), were examined (Chatterjee et al., 2003). Amastigotes were extensively washed and resuspended (1×10^6 cells/tube) in pre-chilled RPMI medium supplemented with 2% bovine serum albumin and 0.1% sodium azide for 1 h at 4°C. Parasites were then incubated with biotinylated SNA and MAA (5 μl, 5 μg/ml) for 30 min at 4°C. Cells were then washed and lectin binding detected by measuring the binding of FITC-conjugated streptavidin. The presence of 9-*OAc*SA was quantified using FITC-Achatinin-H.

To measure the binding of Siglecs to *L. donovani* amastigotes, the Siglecs-Fc were initially complexed with biotinylated goat anti-human antibodies (Fc-specific) by incubation at 25°C for 1 h. Subsequently, amastigotes were incubated with the complexed Siglecs for 30 min on ice (Chatterjee et al., 2003). The cells were then washed and the extent of binding detected by flow cytometry (FACS Scan flow cytometer, Becton Dickinson, Mountain View, USA) using streptavidin-FITC. Laser excitation was at 488 nm; between 10000 and 20000 total events were acquired per sample with a FSC threshold. In all experiments, the background fluorescence was determined by staining the cells with FITC alone. We arbitrarily set a threshold of fluorescence intensity such that $<3\%$ of the cells fluoresced above this level. The percentage of cells stained with the lectins that fluoresced above this threshold was considered as positive frequency.

Sialidase treatment of *L. donovani* amastigotes

To confirm the presence of sialic acids, parasites (1×10^6/100 μl) were incubated with 50 μl of *Arthrobacter ureafaciens* neuraminidase (Roche, Mannheim, Germany; stock solution 10 U/ml) overnight at 37°C in PBS containing 1% bovine serum albumin (BSA). Cells were then washed three times, re-suspended in RPMI and processed for flow cytometric analysis.

9-*O*-acetylesterase treatment of *L. donovani* amastigotes

The presence of *O*-acetylated sialoglycans on parasites was demonstrated by using 9-*O*-acetyl hemagglutinin esterase of influenza C virus (Vlasak et al., 1987). Accordingly, cells (1×10^6) were incubated with a recombinant 9-*O*-acetyl hemagglutinin esterase of influenza C virus (100 μl, 100 U/ml) for 3 h at 37°C. Cells were then washed and processed for FACS analysis.

In addition, amastigotes (1×10^6 cells) were incubated with a recombinant 9-*O*-acetyl hemagglutinin esterase of influenza C virus, (100 U/ml, 100 μl per tube) for 3 h at 37°C; cells were washed, and incubated overnight with 50 μl of *A. ureafaciens* neuraminidase at 37°C. De-*O*-acetylated and de-sialylated cells were then washed and processed for lectin binding studies as described above.

Western blot analysis of parasite membranes

Membrane fractions were purified from *L. donovani* amastigotes (Chatterjee et al., 2003). Membrane proteins (20 μg/lane) before and after sialidase treatment were separated by SDS-PAGE (10%) and the presence of sialoglycoconjugates was visualized colorimetrically as described earlier (Chatterjee et al., 2003).

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