MAJOR ARTICLE

Variable Degree of Alternative Complement Pathway–Mediated Hemolysis in Indian Visceral Leishmaniasis Induced by Differential Expression of 9-*O*–Acetylated Sialoglycans

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Background. Increased expression of linkage-specific 9-*O*–acetylated sialoglycans (9-*O*-AcSGs) has been demonstrated on erythrocytes from patients with visceral leishmaniasis (VL) by use of Achatinin-H. We assessed the capacity of this glycotope to influence hemolysis via activation of the alternative complement pathway in patients with VL, compared with that in healthy control subjects.

Methods. The differential expression of 9-*O*-AcSGs on surfaces of erythrocytes was measured, 9-*O*-AcSGs were affinity purified, and the molecular determinants were identified by Western blotting. The degree of alternative complement pathway–mediated hemolysis was compared with expression of 9-*O*-AcSGs on erythrocytes.

Results. Enhanced expression of linkage-specific 9-*O*-AcSGs was demonstrated on erythrocytes from patients with active VL. Six distinct molecular determinants present only on diseased erythrocytes were affinity purified and were absent after elimination of parasite burden. A correlation ($r^2 = 0.9$) was observed between the presence of 9-*O*-AcSGs and the degree of alternative complement pathway–mediated hemolysis.

Conclusion. The 9-*O*-AcSGs expressed on erythrocytes from patients with VL are potent complement activators, causing enhanced hemolysis via activation of the alternative complement pathway, and may account for the anemia that is a common manifestation of VL.

Leishmaniasis, which is caused by the obligate intracellular protozoan parasite of the genus *Leishmania*, affects >10 million people in the tropical and subtropical regions of the world where the parasite is endemic; 10% of the world's population is at risk of infection [1, 2]. The classic diagnostic criterion requires visualization of the intracellular amastigote form of the parasite in giemsa-stained smears of host tissue samples or of the extracellular flagellated promastigote stage in culture. Unfortunately, with regard to the visceral form of the disease, because of the insensitivity of the diagnostic procedure [3], associated inconvenience, and potential risks, many patients are empirically treated for other coendemic diseases, such as tuberculosis and malaria. Several serodiagnostic methods are gradually acquiring importance as complementary procedures to the existing techniques [4–6]. However, cross-reactivity, the possibility of false-negative results for immunocompromised individuals, and the persistence of antibody levels after cure limit their clinical acceptability. It is therefore important that disease-specific biomarkers be identified, because they are potentially useful for monitoring the status of the disease.

Sialic acids constitute a family of *N*- and *O*-substituted 9-carbon carboxylated monosaccharides commonly referred to as *N*-acetyl neuraminic acid. The most common modification of sialic acid is *O*-acetylation at positions C-7/8/9, which generates a family of

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Table 1. Clinical history of patients with visceral leishmaniasis (VL) and control subjects.

Group, classification	No.	Age, mean (range), years	Splenic score, mean (range)	Sex, M:F	Hb level, mean ± SD, g/dL	Erythrocyte-binding assay, mean ± SD[a]
Patients with VL						
Untreated	30	26 (5–60)	1.92 (1–4)	22:8	6.4 ± 1.4	1.14 ± 0.04
Treated[b]	15	30 (8–60)	2.4 (1–3)	12:3	9.7 ± 0.1	0.25 ± 0.01
Control subjects						
Living in area where VL is endemic	10	36 (30–60)	ND	4:6	12 ± 1.2	0.23 ± 0.03
Living in area where VL is not endemic	10	30 (25–45)	ND	8:2	12 ± 2.2	0.32 ± 0.03

NOTE. Hb, hemoglobin; ND, not detected.

[a] Blood was collected in Alsevers solution, and the presence of 9-*O*-AcSAα2-6GalNAc glycotopes was determined by use of an erythrocyte-binding assay [12].

[b] Blood was obtained after completion of antileishmanial treatment.

O-acetylated sialoglycans (*O*-AcSGs) [7, 8]. There is growing evidence that several physiological and pathological processes, including cell-cell adhesion, signaling, differentiation, and metastasis, may be attributed to the appearance of this *O*-acetylated glycotope [9, 10].

The preferential specificity of Achatinin-H toward *O*-AcSGs allowed us to identify 9-*O*–acetylated sialic acid (9-*O*-AcSA) $\alpha2\rightarrow6$GalNAc as a glycotope on hematopoietic cells from patients with visceral leishmaniasis (VL) [11, 12], since 9-*O*-AcSA$\alpha2\rightarrow6$GalNAc is notably absent in cells of patients with diseases that are coendemic with leishmaniasis, such as malaria and tuberculosis. Sialic acid and its derivatives have also been shown to be components of *Leishmania donovani* promastigotes [13].

The 9-*O*-AcSGs present on the surface of murine erythrocytes and murine erythroleukemia cells have been reported to contribute to their susceptibility to lysis, by activation of the alternative complement pathway [14]. We have observed that mammalian erythrocytes with more linkage-specific 9-*O*-AcSAs, as recognized by Achatinin-H, have greater susceptibility to alternative complement pathway–mediated hemolysis [15]. Anemia is a common manifestation of VL and has been attributed to bone marrow infiltration, hypersplenism, and autoimmune hemolysis [16]. An unanswered question is whether the enhanced presence of 9-*O*-AcSGs on these diseased erythrocytes plays a role in anemia. Accordingly, using Achatinin-H as the analytical tool, we have studied (1) the qualitative and quantitative differential expression of 9-*O*-AcSGs on erythrocytes from patients with VL before and after antileishmanial chemotherapy, (2) the purification and characterization of 9-*O*-AcSGs on erythrocytes from patients with VL, and (3) the correlation, if any, between expression of this glycotope and susceptibility of erythrocytes to alternative complement pathway–mediated hemolysis.

Table 2. Correlation of total 9(8)-*O*–acetylated sialic acids (9-*O*-AcSAs) and percentage of hemolysis induced on erythrocytes from patients with visceral leishmaniasis (VL) and healthy control subjects.

Group, classification	9(8)-*O*-AcSA, %[a]	HU[b]	Achatinin-H–positive cells[c]	Hemolysis, %[d]
Patients with VL				
Untreated	30.6 ± 5.1	142.7 ± 4.7	76.07 ± 6.31	36.0 ± 2.26
Treated	7.5 ± 3.4	ND	5.07 ± 2.86	7.5 ± 1.48
Control subjects				
Living in area where VL is endemic	6.2 ± 2.4	ND	2.58 ± 1.18	7.2 ± 0.21
Living in area where VL is not endemic	5.2 ± 2.2	ND	2.38 ± 1.37	7.1 ± 0.11

NOTE. Data are mean ± SD. Blood for all analyses was collected in Alsevers solution. HU, hemagglutination unit; ND, not detected.

[a] Total percentage of 9(8)-*O*-AcSA present on erythrocyte membranes was fluorimetrically estimated, as described in Subjects, Materials, and Methods [11, 15, 19].

[b] Achatinin-H binding HU is defined as the reciprocal of the highest dilution of Achatinin-H that produced visible agglutination [11].

[c] Achatinin-H–positive cells were determined by flow-cytometric analysis using fluorescein isothiocyanate–labeled Achatinin-H as an analytical probe, as described in Subjects, Materials, and Methods.

[d] The percentage of hemolysis induced by the alternative complement pathway using 50 μL of guinea pig serum as source of complement, as described in Subjects, Materials, and Methods [15].

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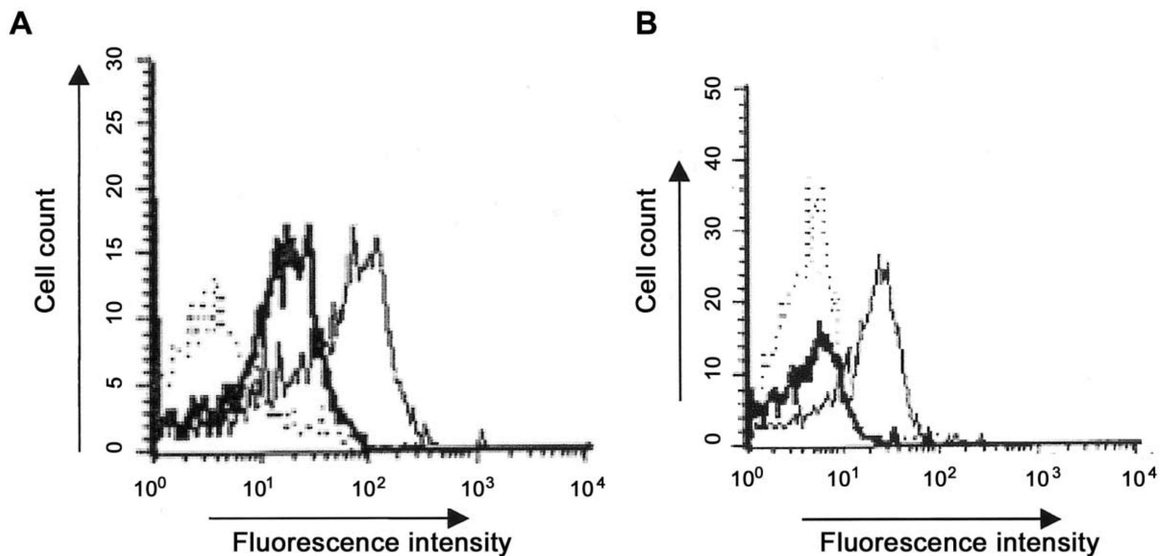

Figure 1. A representative profile of expression of 9-*O*–acetylated sialoglycans on erythrocytes from patients with visceral leishmaniasis (VL), derived by use of flow-cytometric analysis using fluorescein isothiocyanate (FITC)–labeled Achatinin-H. Cells (1×10^6) were incubated with FITC-labeled Achatinin-H and were processed for flow cytometry as described in Subjects, Materials, and Methods. *A,* Erythrocytes from patients with active VL were analyzed before *(thin line)* and after *(bold line)* pretreatment with *O*-acetyl esterase. *Dotted line,* Background binding. *B,* Binding of erythrocytes from patients with VL before *(thin line)* and after *(bold line)* completion of antileishmanial treatment. *Dotted line,* Background binding.

SUBJECTS, MATERIALS, AND METHODS

Study population and study design. The study population included different groups of patients with clinically confirmed VL at presentation ($n = 30$) who completed antileishmanial chemotherapy; they received 15 infusions of amphotericin (1 mg intravenously) on alternate days ($n = 15$), as depicted in table 1. Control subjects included healthy individuals from areas where leishmaniasis is ($n = 10$) or is not ($n = 10$) endemic. Informed consent was obtained either from the patient or the guardian, and the study received approval from the Institutional Human Ethical Committee of the Institute of Medical Sciences, Banaras Hindu University (Varanasi, Indai). The diagnosis of VL was established by microscopic demonstration of amastigotes in bone marrow or splenic aspirates, performed at the Kala-Azar Research Center (Muzaffarpur, Bihar, India), in accordance with recommendations of the World Health Organization [17]. Blood was sent to the Indian Institute of Chemical Biology (Kolkata), where serum was separated, and erythrocytes were immediately processed. The diagnosis was validated by 2 in-house techniques, in which the increased presence of linkage-specific 9-*O*-AcSGs was quantified by use of an erythrocyte-binding assay [12] (table 1), and anti-9*O*-AcSGs were detected by use of an ELISA [5].

Probes. Achatinin-H, a 9-*O*-AcSA binding lectin, was affinity-purified from the hemolymph of the giant land snail *Achatina fulica* by use of bovine submaxillary mucin (BSM) coupled to sepharose-4B as the affinity matrix, on the basis of the long-standing evidence that BSM contains a high percentage of 9-*O*-AcSA [18]. The presence of 9(8)-*O*-AcSA derivatives on BSM was estimated fluorimetrically and was found to be 22.5% of the total sialic acid [11, 15, 19]. This result was further reconfirmed by fluorimetric high-performance liquid chromatography [13]. Furthermore, the specificity of Achatinin-H toward 9-*O*-AcSGs was confirmed through several approaches [20–23]. The binding of Achatinin-H with BSM was reestablished by Western-blot analysis, in which BSM (10 μg) was resolved on 10% SDS–polyacrylamide gel, transblotted onto nitrocellulose, and probed with Achatinin-H (data not shown). Lectin specificity toward *O*-acetylated sialic acid was confirmed by removal of the alkali-labile *O*-acetylated glycotopes by saponification with 0.1 mol/L NaOH, incubation of the blots for 45 min on ice, and neutralization of the excess alkali with 0.1 *N* HCl.

Preparation of erythrocyte membranes and quantification of (9)8-O-AcSAs. Erythrocyte membranes were prepared by resuspending erythrocytes in ice-cold buffer containing digitonin (1 mg/mL) for 20 min on ice. Then the percentage of sialic acid that is *O*-acetylated was estimated fluorimetrically, as described elsewhere [11].

Flow-cytometric analysis. The presence of 9-*O*-AcSA on erythrocyte surfaces was established by use of fluorescein isothiocyanate (FITC)–labeled Achatinin-H. In brief, cells (1×10^6) were extensively washed and incubated with FITC-Achatinin-H (1 μg/tube) for 60 min at 4°C and were fixed with 1% paraformaldehyde. The extent of binding was measured by flow

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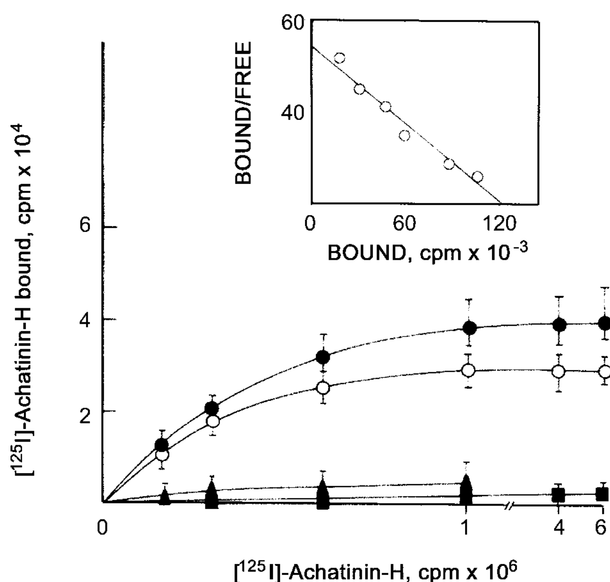

Figure 2. Binding of [^{125}I]-Achatinin-H to erythrocytes from patients with visceral leishmaniasis (VL). A fixed amount of erythrocytes (1×10^6) were incubated with increasing amount of [^{125}I]-Achatinin-H. For evaluating the specific nature of binding, a 50-fold excess of unlabeled Achatinin-H was added. The bound and unbound Achatinin-H were separated at 4°C, and the bound radioactivity was determined as described in Subjects, Materials, and Methods. Specific binding (○) was determined by calculating the difference between total binding (●) and nonspecific binding (■). Post-treatment erythrocytes (▲) were similarly processed for specific binding. Results are expressed as mean ± SD of data from triplicate experiments. $P < .001$, pretreatment erythrocyte binding ligands of Achatinin-H vs. post-treatment erythrocyte binding ligands of Achatinin-H. *Inset,* Scatchard plot of the binding of [^{125} I]-Achatinin-H to erythrocytes from patients with VL.

cytometry (FACScan flow cytometer; Becton Dickinson) by use of normal erythrocytes and FITC-labeled bovine serum albumin (BSA) as the negative control [12].

Esterase treatment of erythrocytes to confirm binding specificity of Achatinin-H. The presence of *O*-AcSGs on erythrocytes was confirmed by preincubating cells with a recombinant 9-*O*-acetyl hemagglutinin esterase of influenza C virus (100 μL) that specifically cleaves the *O*-acetylated component, for 3 h at 37°C [13, 24].

Quantitation of 9-O-AcSGs on erythrocytes from patients with VL, by Scatchard analysis. The purified Achatinin-H was iodinated, according to the method of Hunter [25], by use of chloramine T and Na[^{125}I]. Erythrocytes (1×10^6 cells/25 μL) were incubated for 60 min at 4°C in a total reaction mixture (100 μL) that contained increasing amounts of [^{125}I]-Achatinin-H (specific activity, 1.4×10^6 cpm/μg), $CaCl_2$ (0.3 mol/L; 15 μL), and Tris-buffered saline (TBS) containing BSA (BSA in TBS [pH 7.4]; 0.2%; 40 μL). Nonspecific binding was removed by 3 washes with TBS-BSA, and bound reactivity in the cell pellet was quantified in a Gamma counter (Electronic Corporation of India). For evaluating the specific nature of binding, a 50-fold excess of unlabeled Achatinin-H was added. The K_d and the number of binding sites for Achatinin-H were calculated from the Scatchard plot [26].

Affinity purification of 9-O-AcSGs from erythrocytes. The purified Achatinin-H was coupled to sepharose-4B (1.0 mg of Achatinin-H/mL of gel), and erythrocyte membrane fractions (1.85 mg) were passed through this column (1×2 cm), which had previously been equilibrated with TBS containing 0.03 mol/L $CaCl_2$ at 4°C. After nonspecific washing with TBS containing 0.03 mol/L $CaCl_2$, the bound 9-*O*-AcSGs were eluted with TBS containing 0.04 mol/L sodium citrate (pH 7.2). The eluted material was dialyzed against TBS at 4°C and was stored at −70°C. These affinity-purified 9-*O*-AcSGs were separated by 7.5% SDS–polyacrylamide gel, according to the method of Laemmli [27], and were stained with silver nitrate. The biological activity was tested by use of an ELISA [5], in which equal amounts of both crude and purified 9-*O*-AcSGs (250 ng/100 μL/well) were used separately as coating antigens, were probed with Achatinin-H, and were processed as described elsewhere [13].

Western-blot analysis of 9-O-AcSGs. Erythrocyte ghosts obtained from patients with VL before and after treatment [11] were electrophoresed (30 μg/lane) on 7.5% SDS–polyacrylamide gel. The nonspecific binding sites were transferred onto

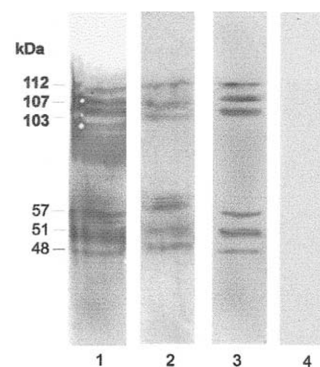

Figure 3. SDS–polyacrylamide gel and Western-blot analysis of crude erythrocyte membranes and affinity-purified 9-*O*–acetylated sialoglycans (9-*O*-AcSGs) eluted from an Achatinin-H affinity matrix. *Lane 1,* Crude membrane proteins (30 μg) on erythrocytes from patients with visceral leishmaniasis (VL) were electrophoresed on a 7.5% SDS–polyacrylamide gel and transblotted onto nitrocellulose. The blots were incubated with purified Achatinin-H and probed with rabbit anti–Achatinin-H and peroxidase-conjugated anti–rabbit IgG, as described in Subjects, Materials, and Methods. *Lane 2,* Purified 9-*O*-AcSGs on erythrocytes from patients with VL (5 μg) were electrophoresed on a 7.5% SDS–polyacrylamide gel and visualized by silver stain. *Lane 3,* Western blotting of purified 9-*O*-AcSGs on erythrocytes from patients with VL, in which the binding of Achatinin-H was detected with rabbit anti–Achatinin-H and peroxidase-conjugated anti–rabbit IgG, as described in Subjects, Materials, and Methods. *Lane 4,* Crude membrane proteins (30 μg) on erythrocytes from patients with VL after treatment were electrophoresed, and Western blotting was performed with Achatinin-H, as described in Subjects, Materials, and Methods.

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Table 3. Purification of 9-*O*–acetylated sialoglycans on erythrocytes from patients with visceral leishmaniasis (VL).

Fraction	Total volume, mL	Protein, mg/mL	Recovery, %	ELISA titer[a]
Crude membrane	0.5	1.85	100	0.57 ± 0.02
Eluate of Achatinin-H and sepharose 4B	6.0	0.75	40.54	1.32 ± .04

[a] Equal amounts of purified and crude fractions on erythrocytes from patients with VL (250 ng/well) were used as coating antigen, and binding with Achatinin-H was monitored, as described in Subjects, Materials, and Methods.

nitrocellulose, blocked with TBS (0.1 mol/L [pH 7.4]) containing 2% BSA (TBS-BSA), and probed with Achatinin-H (5 μg) [13]. Purified 9-*O*-AcSGs were similarly analyzed.

Alternative complement pathway–mediated hemolysis. Before and after chemotherapy, blood was obtained from patients with VL and was placed in PBS containing 0.1% gelatin and 0.04 mol/L EDTA. Activation of the alternative complement pathway was assessed by incubating erythrocytes for 45 min at 37°C in the presence of various amounts of fresh guinea pig serum, which served as the source of complement, as described elsewhere [15].

Statistical analysis. Results are expressed as mean ± SD for individual sets of experiments. Each experiment was performed 2–3 times, and the results are representative of each set of experiments. The 1- or 2-tailed *t* test for significance was performed as applicable in each case. $P < .05$ was considered to be significant. The correlation coefficient was determined between individual titers of 9-*O*-AcSA obtained from the erythrocyte-binding assay (optical density at 405 nm) and percentage of hemolysis (table 1).

RESULTS

Variable amounts of total 9(8)-O-AcSAs on erythrocytes from patients with VL. The increased expression of 9(8)-*O*-AcSA on the surface of erythrocytes was fluorimetrically quantified in cases where the mean ± SD percentage of 9(8)-*O*-AcSA in erythrocytes obtained from patients at presentation ($n = 30$) was 6-fold higher than that in erythrocytes from healthy control subjects ($n = 20$; $P < .001$; table 2). Erythrocytes obtained after chemotherapy ($n = 15$) showed a 4-fold decrease in the percentage of 9-*O*-AcSA, compared with that in erythrocytes from patients with active VL ($P < .001$), similar to that in erythrocytes from healthy control subjects (table 2).

Flow cytometric analysis demonstrated a differential expression of linkage-specific 9-O-AcSGs, as recognized by binding of Achatinin-H. A representative profile of flow-cytometric analysis (figure 1*A*) of erythrocytes from a patient with active VL shows a strong binding with Achatinin-H (76.07% ± 6.31%; $n = 10$). To confirm that this binding was restricted to 9-*O*–acetylated determinants on erythrocytes, cells were incubated with a recombinant *O*-acetylesterase [13, 24]. The de-*O*-acetylation resulted in a drastic reduction of lectin binding, to 9.4% ± 3.4% (figure 1*A*). Patients who had completed their chemotherapy also had a dramatic reduction in their lectin binding (5.07% ± 2.86%; $n = 10$; figure 1*B*). The presence of these glycotopes was negligible on erythrocytes from healthy control subjects (2.38% ± 1.37%; $n = 10$; table 2).

Increased ligands for Achatinin-H on erythrocytes from patients with VL. An increased number of 9-*O*-AcSG molecules was quantified on erythrocytes from patients with active VL ($n = 5$), by measuring the receptor-binding density of Achatinin-H by Scatchard analysis, and was found to be 125,000 ± 552 receptors (figure 2). For evaluating the specific nature of binding, a 50-fold excess of cold, unlabeled Achatinin-H was added, and the apparent K_d was found to be 1.88 ± 0.02 nmol/L. After chemotherapy ($n = 5$), a negligible amount of binding with Achatinin-H was observed, comparable to that with erythrocytes from healthy control subjects, thus reconfirming the absence of these biomarkers.

Molecular analysis of erythrocyte membranes from patients with VL before and after antileishmanial chemotherapy. To pinpoint the molecular identity of glycotopes on erythrocyte membranes that react with Achatinin-H, Western blotting was performed. Six distinct *O*-AcSGs, corresponding to 112, 107, 103, 57, 51, and 48 kDa, were detected on erythrocyte membranes from patients with active VL ($n = 8$; figure 3, *lane 1*). The absence of binding of Achatinin-H after removal of the *O*-acetylated glycotopes by saponification of the membranes confirmed the lectin specificity toward the *O*-acetyl group (data not shown). In parallel, erythrocyte membranes from patients who had completed their antileishmanial chemotherapy ($n = 8$) showed no reactivity to Achatinin-H (figure 3, *lane 4*), similar to erythrocytes from healthy control subjects ($n = 5$; data not shown).

Purification and characterization of 9-O-AcSGs on erythrocytes from patients with VL. Affinity purification of 9-*O*-AcSGs from erythrocytes from untreated patients with VL, using Achatinin-H-sepharose 4B, indicated that 40.54% of the total membrane protein bound with Achatinin-H (table 3). An ELISA using equal amounts of purified and crude fractions of erythrocytes from patients with VL as the coating antigen showed a 2.7-fold increase in lectin binding. The purified material showed 6 bands on SDS–polyacrylamide gel (figure 3,

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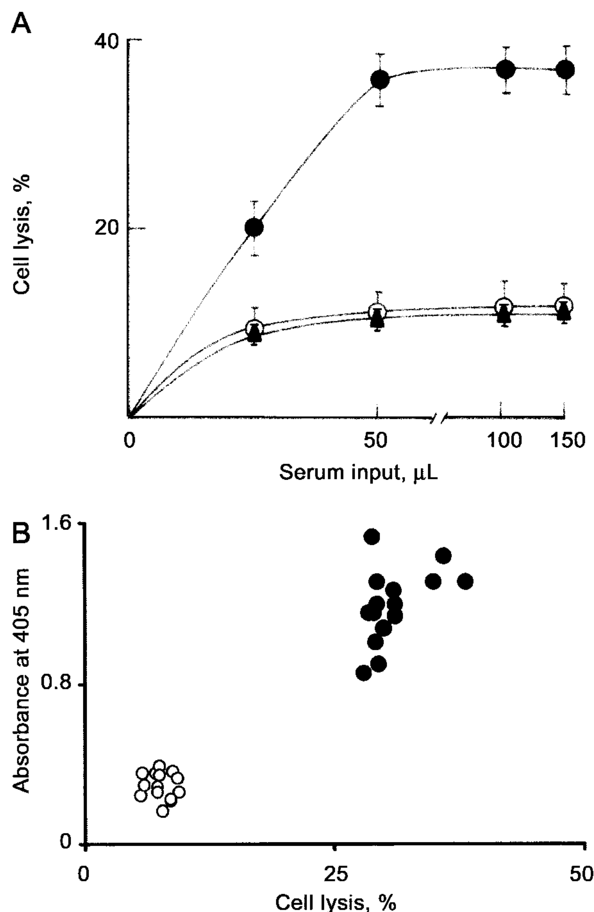

Figure 4. *A,* Alternative complement pathway–mediated hemolysis of erythrocytes from patients with visceral leishmaniasis (VL). Cell-lysis assay of erythrocytes obtained from different groups of patients with VL was performed using increasing amounts of fresh guinea pig serum as the source of complement, as described in Subjects, Materials, and Methods. Erythrocytes included were obtained from patients with active VL (●), patients with VL who had completed treatment (○), and healthy control subjects (▲). Each point is the average of triplicate determinations. Results are expressed as mean ± SD of data from triplicate experiments. $P < .001$, induction of hemolysis in erythrocytes from patients with active VL vs. induction of hemolysis in erythrocytes from patients with VL who had completed treatment and healthy control subjects. *B,* Correlation between individual *O*-acetylated sialoglycan levels, as measured by use of an erythrocyte-binding assay [12], and percentage hemolysis of erythrocytes obtained from patients with VL before treatment (●; $n = 15$) and after completion of treatment (○; $n = 15$).

lane 2), corresponding to 112, 107, 103, 57, 51, and 48 kDa; the binding with Achatinin-H was confirmed by Western-blot analysis (figure 3, *lane 3*).

Enhanced hemolysis of erythrocytes from patients with VL through activation of the alternative complement pathway. Substitution of the OH group at C-9 of sialic acid has been reported to cause enhancement of mammalian erythrocyte lysis, using human serum as complement, through activation of the alternative complement pathway [14, 15]. Accordingly, erythrocytes obtained from patients with VL before ($n = 15$) and after ($n = 15$) completion of chemotherapy were analyzed, to evaluate the contribution of linkage-specific 9-*O*-AcSGs on alternative complement pathway–mediated cytolysis. Erythrocytes from patients with active VL showed a 5-fold higher degree of hemolysis, compared with erythrocytes from healthy individuals residing in an area where leishmaniasis is endemic (36.0% ± 2.26% vs. 7.2% ± 0.21%; figure 4*A* and table 2). However, the degree of hemolysis was significantly decreased in patients who had completed their chemotherapy (7.5% ± 1.48%). In addition, we observed that the addition of esterase to erythrocytes from patients with VL resulted in a decrease in alternative complement pathway–mediated hemolysis to levels comparable with those observed in healthy control subjects (data not shown). The degree of expression of individual 9-*O*-AcSGs on erythrocytes obtained from patients with VL before and after treatment, as determined by use of an erythrocyte-binding assay [12], showed a good correlation ($r^2 = 0.90$) with the percentage of hemolysis (figure 4*B*).

DISCUSSION

Sialic acids have been identified as critical determinants of erythrocyte survival. By virtue of their binding to factor H, they prevent formation of the C3b3b complex and thereby prevent activation of the alternative complement pathway [28, 29]. As proof of this negative regulatory function, Varki and Kornfeld [14] demonstrated that the exocyclic side chain of sialic acid is vital. Studies of murine erythrocytes and murine erythroleukemia cells have shown that, when sialic acid is replaced by its bulky 9-*O*–acetylated derivative, it prevents the binding of sialic acid to factor H, resulting in enhanced activation of the alternative complement pathway and subsequent hemolysis [30]. Furthermore, we have provided evidence that a better correlation exists between the degree of alternative complement pathway–mediated hemolysis and linkage-specific 9-*O*-AcSGs, as identified by Achatinin-H [15].

Because initial studies from our group showed an increased presence of linkage-specific *O*-AcSGs on erythrocytes from patients with active VL [11], we wanted to address the question of whether these diseased erythrocytes have a greater degree of susceptibility to alternative complement pathway–mediated hemolysis. Results of flow-cytometric analyses clearly indicated the increased presence of surface 9-*O*-AcSGs on erythrocytes from patients with VL at presentation, compared with a drastic decrease after chemotherapy (figure 1*A*). The specificity of Achatinin-H was substantiated by removal of the *O*-acetyl moiety with *O*-acetylesterase, which resulted in decreased lectin binding (figure 1*B*). These findings were validated by fluorimetric quantitation of surface 9-*O*-AcSA, in which a 6-fold increase was observed in patients with VL, compared with

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control subjects, which completely decreased with successful chemotherapy (table 2). The quantification of ligands for Achatinin-H was undertaken because they indirectly serve as a measure of the linkage-specific 9-*O*-AcSGs present on the erythrocyte surface. Here again, erythrocytes from patients with VL had an abundance of Achatinin-H ligands, which decreased significantly, almost to background levels, after effective chemotherapy (figure 2). Taken together, our data clearly indicate that 9-*O*-AcSGs can serve as an effective biomarker for monitoring the disease status and, possibly, can serve to develop better chemotherapeutic strategies.

Affinity purification of 9-*O*-AcSGs present on the erythrocyte surface indicated that a large proportion of these glycotopes are present, accounting for 40% of the total protein content (table 3). Western-blot analysis of these purified 9-*O*-AcSGs corroborated findings with crude membrane fractions that showed similar reactivity with Achatinin-H (figure 3, *lane 1*). The identification of 6 distinct molecular determinants on diseased erythrocytes was notably absent on erythrocytes from which the parasite burden had been eliminated (figure 3, *lane 4*). Thus, these purified *O*-AcSGs can be used to develop an ELISA in which the presence of antibodies against 9-*O*-AcSGs can be monitored. Previous studies showed the increased presence of anti–9-*O*-AcSGs by use of BSM as the coating antigen [5]. It may be hypothesized that replacing BSM with these purified 9-*O*-AcSGs might help to enhance the sensitivity and specificity of BSM-ELISA. It would be especially relevant for monitoring the reappearance of the disease in cases in which measurement of specific anti–9-*O*-AcSGs may be a better option than measurement of total antileishmanial antibody titers, which have the disadvantage of persistence after cure; such studies are ongoing.

We have recently demonstrated that differential expression of linkage-specific 9-*O*-AcSGs strongly correlates with the susceptibility of mammalian erythrocytes to lysis, by the alternative complement pathway [15]. Although the presence of *O*-acetylated gangliosides (CDw60) has been detected in leukocytes [31], their presence on erythrocytes has not been reported to date, and, accordingly, their biological role remains unknown. In the present study, we have examined whether this hypothesis can be extrapolated to erythrocytes from patients with VL, which have an increased expression of these linkage-specific 9-*O*-AcSGs. Indeed, the enhanced presence of this glycotope does cause enhanced hemolysis (figure 4*A* and 4*B*), as evidenced by an excellent correlation ($r^2 = 0.90$) between the presence of this glycotope, as recognized by binding of Achatinin-H, and the degree of hemolysis mediated by the alternative complement pathway. We, therefore, contend that these sialoglycans contribute to the anemia commonly associated with VL. Anemia contributes significantly to the morbidity associated with VL, and it remains to be seen whether, in the future, treatment for VL could include methods where the selective expression of glycotopes can be reduced.

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