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# Identification of 9-*O*-acetylated sialoglycans on peripheral blood mononuclear cells in Indian Visceral Leishmaniasis

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**Although the existence of *O*-acetylated sialic acids is well known, it is only in recent years that steady refinement of analytical techniques have enabled detailed mapping of their structural diversity [1]. Fluorimetric analysis of peripheral blood mononuclear cells (PBMC) of patients with Visceral Leishmaniasis (VL) showed six fold increase in the percentage of surface 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) as compared to normal human donors. Using Achatinin-H, a 9-*O*-acetyl sialic acid- binding lectin, an enhanced presence of 9-*O*-AcSGs in an $\alpha 2 \rightarrow 6$ linkage was demonstrated by flow cytometry; abolition of its binding by pretreatment with a recombinant 9-*O*-acetylesterase corroborated the presence of this glycotope. Western blotting of PBMC from VL patients indicated the presence of five *O*-acetylated sialoglycans corresponding to 144, 65, 56, 36 and 19 kDa as compared to 144 and 36 kDa in normal individuals. Taken together our data indicates that during active disease, there is an overexpression of 9-*O*-AcSGs on the surface of PBMC of VL patients, thus opening up new research avenues wherein the expression of this biomarker could be exploited to monitor the clinical status of VL patients.**
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***Abbreviations:*** **ABTS, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ammonium salt; ALL, acute lymphoblastic leukemia; BSM, Bovine submaxillary mucin; DIG, digoxigenin enzyme assay; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, Horse radish peroxidase; Neu5Ac, *N*-acetyl neuraminic acid; *O*-AcSGs, *O*-acetylated sialoglycoconjugates; PBMC, Peripheral blood mononuclear cells; VL, Visceral Leishmaniasis.**

## Introduction

Protozoan parasites of the genus *Leishmania* are obligate intracellular protozoan parasites responsible for a wide spectrum of clinical manifestations resulting in substantial morbidity and mortality placing a 10th of the world's population at risk [2]. The visceral form of the disease or kala-azar is widely prevalent in Eastern India, and, owing to its typical features of irregular fever, progressive hepatosplenomegaly, anemia, immunosuppression and hypergammaglobulinemia, patients are often empirically treated for other co-endemic diseases like tuberculosis and malaria. The presence of high anti-leishmanial titers has allowed for the development of a number of serodiagnostic methods; however, the possibility of cross reactivity, false negativity in immunocompromised individuals, and persisting antibody titers following cure limits its clinical acceptability [3]. Therefore, it is relevant to identify biomarkers whose presence can be harnessed to evaluate the disease status since they are observable end points in a continuum of events leading from exposure to toxic agents to diseases that ultimately result from exposure.

The preferential affinity of Achatinin-H, a lectin that binds preferentially to glycoconjugates having terminal 9-*O*-acetylated sialic acid derivatives present in an $\alpha 2 \rightarrow 6$ linkage to subterminal GalNAc [4–6], allowed us to identify the

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presence of the glycotope having terminal 9-*O*-AcSGs on erythrocytes of patients with VL [7,8] as also on the surface of *Leishmania donovani* promastigotes [9]. As the concomitant presence of this *O*-acetylated glycotope both on parasites and erythrocytes of VL patients suggests a common mechanism responsible for induction of *O*-acetylation, this study was undertaken to evaluate the status of *O*-acetylated sialoglycans on peripheral blood mononuclear cells of patients with VL.

## Materials and methods

### Study population

Peripheral blood (5 ml) was collected from clinically confirmed VL patients admitted at the Kala-azar Medical research center, Muzaffarpur, Bihar. PBMC were separated by Ficoll-Hypaque density centrifugation; plasma was separated and stored at $-20^{\circ}$C. Informed consent was taken either from the patient or guardian of the patient. The study was approved by the institutional human ethical committee as per protocol of Indian Council of Medical Research.

### Probes for detection of 9-*O*-acetylated sialoglycans

Achatinin-H was affinity purified from the hemolymph of the African giant snail, *Achatina fulica*, using bovine submaxillary mucin (BSM) [6]. Our strategy to employ BSM was based on established evidence that it contains a high proportion of 9-*O*-AcSGs [10]. The percentage of 9-*O*-AcSGs present in BSM was fluorimetrically estimated [11] and found to be 22.0%.

In an alternate approach, serum components from VL patients having a preferential affinity for 9-*O*-AcSGs *i.e.* *O*-AcSG binding proteins were affinity- purified from serum of VL patients. Briefly, serum (5.0 ml) following a 33% ammonium sulfate fractionation was passed through an asialo BSM column. The resultant pass-through was loaded onto a BSM column where the specific proteins were eluted [12].

### Qualitative analysis of glycosylation

Membrane fractions were prepared from VL patients ($PBMC_{VL}$) and normal donors ($PBMC_N$) [13], and the degree of glycosylation was detected by a digoxigenin (DIG) enzyme assay using a commercially available DIG-glycan kit (Cat no: 1142372, Roche Molecular Biochemicals). To measure the degree of sialylation, samples were exposed to mild oxidation with sodium metaperiodate (1 mM) for 20 min at $0^{\circ}$C as per the manufacturer's instructions.

### Quantitation of percentage 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs)

The percentage of 9-*O*-AcSGs on $PBMC_{VL}$ ($n = 19$) and $PBMC_N$ ($n = 3$) was determined [7,11] using a Hitachi spectrofluorimeter by subtracting the percentage of unsubstituted sialic acids obtained after de-*O*-acetylation. The membrane integrity of the cells was confirmed using trypan blue.

### Enzyme-linked immunosorbent assay (ELISA)

Membrane fractions purified from $PBMC_{VL}$ and $PBMC_N$ served as the coating antigen (1 $\mu$g/100 $\mu$l/well in phosphate buffer, pH 7.8) in 96-well flat-bottomed polystyrene microtiter plates. The binding of *O*-AcSG binding proteins purified from serum of VL patients (0.1 mg/ml, 100 $\mu$l per well) was detected using Horseradish peroxidase (HRP) conjugated Protein A (diluted 1:10,000) as previously described [12].

### Flow cytometric analysis

Achatinin-H was conjugated with fluorescein isothiocyanate (FITC) [14]. $PBMC_{VL}$ and $PBMC_N$ ($1 \times 10^6$ cells/100 $\mu$l/tube) were washed with phosphate-buffered saline (PBS, 0.02 M, pH 7.4), and the binding of FITC-Achatinin-H (1 $\mu$g) and FITC- labeled desialylated bovine serum albumin (BSA) as the negative control was analyzed on a FACS Calibur flow cytometer.

The binding specificity of Achatinin-H toward 9-*O*-acetylated sialoglycans was confirmed by measuring the extent of binding following pre-treatment with *O*-acetylesterase known to specifically cleave *O*-acetylated sialic acid derivatives [9,15]. Accordingly, cells were pre-treated for 3 h at $37^{\circ}$C with 100 $\mu$l of culture supernatant.

### SDS PAGE and Western blot analysis

Membrane fractions of $PBMC_{VL}$ and $PBMC_N$ were electrophoresed and transferred onto nitrocellulose. The blots were then probed with Achatinin-H (0.1 mg/ml) and binding of Achatinin-H was detected using rabbit anti-Achatinin-H (diluted 1:100), followed with HRP-conjugated anti-rabbit (diluted 1:500), and the binding was colorimetrically detected.

## Results

### Achatinin-H recognizes terminal 9-*O*-AcSA $\alpha$2-6 GalNAc glycotopes

Hemagglutination inhibition assays using 9-*O*-AcSA and various sialoglycoproteins, mainly BSM having 20% 9-*O*-AcSA, have previously confirmed that Achatinin-H selectively binds to sialoglycoconjugates having terminal 9-*O*-AcSA in an $\alpha 2 \rightarrow 6$ linkage to a subterminal GalNAc of the underlying structure [6].

### Increased degree of surface sialylation on PBMC of VL patients

A higher degree of surface glycosylation was demonstrated in $PBMC_{VL}$ as compared to $PBMC_N$ using the DIG kit (Figure 1A) and was associated with a higher amount of surface sialylation (Figure 1B). Quantification by densitometric scanning of these blots revealed a four-fold increase in the degree of glycosylation

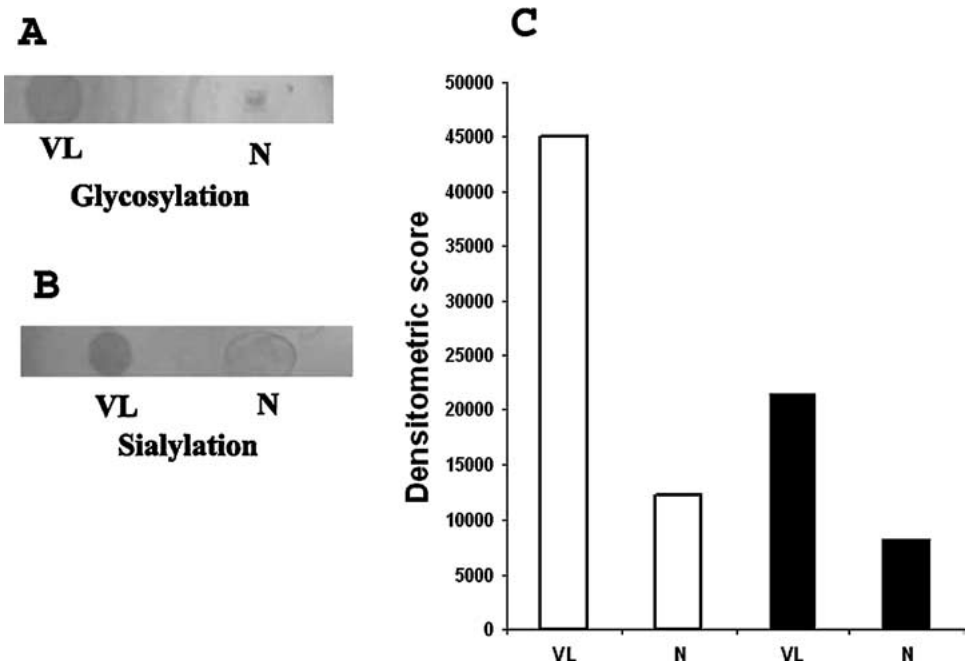


**Figure 1.** Representative profile of glycosylation and sialylation as analysed by digoxigenin enzyme immuno assay. (A) Dot blot analysis of glycosylation profile of membrane fractions from $PBMC_{VL}$ (VL) and $PBMC_N$ (N). (B) Dot blot analysis of sialylation profile of membrane fractions from $PBMC_{VL}$ (VL) and $PBMC_N$ (N). (C) Densitometric scoring of glycosylation (□) and sialylation (■) profiles of membrane fractions from $PBMC_{VL}$ (VL) and $PBMC_N$ (N).

and a two-fold increase in sialylation in membrane fractions of $PBMC_{VL}$ vs. $PBMC_N$ (Figure 1C).

### Membrane fractions from VL patients have an increased percentage of surface 9-*O*-acetylated sialoglycans

Fluorimetric analysis of PBMC revealed a six-fold increase of 9-*O*-AcSGs present in $PBMC_{VL}$ as compared to $PBMC_N$, Mean ± S.E. being 30.0 ± 2.0 vs. 5.0 ± 3.0, respectively (Table 1).

### Flow cytometric analysis demonstrated increased binding of Achatinin-H to PBMC of VL patients

Flow cytometric studies were undertaken to measure the expression of glycoconjugates having terminal 9-*O*-AcSA $\alpha 2 \rightarrow 6$ GalNAc epitopes specifically recognized by Achatinin-H [6]. A higher presence of this glycotope on $PBMC_{VL}$ was clearly evident from the higher binding of Achatinin-H (97%) as compared to a minimal binding in $PBMC_N$ (10%) (Figure 2). To validate that this binding is due to an increased amount of *O*-acetylation, binding of Achatinin-H was also examined following treatment with a recombinant *O*-acetylesterase, known to hydrolyse *O*-acetyl esters. $PBMC_{VL}$ when incubated with this chimeric esterase caused a near total abolition of lectin-binding from 97.0% to 13.0% (Figure 3A-B).

### ELISA based detection of surface 9-*O*-AcSGs in PBMC of VL patients

Binding of the immunoglobulin component of a BSM eluted serum fraction containing 9-*O*-AcSG binding proteins to $PBMC_{VL}$ and $PBMC_N$ was measured using Protein A. A 13-fold higher binding with membrane fractions from $PBMC_{VL}$

**Table 1.** Clinical data for VL patients along with their cell surface 9-*O*-AcSA content

| *Categories* | [a]*% 9-O-AcSGs Mean ± S.E. (Range)* | *Duration of illness (days), Mean ± S.E. (Range)* | [b]*Splenic score (Range)* | *Age range (Years)* | *Male:Female* |
|---|---|---|---|---|---|
| VL | 30.0 ± 2.0 (15–43.3) | 53.94 ±11.28 (10–210) | 4+ –1+ | 5–50 | 13:6 |
| Normal donors | 5.4 ± 3.3 (1.2–12) | – | N.A. | 25–50 | 2:1 |

[a]As determined by fluorimetric analysis [11].
[b]Microscopic examination of Giemsa-stained splenic aspirates.

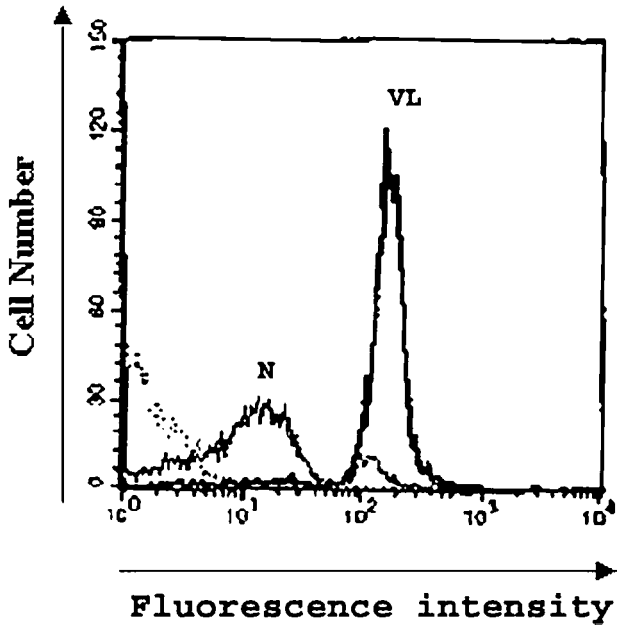


**Figure 2.** Single color fluorescence analysis to demonstrate selective binding of Achatinin-H to PBMC of VL patients. Surface expression of 9-*O*AcSG was determined by flow cytometric analysis using FITC-Achatinin-H on PBMC from VL patients (VL) and normal donors (N) respectively as compared to the negative control (dotted line).

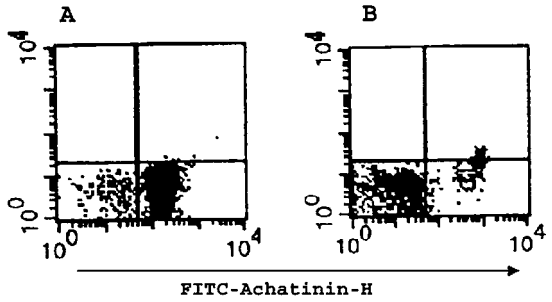


**Figure 3.** Demonstration of binding specificity of Achatinin-H to 9-*O*-acetylated sialic acids by single color fluorescence analysis. Surface expression of 9-*O*AcSG was determined by flow cytometric analysis using FITC-Achatinin-H (A) and following pre-treatment with *O*-acetyl esterase (B).

than $PBMC_N$ was obtained, Mean $\pm$ S.D. of absorbances at 405 nm being $1.94 \pm 0.45$ vs. $0.15 \pm 0.03$ respectively.

### Molecular characterization of the glycotope recognized by Achatinin-H

To characterize the *O*-acetylated sialoglycans present on $PBMC_{VL}$, Western blotting was performed. Achatinin-H bound to *O*-acetylated glycotopes corresponding to 144, 65, 56, 36 and 19 kDa present on $PBMC_{VL}$ as compared to two bands in $PBMC_N$ at 144 and 36 kDa (Figure 4).

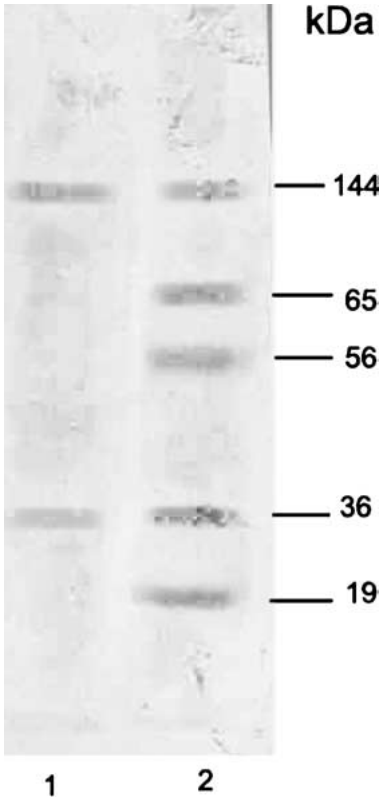


**Figure 4.** Molecular characterization of cell surface 9-*O* acetylated sialoglycans that bind to Achatinin-H. Membrane proteins from $PBMC_N$ (lane 1) and $PBMC_{VL}$ (lane 2) were analysed on SDS-PAGE (10%), transferred onto nitrocellulose, and the extent of binding of Achatinin-H was measured by Western blot analysis as described in Material and Methods.

## Discussion

Sialic acids are a family of *N*- and *O*-substituted derivatives of neuraminic acid, a 9-carbon carboxylated monosaccharide commonly referred to as *N*-acetyl neuraminic acid or Neu5Ac [1]. Its terminal position provides it accessibility reflected in its regulation of a multitude of cellular and molecular interactions [16]. The most common modification of the parent sugar Neu5Ac is *O*-acetylation at position C-7/8/9 to form *N*-acetyl-7/8/9-*O*-acetyl neuraminic acid or *O*-acetylated sialoglycoconjugates [17]. Growing evidence, suggests a number of physiological and pathological processes ranging from cell-cell adhesion, signaling, differentiation and metastasis may be attributed to the appearance of *O*-AcSGs [16]. Accordingly, study of their occurrence, metabolism, quantitative and qualitative analyses will provide an insight into the structure and function of these fascinating molecules.

The increased presence of 9-*O*AcSGs on both erythrocytes of VL patients [7,8] as also *Leishmania donovani* promastigotes [9] prompted us to assess the status of this glycotope

on PBMC of VL patients. We observed a marked increase in surface glycosylation in $PBMC_{VL}$ concomitantly associated with a higher amount of surface sialylation in comparison with $PBMC_N$ (Figure 1).

To date, study of the cell biology and biochemistry of 9-*O*-acetylated sialic acids remains limited as their accurate quantification requires their previous release from glycosidic linkages, which is often not totally satisfactory [18]. Currently available tools for detecting these alkali labile *O*-AcSGs include the Influenza-C-virus or its recombinant soluble form with the Fc portion of human IgG [17]. These tools can detect 9-*O*-AcSGs irrespective of their linkage and the subterminal sugar. Lectins available include the Californian coastal crab *Cancer antennarius* that binds to *O*-acetylated sialic acids both at C-4 and C-9 positions [17] and the snail lectin Achatinin-H whose glycotope has been defined as 9-*O*-AcSA $\alpha 2 \rightarrow 6$ GalNAc [6].

To examine the extent of *O*-acetylation induced on $PBMC_{VL}$, flourimetric analysis was undertaken to measure the percentage of sialoglycans shown to be 9(8)-*O*-acetylated irrespective of the linkage or the subterminal sugar. A six-fold enhancement in the levels of *O*-AcSA on $PBMC_{VL}$ was observed as compared to $PBMC_N$ (Table 1).

Flow cytometric analysis demonstrated a high rate of binding of Achatinin-H, a 9-*O*-AcSA $\alpha$2-6 GalNAc binding-lectin to $PBMC_{VL}$ (97%). In contrast, $PBMC_N$ showed minimal binding (10%) in concordance with previous reports that lymphocytes contain low levels of 9-*O*-acetylated sialic acids [19] (Figure 2). To further establish that the *O*-AcSA glycotope present on $PBMC_{VL}$ is vital for recognition by Achatinin-H, its binding was re-examined following selective removal of cell surface *O*-acetyl sialoglycan by a chimeric *O*-acetyl esterase [15]. The substantial decrease in lectin binding validated that a higher proportion of glycotopes containing terminal *O*-AcSA are present on $PBMC_{VL}$ (Figure 3A–B). This was further corroborated by ELISA studies using membrane fractions from $PBMC_{VL}$ and $PBMC_N$ as the coating antigens, where a significantly higher binding of purified human antibodies raised against 9-*O*-AcSG epitopes to $PBMC_{VL}$ was demonstrated.

Western blot analysis of $PBMC_{VL}$ membranes by the probe Achatinin-H identified the presence of three distinct disease-specific protein corresponding to 65, 56, and 19 kDa on $PBMC_{VL}$ (Figure 4). Although 9-*O*-AcSGs have been shown to be present on PBMC of patients with acute lymphoblastic leukemia (ALL) [19,20], the *O*-AcSGs have a different molecular weight of 135, 120, and 90 kDa suggesting that, although O-acetylation is occurring, distinctly different glycoproteins are *O*-acetylated. A direct relationship has been established between the presences of *O*-AcSGs on lymphoblasts of children with ALL using a noninvasive, lymphoproliferation assay; it may be envisaged that similar to ALL, monitoring these disease specific biomarkers may help in assessing the clinical status of VL patients [20].

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