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# Identification and characterization of adsorbed serum sialoglycans on *Leishmania donovani* promastigotes

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**Sialic acids as terminal residues of oligosaccharide chains play a crucial role in several cellular recognition events. The presence of sialic acid on promastigotes of *Leishmania donovani*, the causative organism of Indian visceral leishmaniasis, was demonstrated by fluorimetric high-performance liquid chromatography showing Neu5Ac and, to a minor extent, Neu5,9Ac$_2$. The presence of Neu5Ac was confirmed by GC/MS analysis. Furthermore, binding with sialic acid-binding lectins *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), and Siglecs showed the presence of both α2,3- and α2,6-linked sialic acids. No endogenous biosynthetic machinery for Neu5Ac could be demonstrated in the parasite. Concomitant western blotting of parasite membranes and culture medium with SNA demonstrated the presence of common sialoglyconjugates (123, 90, and 70 kDa). Similarly, binding of MAA with parasite membrane and culture medium showed three analogous sialoglycans corresponding to 130, 117, and 70 kDa, indicating that α2,3- and α2,6-linked sialoglycans are adsorbed from the fetal calf serum present in the culture medium. *L. donovani* promastigotes also reacted with Achatinin-H, a lectin that preferentially identifies 9-*O*-acetylated sialic acid in α2 → 6 GalNAc linkage. This determinant was evidenced on parasite cell surfaces by cell agglutination, ELISA, and flow cytometry, where its binding was abolished by pretreatment of cells with a recombinant 9-*O*-acetylesterase derived from the HE1 region of the influenza C esterase gene. Additionally, binding of CD60b, a 9-*O*-acetyl GD3-specific monoclonal antibody, corroborated the presence of terminal 9-*O*-acetylated disialoglycans. Our results indicate that sialic acids (α2 → 6 and α2 → 3 linked) and 9-*O*-acetyl derivatives constitute components of the parasite cell surface.**

*Key words:* Achatinin-H/*Leishmania donovani*/*O*-acetylated sialic acid/sialic acid/UDP-GlcNAc2-epimerase

## Introduction

Sialic acids 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; Angata and Varki, 2002). Among over 40 diverse structural modifications of the parent molecule, the most common are *O*-acetyl substitutions at the C-4, C-7, and C-9 positions (Schauer and Kamerling, 1997). Because *O*-acetyl esters at the C-7 position are known to migrate to the C-9 position, 9-*O*-acetylated sialic acids usually predominate on cell surface glycoconjugates generating a family of 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) (Varki, 1992; Schauer, 2000).

Kinetoplastids represent a prominent order of flagellated protozoa, especially among the genera *Trypanosoma* and *Leishmania*, containing several species that threaten humans and domestic animals. Studies have shown the presence of *N*-acetyl and *N*-glycolyl neuraminic acid on *Trypanosoma* species (Schauer *et al*., 1983). For glycobiologists and parasitologists, this was considered a landmark event because it was not simply demonstration of a sugar molecule but, more importantly, provided persuasive evidence that *T. cruzi* parasites do not contain the requisite machinery for synthesizing their own sialic acid (Schauer *et al*., 1983). Instead they scavenge sialic acids from host sialoglycoconjugates without metabolic energy requirements and mediate the transglycosylation reaction through a novel group of sialidases, the trans-sialidases (Cross and Takle, 1993). These surface trans-sialidases are capable of cleaving bound sialic acids from host-derived glycans, which are then incorporated mostly into mucin-like molecules attached to the parasite membrane through a glycophosphatidyl inositol anchor (Schenkman *et al*., 1991).

Released and cell membrane-bound carbohydrate determinants are very important in the biology of *Leishmania* parasites because they support parasite survival and

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proliferation. Most of these specialized molecules are members of a family of phosphoglycans, whereas others are a family of glycoinositol phosholipids (Ferguson, 1999). However, the topography of *Leishmania* parasites with regard to their sialoglycan profile remains a poorly investigated area. To address this question indirectly, surface trans-sialidases have been demonstrated in the *Kinetoplastida* family where the occurrence of cell-bound sialic acid was found to coincide with trans-sialidase activity (Engstler *et al.*, 1995).

Exploiting the narrow binding specificity of Achatinin-H, a 9-*O*-acetylated sialic acid-binding lectin, we have demonstrated the presence of 9-*O*-acetylated sialic acid derivatives (9-*O*-AcSA) on erythrocytes of patients with visceral leishmaniasis (VL) as compared to normal human erythrocytes, which notably do not contain this *O*-acetylated glycotope (Sharma *et al.*, 1998). Subsequently, we reported an enhanced level of antibodies against *O*-acetylated sialic acids (*O*-AcSA) in VL patients absent in patients with coendemic diseases, such as malaria and tuberculosis, and normal individuals. Importantly, a decline in the anti *O*-AcSA antibody levels was observed following conventional antimonial treatment in those patients who responded to chemotherapy. In contrast, VL patients who were drug nonresponsive continued to maintain high levels of circulating anti *O*-AcSA antibodies (Chatterjee *et al.*, 1998). Our data suggested that anti *O*-AcSA levels correlated with the amount of circulating *Leishmania* parasite.

In light of these observations, this study was undertaken to assess the sialoglycoconjugate profile of *L. donovani* promastigotes. We describe the identification of both $\alpha 2 \rightarrow 6$- and $\alpha 2 \rightarrow 3$-linked sialic acids as also its 9-*O*-acetylated derivative on the parasite surface. We also provide evidence that these sialoglycans are derived by direct adsorption from fetal calf serum (FCS) used in the cell culture medium.

## Results

### *Identification of sialoglycans on* L. donovani *promastigotes*

The presence of sialic acids on *L. donovani* promastigotes was demonstrated by preparing acid hydrolysates of parasites that were analyzed by fluorimetric high-performance liquid chromatography (HPLC). As shown in Figure 1, the chromatogram exhibited well-resolved peaks that coincided with that of *N*-acetyl neuraminic acid (Neu5Ac) and one, comigrating with Neu5,9$Ac_2$, which resembles 7.7% of total sialic acids as demonstrated by its sensitivity to saponification. To confirm that these peaks represented sialic acids and not unidentified α-keto acids, the samples were incubated with sialate pyruvate lyase prior to derivatization. Following this enzymatic treatment, diminution of the corresponding peaks confirmed the presence of Neu5Ac and Neu5,9$Ac_2$ on parasites. To exclude that sialic acids were not loosely adherent to the cell surface, the parasites were extensively washed three times (50 ml per wash) with phosphate buffered saline (PBS), and the washes were also examined for their sialic acid content. The final wash contained only 13 ng (1.7%) of Neu5Ac. The cells contained

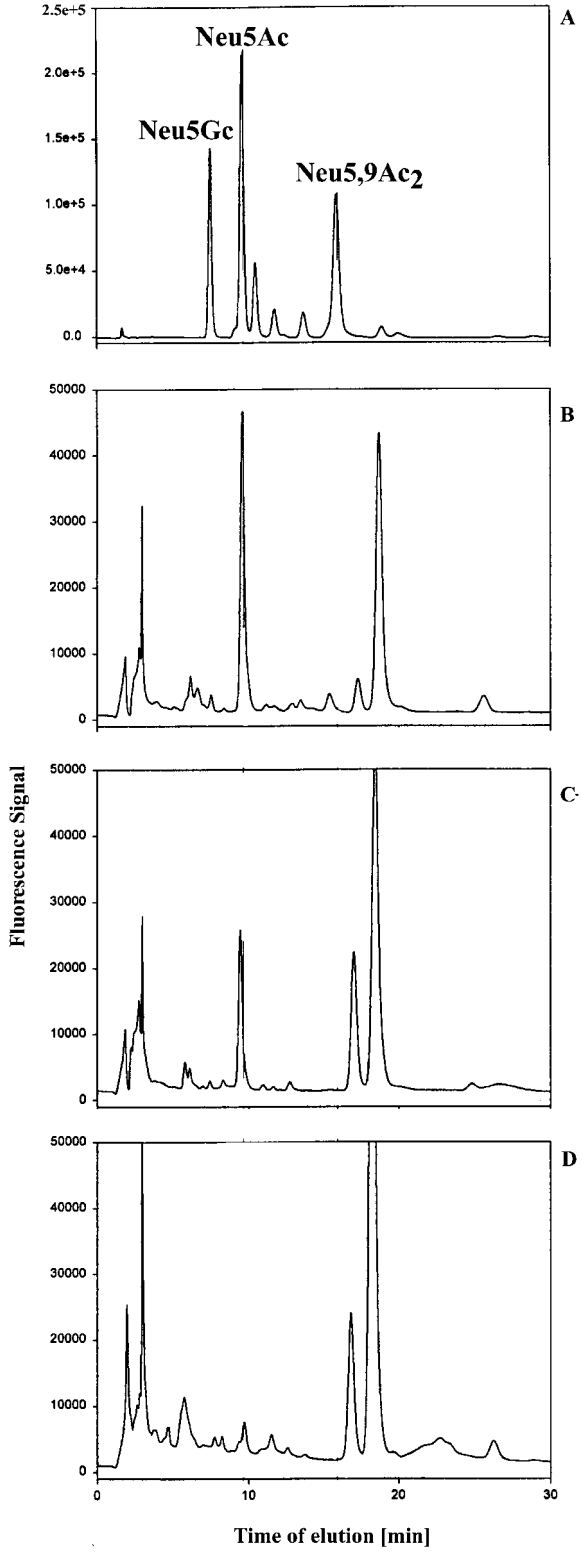


**Fig. 1.** Determination of sialic acid (Neu5Ac) and its principal derivatives by fluorimetric HPLC. Representative profile of a HPLC chromatogram of fluorescent derivatives of free sialic acids derived from (**A**) bovine submandibular gland mucin, *L. donovani* promastigotes before (**B**) and after preincubation with with 0.1 M ammonia vapor (**C**) and sialate pyruvate lyase (**D**). Glycosidically bound sialic acids were subjected to acid hydrolysis, derivatized with 1,2 diamino-4,5-methylenedioxybenzole, and analyzed as described in *Materials and methods*.

about 800 ng of sialic acid in $2 \times 10^9$ cells corresponding to $7 \times 10^5$ molecules of sialic acid per cell.

To confirm this data, mass spectrometry (MS) analysis of trimethylsilyl (TMS)-methylester derivatives of the same samples was done. Due to contaminants and small amount of sample material, the only sialic acid to be clearly detected in the parasite by mass fragmentography was Neu5Ac showing fragment ions (*m*/*z*) at 668, 624, 478, 400, 317, and 298, respectively.

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

The surface density of sialoglycoconjugates present on *L. donovani* promastigotes was examined by flow cytometric analysis using two sialic acid-binding plant lectins, *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), that recognize $\alpha2 \rightarrow 6$ and $\alpha2 \rightarrow 3$ sialylgalactosyl residues, respectively. As demonstrated in Figure 2, MAA showed a relatively lower binding than SNA ($42.5 \pm 5.4\%$ versus $70.1 \pm 11.84\%$, respectively). Its specificity toward Neu5Ac was confirmed by diminished binding after sialidase treatment (Figure 2, Table I). High amounts of SNA binding glycans were expressed on the parasite indicating the predominance of $\alpha2 \rightarrow 6$-linked sialic acids (Figure 2). The binding of SNA remained unchanged despite alterations of sialidase concentration, pH, incubation time, and temperature (Table I). A marginal reduction (23%) in SNA binding was observed with *Arthrobacter ureafaciens* neuraminidase. Positive controls used were CEM-C7 cells (T-acute lymphoblastic leukemia), where sialidase pretreatment abolished SNA binding (data not shown).

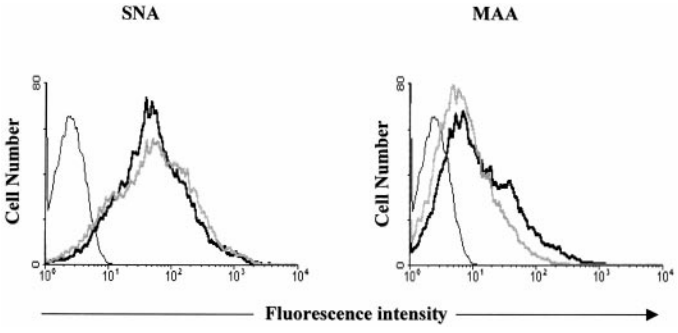


**Fig. 2.** Representative profiles of cell surface expression of $\alpha2 \rightarrow 6$- and $\alpha2 \rightarrow 3$-linked sialoglycans on *L. donovani* promastigotes. Parasites were incubated in the absence (dotted line) and presence (bold line) of biotinylated SNA and MAA, whereas the thin line represents cells pretreated with VCN. Binding was detected by FITC-streptavidin as described in *Materials and methods*.

### *Binding of Siglecs indicates presence of both $\alpha2 \rightarrow 3$- and $\alpha2 \rightarrow 6$-linked sialic acids*

Corroborative evidence for the presence of $\alpha2 \rightarrow 3$ and $\alpha2 \rightarrow 6$ linked sialoglycans on the *L. donovani* promastigotes was provided by the binding of various recombinant sialic acid binding lectins (Siglecs). Although the binding pattern appeared complex, most Siglecs tested showed some degree of binding (Table II). Siglecs exhibit widely differing preferences for sialic acid linkage to subterminal sugars. For example, CD22/Siglec-2 binds only to $\alpha2 \rightarrow 6$-linked sialic acids, whereas sialoadhesin/Siglec-1 prefers $\alpha2 \rightarrow 3$-linked sialic acids and Siglec-5 binds both linkages. Taken together, our results support the idea that sialic acids, both $\alpha2 \rightarrow 3$ and $\alpha2 \rightarrow 6$ linked, are present on *Leishmania* parasites.

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

For molecular characterization of sialoglycans present on the parasite surface, reactivity of two plant lectins, SNA and MAA, was examined by western blotting. Using SNA, the presence of three sialoglycoproteins corresponding to 123, 90, and 70 kDa were identified on parasite membranes (Figure 3A, lane 1). As compared to 90 and 70 kDa, the expression of 123 kDa was much weaker. In case of MAA, five sialoglycans were identified that corresponded to 130, 117, 106, 70, and 61 kDa (Figure 3A, lane 3). The binding specificity of SNA and MAA was confirmed by the absence of binding following prior neuraminidase treatment.

**Table I.** Sialidase treatment of *L. donovani* promastigotes

| Enzyme | Dose (U) | Temp. (°C) | pH | Incubation time (min) | % Binding of SNA | | % Binding of MAA | |
|---|---|---|---|---|---|---|---|---|
| | | | | | − | + | − | + |
| VCN | 0.15 | 37 | 7.2 | 60 | $72.0 \pm 14.3$ | $67.0 \pm 12.0$ | $41.0 \pm 6.3$ | $27.0 \pm 4.2$ |
| VCN | 1.5 | 37 | 6.5 | 60 | 59.0 | 55.0 | 39.0 | 21.0 |
| VCN | 1.5 | 37 | 7.2 | 60 | 75.0 | 76.0 | 42.0 | 21.5 |
| VCN | 1.5 | 20–25 | 7.2 | Overnight | 59.0 | 90.3 | 39.0 | 36.0 |
| AF | 0.5 | 20–25 | 7.2 | 30 | 61.0 | 47.0 | 50.0 | 30.0 |
| Esterase | 100 μU | 20–25 | 7.2 | 60 | 60.0 | 30.0 | 31.0 | 30.0 |

*L. donovani* promastigotes were pretreated with the sialidases *Vibrio cholerae* neuraminidase, 1.0 U/ml; *Arthrobacter ureafaciens* neuraminidase, 10 U/ml; or esterase. The binding of sialic acid-binding lectins SNA or MAA was measured by flow cytometry as described in *Materials and methods*. − and + indicate the absence and presence of sialidase or esterase pretreatment, respectively.

**Table II.** Binding of Siglecs to *L. donovani* promastigotes

| Probe | Linkage specificity | Occurrence | − VCN | + VCN | % Inhibition |
|---|---|---|---|---|---|
| Siglec-1 | $\alpha2\rightarrow3 > \alpha2\rightarrow6$ | Macrophages | $17.3 \pm 3.7$ | $6.0 \pm 0.1$ | 65.3 |
| Siglec-2 | $\alpha2\rightarrow6 \gg \alpha2\rightarrow3$ | B cells | $50.5 \pm 11.4$ | $34.3 \pm 5.8$ | 49.8 |
| Siglec-5 | $\alpha2\rightarrow3 = \alpha2\rightarrow6$ | Neutrophils, myeloid cells | $31.4 \pm 3.7$ | $12.0 \pm 4.7$ | 61.8 |
| Siglec-7 | $\alpha2\rightarrow6 > \alpha2\rightarrow3$ | NK cells, monocytes | $19.0 \pm 4.3$ | $8.6 \pm 2.6$ | 54.7 |
| Siglec-8 | $\alpha2\rightarrow3$ | Eosinophils | $28.65 \pm 4.2$ | $7.6 \pm 2.8$ | 73.5 |
| Siglec-10 | $\alpha2\rightarrow3 = \alpha2\rightarrow6$ | Myeloid cells | $32.5 \pm 1.5$ | $15.6 \pm 4.1$ | 52.0 |

*L. donovani* promastigotes were pretreated with VCN and binding of Siglecs was measured by flow cytometry (mean ± SD of three independent determinations) as described in *Materials and methods*. – and + indicate the absence and presence of sialidase pretreatment, respectively.

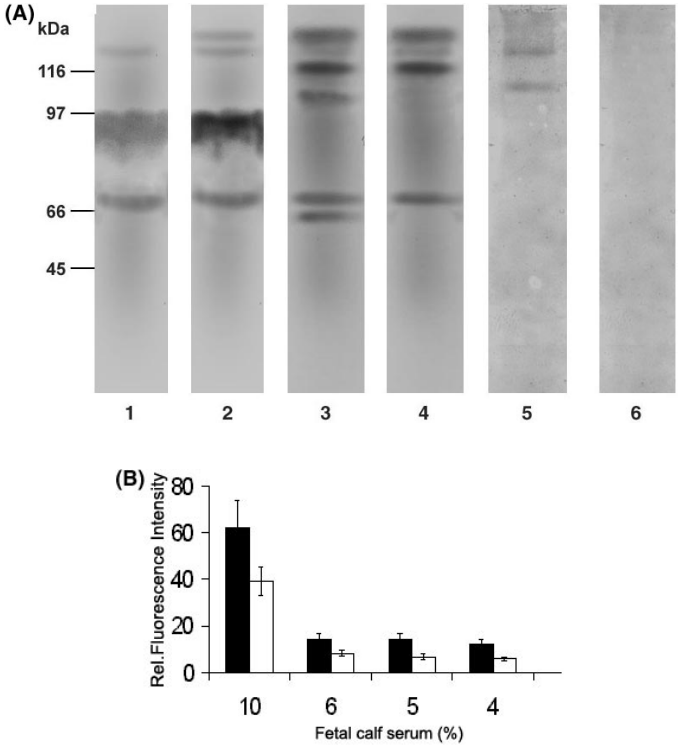


**Fig. 3.** (**A**) Molecular characterization of sialoglycoproteins present on *L. donovani* promastigotes. Membrane proteins from Ag83 promastigotes were electrophoresed (7.5% SDS–PAGE) and, following transfer onto nitrocellulose membranes, were incubated with SNA (lane 1), MAA (lane 3), or Achatinin-H (lane 5). Binding was detected as described in *Materials and methods*. Similarly, western blot was carried out to demonstrate the binding of SNA (lane 2), MAA (lane 4), and Achatinin-H (lane 6) to medium M199 containing 10% FCS. (**B**) Differential adsorption of serum sialoglycans onto *L. donovani* promastigotes. Parasites were cultured in varying concentrations of FCS. Parasite binding to SNA (filled bars) and MAA (open bars) was examined by flow cytometry as described in *Materials and methods*.

### *Achatinin-H preferentially binds to 9-*O*-AcSGs*

Hemagglutination and hemagglutination inhibition assays confirmed that Achatinin-H preferentially bound to bovine submandibular mucin (BSM) known to have terminal 9-*O*-AcSA and a subterminal GalNAc in an $\alpha2\rightarrow6$ linkage (Reuter *et al.*, 1983). The strong inhibition of binding with purified 9-*O*-AcSA and absence of inhibition with 4-*O*-AcSA pointed toward its specificity toward 9-*O*-AcSA (Mandal and Basu, 1987; Mandal *et al.*, 1989; Mandal and Mandal, 1990). No inhibition occurred with de-*O*-acetylated BSM and asialo-BSM, reconfirming lectin specificity toward *O*-AcSA derivatives; other sialoglycoproteins, for example, human chorionic gonadotrophin, fetuin and $\alpha_1$-acid glycoprotein, and sheep submaxillary mucin having terminal sialic acid either in $\alpha2\rightarrow6$ or $\alpha2\rightarrow3$ linkages, did not show any inhibition, indicating that presence of the *O*-acetyl derivative is crucial for lectin binding (Sen and Mandal, 1995).

### *Achatinin-H agglutinates* L. donovani *promastigotes*

Previous studies from our group have demonstrated the increased presence of antibodies directed against *O*-AcSA in serum of VL patients. Accordingly, we wished to examine the possible presence of this *O*-AcSA moiety on the cell surface of *L. donovani* promastigotes. Exploiting the preferential affinity of Achatinin-H toward 9-*O*-AcSA, presence of these derivatives was evidenced by a dramatic increase in parasite agglutination with lectin concentrations above 4.0 μg/well (Figure 4A). Care was taken to adequately disperse the cells. Minimal inherent agglutination was observed in the absence of lectin. With higher concentrations of Achatinin-H, the agglutination pattern changed in that the degree of clumping was so extensive that it prevented counting of individual parasites. Accordingly, nonagglutinated parasites were counted and the percentage of agglutination extrapolated. The observation that Achatinin-H agglutinated the cells through binding to surface 9-*O*-AcSA was confirmed by inhibition of agglutination in the presence of esterase and BSM (data not shown). The selective binding of Achatinin-H was further demonstrated by enzyme-linked immunosorbent assay (ELISA) using membrane preparations of *L. donovani* promastigotes as the coating antigen. The extent of lectin binding was quantitatively estimated by polyclonal anti-Achatinin-H (Figure 4B).

### *Abolition of binding of Achatinin-H by esterase treatment reconfirmed parasite membranes have glycoconjugates with terminal 9-*O*-AcSA*

Flow cytometry demonstrated the presence of cell surface 9-*O*-AcSA on *L. donovani* promastigotes through the strong

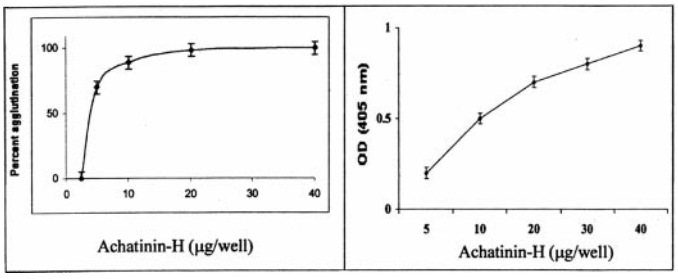


**Fig. 4.** Cell surface expression of *O*-acetyl sialic acids using Achatinin-H, a 9-*O*-acetylated sialic acid binding lectin. (**A**) Parasites ($1 \times 10^7$/ml, 100 μl) were incubated with increasing concentrations of Achatinin-H at 20–25°C for 15 min. Cells were then examined microscopically and the number of nonagglutinated cells counted; accordingly, the degree of agglutination was extrapolated. Each point is the average of three independent experiments. (**B**) To demonstrate lectin binding to parasite membranes, membrane lysates were coated on 96-well plates and incubated with increasing concentrations of Achatinin-H. Binding of lectin was detected colorimetrically as described in *Materials and methods*. Each point is the average of three independent experiments.

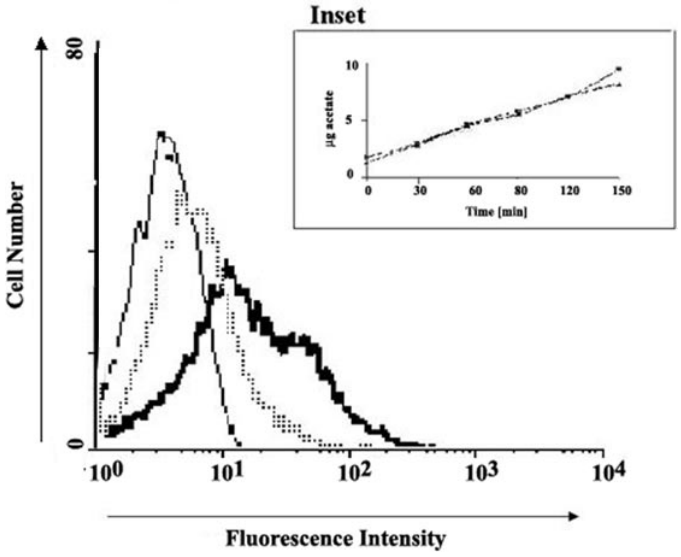


**Fig. 5.** Demonstration of 9-*O*-acetylated sialic acids on cell surface of *L. donovani* promastigotes by flow cytometric analysis. Binding of FITC-Achatinin-H to parasites in the absence (bold line) and presence (gray line) of recombinant 9-*O*-acetylesterase derived from the HE1 region of the influenza C esterase gene as described in *Materials and methods*. Thin line represents untreated cells. Inset: Acetylesterase activity of recombinant 9-*O*-acetylesterase derived from the HE1 region of the influenza C esterase gene HE-1-eGFP (dots) as compared with influenza C virus, C/JJ/50 (triangles) using BSM type IS as substrate. Release of acetate was determined with a commercial test kit as previously described (Vlasak *et al*., 1988). One unit was defined as the amount of enzymatic activity resulting in hydrolysis of 1 μmol of *p*NPA per min.

binding of Achatinin-H (44.3%) (Figure 5). To demonstrate this binding specificity toward the 9-*O*-acetyl moiety, cells were incubated with a recombinant acetylesterase. This recombinant chimeric protein consists of the HE1 domain fused at the C-terminus to eGFP. It was devoid of the HE2 domain, including the transmembrane anchor, and was efficiently being secreted into the culture supernatant of SF9 cells. Typical yields were 1–2 mU/ml of specific *O*-acetylesterase activity. To verify whether this chimeric esterase exhibited a similar specificity as the authentic virus-bound enzyme, both were incubated with BSM. As shown in Figure 5 (inset), the recombinant protein hydrolyzed *O*-acetyl esters present on BSM at a rate comparable with that of the influenza C virus, indicating that the HE1-eGFP protein exhibits the same specificity for 9-*O*-AcSA as the viral HE protein.

When we incubated *L. donovani* parasites with this chimeric esterase, the resultant de-*O*-acetylation of parasite membranes caused a near total abolition of lectin binding, from 44.3% to 9.4%. This reconfirmed the presence of 9-*O*-AcSA glycotope on the parasite surface (Figure 5). The presence of 9-*O*-acetylated determinants on *L. donovani* promastigotes was also confirmed using the CD60b-specific monoclonal antibody UM4D4, whose epitope has been defined as 9-*O*-acetylated ganglioside GD3 and related structures (Schwartz-Albiez, 2001). The binding of fluorescein isothiocyanate (FITC)-labeled CD60b on *L. donovani* promastigotes was examined by flow cytometric analysis and three independent experiments showed the mean ± SD of binding was 34.1 ± 2.9% as compared to 6.85 ± 2.8% in isotype controls (IgM).

Recent evidence suggests that SNA binds not only to α2,6-linked sialic acids but also to its 9-*O*-AcSA derivative (Brinkman-van der Linden *et al*., 2002). Examination of SNA binding on *L. donovani* promastigotes before and after esterase treatment demonstrated a distinct decrease in binding from 60% to 30% (Table I), but no alteration in binding with MAA was observed. Considered together, our data strongly indicates the presence of 9-*O*-AcSA in an α2→6 linkage.

### *Molecular characterization of the epitope recognized by Achatinin-H*

To further characterize the *O*-acetylated sialoglycoproteins present on *L. donovani* promastigote membranes, western blotting was performed. Achatinin-H bound to two *O*-acetylated sialoglycoproteins corresponding to 123 and 109 kDa (Figure 3A, lane 5).

### *Absence of sialic acid biosynthesis machinery in* L. donovani *promastigotes*

To examine whether *L. donovani* possesses a sialic acid biosynthetic machinery, activity of UDP-GlcNAc 2-epimerase, the key enzyme in mammalian sialic acid biosynthesis (Keppler *et al*., 1999a) was analyzed in cytosolic fraction of parasites. No UDP-GlcNAc 2-epimerase activity could be detected in *L. donovani* (<2 μU/mg of protein), indicating that they do not perform sialic acid biosynthesis. For comparison, mammalian hematopoietic cell lines, CEM-C7 (T-cell acute lymphoblastic leukemia) and REH (pre-B-cell leukemia) expressed Neu5Ac on their cell surfaces (26 ± 3 and 32 ± 2 nmol Neu5Ac/mg of protein, respectively) and showed UDP-GlcNAc 2-epimerase activity (125 ± 10 and 130 ± 15 μU/mg of protein, respectively). However, parasites showed no detectable sialic acid by the method of Warren (1959).

*Adsorption of serum sialoglycans onto cell surface of* L. donovani *promastigotes from the culture medium*

Because our data indicates the absence of an endogenous biosynthetic pathway in *L. donovani* promastigotes, we wished to examine whether these newly identified sialoglycans on the parasite surface were derived from sialoglycoproteins present in the medium. Accordingly, binding of SNA and MAA with M199 medium containing 10% FCS was examined. SNA bound to serum sialoglycans (130, 123, 90, and 70 kDa, Figure 3A, lane 2) similar to three sialylated glycotopes identified on the parasite membrane corresponding to 123, 90, and 70 kDa (Figure 3A, lane 1). Similarly, MAA bound to serum sialoglycoproteins corresponding to 130, 117, and 70 kDa (Figure 3A, lane 4) were also present on the parasite membrane (Figure 3A, lane 3). The parasite membrane had two additional α2→3-linked sialoglycans corresponding to 106 and 61 kDa absent in serum, whose source remains to be investigated. However, under these experimental conditions, the presence of Achatinin-H binding *O*-acetylated sialoglycoproteins could not be identified in serum (Figure 3A, lane 6).

To provide further evidence that these sialoglycans on *L. donovani* promastigotes are derived from FCS, parasites were cultured in decreasing concentrations of FCS (10–4%), and its binding to SNA and MAA was examined by flow cytometry. As shown in Figure 3B, a marked decrease in the binding of both SNA and MAA was observed, corroborating that parasite sialoglycans are directly transferred from the culture medium. The experiment in the complete absence of FCS was not feasible because cell viability was adversely affected.

## Discussion

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 one-tenth of the world 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).

Currently, a growing interest in the pathophysiological role of microbial sialoglycoconjugates has arisen following the identification of sialic acid and its 9-*O*-acetylated derivatives on the cell surface of viruses, bacteria, fungi, and protozoans (Crocker and Varki, 2001). Leishmanial parasites have adapted, not only to survive but also to proliferate, largely due to protection conferred by unique glycoconjugates; the principal participants include a family of phosphoglycans and glycoinositol phospholipids whose principal features are the presence of Gal β1 → 4 Man α1 → $PO_4$ repeating units (Ferguson, 1999; Turco *et al*., 2001).

Assessment of the sialoglycan profile of *Leishmania* parasites remains a relatively neglected domain of parasite glycobiology. In this study, we have addressed this aspect and report the presence of sialic acids as also its 9-*O*-acetyl derivative on the cell surface of *L. donovani* promastigotes. To investigate the linkage specificity of these sialoglycans, sialic acid-binding lectins with defined linkages to its subterminal sugars were used. The plant lectins SNA and MAA prefer Neu5Acα2 → 6 Gal/GalNAc and Neu5Acα2 → 3 Gal, respectively (Shibuya *et al*., 1987; Wang and Cummings, 1988); also Siglecs, members of the immunoglobulin superfamily, bind to sialic acids and are mainly expressed by cells of the hematopoietic system (Powell and Varki, 1994). Although both plant lectins showed binding, the binding of SNA was distinctly higher, indicating predominance of this linkage (Figures 2 and 3).

The presence of α2 → 6 linked sialic acids on the parasite cell surface was consistent with their binding to Siglec 2 (CD22), known to require sialic acids α2 → 6 linked to Galβ1 → 4GlcNAc sequences for recognition (Powell *et al*., 1993) (Table II). Like MAA, the lower binding with Siglec-1 and Siglec-8 that prefer α2 → 3-linked sialic acids points toward the predominance of α2 → 6 linked sialic acids. Binding of Siglec-1, -2, -5, -7, -8, and -10 exhibit a complex pattern of binding specificities (Table II). This is in contrast to another member of the Kinetoplastid family, the trypanosomes, where α2 → 3-linked sialic acids predominate (Engstler *et al*., 1995) and the best substrate identified for trans-sialidase was found to be α2 → 3-linked sialyllactose (Schenkman *et al*., 1991).

The binding specificity of MAA toward Neu5Ac was verified by sialidase pretreatment that caused 60% reduction in MAA binding (Table I). However, prior sialidase treatment caused little or no reduction of SNA binding despite multiple variations in the enzyme treatment (Table I). The presence of α2 → 6-linked sialic acids on the parasite cell surface was, however, reconfirmed through its binding with CD22 (Siglec-2) (Powell and Varki, 1994) (Table II). A similar scenario was reported by Keppler *et al*. (1999b) where sialidase treated cells continued to show high binding with SNA. This could be a specific feature of the SNA-binding glycotope present on the parasite surface and needs to be explored. However, western blotting of parasite membrane following sialidase treatment resulted in abolition of SNA binding (data not shown). It may be envisaged that SNA-binding glycotopes are cryptic, rendering them inaccessible to neuraminidase treatment in intact cells. Additionally, the 50% decrease in SNA binding following esterase treatment suggests that SNA binds not only to α2 → 6-linked sialic acids but also to glycotopes bearing terminal 9-*O*-AcSA derivatives (Table I) and corroborates similar findings reported by Brinkman-van der Linden *et al*. (2002).

Detection of these sialic acids raises the obvious question regarding the mechanism(s) adopted by the parasite to acquire these terminal sugar molecules. No biosynthetic machinery for sialic acid has been elucidated in *Trypanosoma* parasites possessing sialic acids on their cell surface (Schauer *et al*., 1983). Barring a few bacteria, biosynthesis of sialic acids is restricted to multicellular organisms, the key enzyme being UDP-GlcNAc 2-epimerase, which catalyzes the first step of this pathway and shows a strong feedback inhibition (Keppler *et al*., 1999a). Therefore, it follows that, if *L. donovani* should have its own sialic

acid biosynthesis, expression of UDP-GlcNAc 2-epimerase activity would be necessary. Our data clearly shows that *L. donovani* has no UDP-GlcNAc 2-epimerase activity and consequently does not possess a machinery for sialic acid biosynthesis.

Trypanosomal parasites possess trans-sialidases, which enable them to transfer glycosidically linked sialic acids from the environment (e.g., serum sialoglycoconjugates onto parasite surface molecules) (Zingales *et al*., 1987). However, among *Leishmania* species, the presence of such trans-sialidases has not been demonstrated (Engstler *et al*., 1995). The presence of serum trans-sialidases is still a matter of debate, and it would be interesting to analyze whether such trans-sialidases are operative in leishmaniasis, accounting for parasite sialylation. Alternatively, another approach that the parasite might well utilize is ecto-sialyl transferases or serum sialyl transferases that would catalyze the transfer of sialic acid from the nucleotide sugar donor CMP-Neu5Ac onto acceptor glycoconjugates (Gross *et al*., 1996). However, such enzymatic reactions would require the presence of CMP-Neu5Ac, whose presence in serum of VL patients is yet to be substantiated.

Another option that the parasite may adopt is to acquire Neu5Ac from the growth medium either by transglycosylation or by incorporation of serum components to the parasite polyanionic lipophosphoglycan, LPG/proteophosphoglycan-rich cell surface (Pereira-Chiocola *et al*., 2000). However, this maybe ruled out because the western blotting showed discrete glycoprotein bands (Figure 3A) and not a smear (10 to 60 kDa) characteristic of lipophosphoglycan (Ferguson, 1999). Parasitologists are limited by 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 still remains unanswered (Angata and Varki, 2002).

Our studies indicate that there is a direct transfer, that is, adsorption of certain sialoglycoproteins from culture medium onto the parasite surface, as binding of SNA and MAA to parasite membranes and culture medium demonstrated the presence of analogous sialoglycans (Figure 3A). This was reconfirmed with the decreased binding of SNA and MAA to parasites when cultured in decreasing concentrations of FCS (Figure 3B). Our investigations therefore demonstrate that the parasite is "borrowing" sialoglycans from the culture medium through simple adsorption to possibly compensate for the deficient sialic acid. It may be speculated that incorporation of serum sialoglycoconjugates onto the parasite surface might be related to the membrane architecture, and they are accordingly adsorbed under different stimuli or stress conditions (Schwarzkopf *et al*., 2002). However, what remains to be investigated is whether these adsorbed components of FCS are transferred wholly or partially following fragmentation by cellular enzymes or are degraded otherwise. However, with regard to *O*-acetylated sialoglycans, analysis of FCS by western blotting (Figure 3A, lane 6) and HPLC (data not shown) did not indicate the presence of this derivative and therefore its source remains an open-ended question.

The presence of *O*-acetylation of sialoglycoconjugates depends on *O*-acetyl transferases, but their role is yet to be substantiated because these enzymes have so far proven intractable to purification and molecular cloning. In this regard, Shi *et al*. (1996) have elegantly demonstrated that transfection of $\alpha2\rightarrow6$ and $\alpha2\rightarrow8$ sialyltransferases in Chinese hamster ovary–Tag cells led to expression of not only $\alpha2\rightarrow6$- and $\alpha2\rightarrow8$-linked sialic acids respectively but also that of surface 9-*O*-acetylated sialic acids on *N*-linked glycoconjugates.

Although the presence of Neu5Ac and Neu5, 9Ac2 in *L. donovani* was detectable by HPLC (Figure 1), analysis of the *O*-acetylated derivative by MS was not achievable. Analytical methods for quantification of these alkali-labile *O*-AcSGs are prone to errors (Reuter *et al*., 1980). Therefore, in practical terms, accurate detection of these saponifiable groups as also information especially with regard to their presence in the sterical context of the intact cell surface is only feasible by using noninvasive approaches, such as lectins or antibodies (Sinha *et al*., 2000). The most common probe used for detecting the presence of 9-*O*-AcSGs irrespective of their linkage and subterminal sugar is the influenza C virus or its recombinant soluble form with the Fc portion of human IgG (CHE-Fc) (Herrler *et al*., 1985).

In another approach we used Achatinin-H isolated from African giant land snail *Achatina fulica*, which preferentially binds to 9-*O*-AcSGs in an $\alpha2\rightarrow6$ linkage to GalNAc (Sen and Mandal, 1995). The detectable agglutination by Achatinin-H (Figure 4A) and its binding to parasite membranes (Figures 3A, 4B) indicated that 9-*O*-AcSGs are present on the cell surface of *L. donovani* promastigotes. Flow cytometric analysis confirmed the lectin binding specificity was toward surface 9-*O*-AcSGs because there was 77.0% reduction of binding following 9-*O*-esterase treatment (Figure 5). Interestingly, a 50% reduction in SNA binding following 9-*O*-esterase treatment reconfirmed the presence of surface 9-*O*-AcSGs in α2-6 linkages (Table I). This was further substantiated by the binding of CD60b antibodies, known to bind to 9-*O*-acetyl disialoglycans (Schwartz-Albiez, 2001). To the best of our knowledge, this is the first report on the presence of 9-*O*-AcSGs on *Leishmania* parasites.

The current problem in Indian leishmaniasis is the increasing unresponsiveness to first-line treatment with pentavalent antimonial drugs, namely sodium antimony gluconate (Guerin *et al*., 2002). In view of the alarming increase in antimonial unresponsiveness, it would be interesting to study whether surface sialoglycans of antimonial resistant parasites are differentially adsorbed. They would then potentially serve as molecular targets helpful in distinguishing drug-responsive from drug-unresponsive patients and allowing development of new drug strategies (Mandal *et al*., 2000). In the future, it will be important to find whether these sialylated determinants, especially the 9-*O*-acetylated derivative, are present in other *Leishmania* strains, whether their expression is stage-specific, and what is the machinery that regulates sialylation and *O*-acetylation of the *Leishmania* parasites. The potential payback is a greater understanding of the endogenous roles of sialoglycans that may be relevant with regard to the host–parasite relationship.

## Materials and methods

### *Parasites*

Promastigotes of an Indian *L. donovani* strain MHOM/IN/83/AG83 (Chatterjee *et al*., 1998) were grown at 22–25°C in M199 medium containing *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (20 mM, pH 7.5) supplemented with 10% heat-inactivated FCS and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml).

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

Bovine submandibular glands, procured locally, were used as a source to purify BSM according to the method of Chatterjee *et al*. (1998). Percentage of 9-*O*-AcSA was determined by subtracting the relative unsubstituted sialic acids from that obtained after de *O*-acetylation using the method of Sharma *et al*. (1998). BSM was then coupled to Sepharose 4B using the method of Kohn and Wilchek (1982). Achatinin-H was purified from the hemolymph of the common giant African land snail *A. fulica* by affinity chromatography using BSM coupled to Sepharose 4B as previously described (Sen and Mandal, 1995). Lectin binding of Achatinin-H was checked by hemagglutination (Sen and Mandal, 1995). Its carbohydrate binding specificity toward 9-*O*-AcSA was examined by hemagglutination inhibition using mono- and disaccharides as well as several sialoglycoproteins as inhibitory reagents (Sinha *et al*., 1999; Pal *et al*., 2000).

### *Fluorimetric HPLC for estimation of sialic acid (Neu5Ac) and its principal derivatives*

Cultured promastigotes of *L. donovani* were extensively washed in PBS (0.02 M, pH 7.2), and the cell pellet was resuspended in 1.0 ml double distilled water. Cell lysis was completed by sonication (three pulses of 16 s each and lysates were kept on ice in between). Glycoconjugates were then subjected to acid hydrolysis with an equal volume of 4 M proprionic acid to release sialic acids. Samples were heated to 80°C for 4 h, cooled on ice for 10 min, separated into three fractions, and then lyophilized (Mawhinney and Chance, 1994). Controls included (1) saponification of sialic acids by incubating the lyophilized sample with 100 μl 0.1 M ammonia for 1 h at 37°C with subsequent lyophilization and (2) sialate pyruvate lyase treatment by resolving the samples in 200 μl 50 mM phosphate buffer, pH 7.2, including 25 mU acylsialate neuraminate lyase (EC 4.1.3.3) and incubated for 2 h at 37°C. Samples were then derivatized with 1,2-diamino-4,5-methylenedioxybenzene for fluorimetric HPLC analysis (Hara *et al*., 1989).

Analysis of the derivatized sialic acids was done on a RP-18 column (4 × 250 mm, Lichrospher RP-18, Merck, Darmstadt, Germany) using isocratic elution with water/acetonitrile/methanol (84/9/7, v/v/v) at a flow rate of 1 ml/min and compared with authentic standard sialic acids purified from BSM. Fluorescence detection was performed using an excitation wavelength of 373 nm and emission wavelength of 448 nm. In parallel, the three washes of the parasite cultures were similarly analyzed as described.

### *GC/MS analysis of sialic acids as TMS-methylester derivatives*

Lyophilized samples were dissolved in dry methanol (0.5 ml) and 80 μl Dowex $H^+$ in methanol was added. The samples were filtered over cotton wool to remove the Dowex and subsequently treated with diazomethane in ether for 5 min at room temperature. 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 gas chromatography/mass spectrometry (GC/MS), respectively (Schauer and Kamerling, 1997).

The following equipment and parameters were used for GC/MS analysis: a Fisons Instruments GC 8060/MD800 system (Interscience, Breda, The Netherlands), an AT-1 column (30 m × 0.25 mm, Alltech, Breda, The Netherlands); the temperature program was 220°C for 25 min, 6°C/min to 300°C, 6 min; the injector temperature was 230°C; and the detection was done by electron impact MS with a mass range of 150–800 *m/z*.

### *Flow cytometric analysis*

To establish the presence of sialic acids on the parasite surface, two sialic acid binding lectins, SNA and MAA (Vector Labs, Burlingame, CA), were used. Parasites were extensively washed and resuspended ($1 \times 10^7$ cells/ml) in prechilled RPMI medium supplemented with 2% bovine serum albumin (BSA) and 0.1% sodium azide (medium A) for 1 h at 4°C in the dark. 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. To demonstrate the presence of modified sialic acid derivatives, parasites were incubated for 1 h at 4°C with FITC-labeled Achatinin-H and CD60b-specific antibody UM4D4. Conjugation of Achatinin-H with FITC was carried out as described by Coligan *et al*. (1993). FITC-IgM served as a control for the binding of parasite with FITC-anti-CD60b. Cells were washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry (FACS Scan flow cytometer, Becton Dickinson, Mountain View, CA).

To measure the binding of Siglecs to *L. donovani* promastigotes, the Siglecs-Fc were initially complexed with biotinylated goat anti-human antibodies (Fc-specific) by incubating at room temperature for 1 h. Subsequently, promastigotes (either untreated or pretreated with *Vibrio cholerae neuraminidase*, VCN) were incubated with the complexed Siglecs for 30 min on ice. The cells were then washed and the extent of binding detected by flow cytometry using streptavidin-FITC.

### *Sialidase treatment of* L. donovani *parasites*

To confirm the presence of sialic acids, parasites ($1 \times 10^6$/100 μl) were incubated for different time periods with different concentrations of VCN (Dade Behring, Marburg, Germany, stock solution 1.0 U/ml) in PBS containing 1.0% BSA for 1 h at 37°C. Cells were then washed three times, resuspended in medium A, and processed for flow cytometric analysis as described. Cells ($1 \times 10^6$/100 μl) were also

treated with 50 µl of *Arthrobacter ureafaciens* neuraminidase (Roche, Mannheim, Germany, stock solution 10 U/ml) for 30 min at 20–25°C, washed, and processed as mentioned.

*Esterase treatment of* L. donovani *parasites*

Presence of *O*-acetyl sialoglycan group on parasites was demonstrated by taking advantage of the 9-*O*-acetyl hemagglutinin esterase of influenza C virus. It had been originally cloned in an SV40 vector (Vlasak *et al*., 1987) to construct a gene consisting of the influenza C virus HE1 domain fused to the eGFP gene. Briefly, the entire HE1 coding region was isolated as a Sac I/Cla I restriction fragment. The Cla I site was filled in to allow blunt end ligation with the filled in BamH I site immediately upstream of the eGFP gene derived from plasmid pEGFP-N3 (Clontech Laboratories, Austria). The resulting chimeric gene contains the entire HE1 domain and the first four codons of the HE2 domain linked via a five-codon spacer to the coding region of eGFP. This construct was ligated into the recombination vector pBakPAK8 (Clontech). The resulting plasmid pBacPAK-CHE1-eGFP was cotransfected with baculovirus DNA (Pharmingen, San Diego, CA) into Sf9 cells. Recombinant baculovirus Bak-CHE1-eGFP was plaque-purified and used to express the recombinant HE1-eGFP fusion protein. The expression of HE-1 domain was sufficient to obtain a specific 9-*O*-acetyl esterase activity that was determined with *p*-nitrophenyl acetate (*p*NPA) as previously described (Vlasak *et al*., 1987). One unit was defined as the amount of enzymatic activity resulting in hydrolysis of 1 µmol of *p*NPA per min. Release of acetate from BSM type I-S (Sigma-Aldrich) was determined with a commercial kit as previously described (Vlasak *et al*., 1998).

Accordingly, $1 \times 10^6$ cells were incubated with 100 µl of the culture supernatant containing recombinant protein for 1 h at 20–25°C. Cells were then washed and processed as described.

*Western blot analysis of parasite membranes*

Membrane fractions were purified from mid-log phase *L. donovani* promastigotes. After the cells were harvested, they were resuspended in lysis buffer (Chatterjee *et al*., 1998), sonicated, and then centrifuged at 10,000 rpm at 4°C for 10 min. Membrane proteins (60 µg/lane) before and after sialidase treatment were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (7.5%) according to Weismann *et al*. (1988) and transferred to nitrocellulose. After the nonspecific binding sites were blocked with 2% BSA in Tris buffered saline (0.05 M, pH 7.3), the presence of sialoglycoconjugates was determined by overnight incubation at 4°C with biotinylated SNA and MAA (0.04 mg/ml, diluted 1:200) that were detected by peroxidase-conjugated avidin. The *O*-acetylated sialoglycoproteins reacting with Achatinin-H were colorimetrically detected with a peroxidase conjugated anti-rabbit IgG (Chatterjee *et al*., 1998). Medium containing 10% FCS (60 µg/lane) were run in parallel for western blot analysis using all three lectins.

*Parasite agglutination assay*

To assess the degree of parasite binding, parasites were harvested, washed three times with PBS, and resuspended in a concentration of $1 \times 10^7$/ml. To 100 µl of parasite suspension serially diluted Achatinin-H was added and incubated at 20–25°C for 15 min. Cells were then examined microscopically and the number of nonagglutinated cells counted; accordingly, the degree of agglutination was extrapolated.

*ELISA*

Log phase cultures of Ag83 were harvested and washed with PBS; the cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 40 mM NaCl, pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 5 mM ethylenediamine tetraacetic acid (EDTA), and 5 mM iodoacetamide (Chatterjee *et al*., 1998). The crude parasite lysate was used as the coating antigen (5 µg/ml, 50 µl/well in 0.02 M phosphate buffer, pH 7.8), and binding of Achatinin-H was determined by measuring binding of rabbit anti-Achatinin-H using horseradish peroxidase-conjugated anti-rabbit IgG (Chatterjee *et al*., 1998).

*Estimation of UDP-N-acetylglucosamine 2-epimerase in* L. donovani *promastigotes*

Promastigotes ($1 \times 10^7$ cells) were harvested, washed once with PBS, and lysed by hypotonic shock in 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for 15 min at 4°C. The crude membrane fraction was pelleted by centrifugation at $100{,}000 \times g$ for 30 min, and the resultant supernatant was assayed for UDP-GlcNAc 2-epimerase as previously described (Hinderlich *et al*., 1997). Briefly, assays were performed in a final volume of 200 µl, containing 35 mM sodium phosphate, pH 7.5, 4 mM $MgCl_2$, 0.5 mM UDP-GlcNAc, and 1 µCi [$^3$H]-UDP-GlcNAc. Incubations were carried out at 37°C for 3 h and stopped by addition of 300 µl ethanol. Radiolabeled substrates were separated by descending paper chromatography and quantified by liquid scintillation analysis. Controls included 100 µM CMP-Neu5Ac, specifically inhibiting UDP-GlcNAc 2-epimerase activity. Protein concentration was determined according to the method of Bradford (1976) using BSA as standard.

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**Abbreviations**

AcSGs, acetylated sialoglycoconjugates; BSA, bovine serum albumin; BSM, bovine submaxillary mucin; EDTA, ethylene diamine tetra acetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluoroscein isothiocyanate; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid

chromatography; MAA, *Maackia amurensis* agglutinin; MS, mass spectrometry; PBS, phosphate buffered saline; *p*NPA, *p*-nitrophenyl acetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNA, *Sambucus nigra* agglutinin; TMS, trimethylsilyl/VCN, *Vibrio cholerae* neuraminidase; VL, visceral leishmaniasis.

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