Sialoglycosylation of RBC in Visceral Leishmaniasis Leads to Enhanced Oxidative Stress, Calpain-Induced Fragmentation of Spectrin and Hemolysis

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Abstract

Visceral leishmaniasis (VL) caused by the intracellular parasite Leishmania donovani accounts for an estimated 12 million cases of human infection. It is almost always associated with anemia, which severely complicates the disease course. However, the pathological processes leading to anemia in VL have thus far not been adequately characterized to date. In studying the glycosylation patterns of peripheral blood cells we found that the red blood cells (RBC) of VL patients (RBCVL) express eight 9-O-acetylated sialoglycoproteins (9-O-AcSGPs) that are not detected in the RBC of healthy individuals (RBCN). At the same time, the patients had high titers of anti-9-O-AcSGP IgG antibodies in their sera. These two conditions appear to be linked and related to the anemic state of the patients, as exposure of RBCVL but not RBCN to anti-9-O-AcSGPs antibodies purified from patient sera triggered a series of responses. These included calcium influx via the P/Q-type but not L-type channels, activation of calpain I, proteolysis of spectrin, enhanced oxidative stress, lipid peroxidation, externalization of phosphatidyl serine with enhanced erythrophagocytosis, enhanced membrane fragility and, finally, hemolysis. Taken together, this study suggests that the enhanced hemolysis is linked to an impairment of membrane integrity in RBCVL which is mediated by ligand-specific interaction of surface 9-O-AcSGPs. This affords a potential explanation for the structural and functional features of RBCVL which are involved in the hemolysis related to the anemia which develops in VL patients.

Introduction

Leishmania donovani, the causative organism of visceral leishmaniasis (VL), is an obligatory intracellular parasite that resides and proliferates within the hostile environment of host macrophages [1]. Approximately 12 million humans suffer from VL with an incidence of 0.5 million cases per year and increasing prevalence on the Indian subcontinent [1]. The clinical spectrum of VL ranges from asymptomatic infection to mortality, if untreated.

VL is usually associated with severe anemia which severely complicates the clinical courses and adds to the patients’ suffering [2]. In general, the average life span of the erythrocytes of patients with VL (RBCVL) is significantly reduced [3]. Accordingly the hemoglobin content in blood of these patients is lower than in normal healthy individuals. Despite its profound impact on the patients’ fate and chances for recovery, little is known about the pathological processes contributing to the hemolysis and anemia.

Sialic acids (SA) are 9-carbon acidic sugars that constitute a family of monosaccharides and terminal components of glycoproteins and glycolipids that play very crucial roles in intercellular communication and defense against different pathogens under various pathological conditions [4–7]. Earlier we have demonstrated exclusive presence of eight VL-associated 9-O-acetylated sialoglycoproteins (9-O-AcSGPs) on RBCVL not detected on erythrocytes of healthy individuals [8–11]. Also the white blood cells of VL patients display different derivatives of sialic acids [12–21]. Additionally, high titers of anti-9-O-AcSGP IgG were found in the patients’ sera [18,22] whereas only a fraction of the polyclonal IgG2 purified from normal human serum shows specificity for 9-O-AcSA [23–24].

The structural integrity of mammalian erythrocyte is supported by a complex network of different cytoskeleton proteins which comprises of five to seven spectrin subunits linked to actin filaments [25]. Structural and biochemical changes of RBC that cause alterations in the cytoskeleton proteins may lead to degradation of the cell. Modifications like glycation and oxidation of spectrin have been documented in diabetes mellitus and associated to erythrocyte membrane changes [26–27]. We recently could demonstrate glycosylation and proteolytic cleavage of spectrin in RBC of VL patients [11].

With the present study we addressed the possible contribution of 9-O-AcSGPs associated with RBCVL and of anti-9-O-AcSGP
IgG<sub>VL</sub> antibodies in VL. We report that anti-9-O-AcSGP IgG purified from serum of VL patients trigger a series of pathological events in RBC<sub>VL</sub> including (1) altered membrane properties as indicated by increased osmotic fragility, hydrophobicity, morphological changes like vesiculation, cell shrinkage, (2) influx of extracellular calcium ion (Ca<sup>2+</sup>) into the cell through P/Q-type channel and elevation of intracellular Ca<sup>2+</sup> level, (3) activation of calpain I caused by elevated cytosolic Ca<sup>2+</sup> accompanied by enhanced fragmentation of human erythrocytic s-tropinin to a 60 kDa 9-O-AcSGP (SGP-60) fragment, (4) enhanced production of reactive oxygen species (ROS) and lipid peroxidation, and (5) externalization of phosphatidyl serine (PS) and erythropagocytosis of sensitized RBC<sub>VL</sub>. This study thus suggests a mechanism of hemolysis in VL patients that may relate to their anemic state.

Materials and Methods

Clinical samples

The study involved clinically confirmed VL patients (Table 1, n = 40; 20 males, 20 females; median age: 30 years) admitted to the School of Tropical Medicine, Kolkata. The diagnosis of VL was based on WHO recommended microscopic demonstration of Leishmania sp. amastigotes in splenic aspirates [28]. Blood was sent to the Indian Institute of Chemical Biology where it was processed immediately and the diagnosis validated by two in-house techniques in which the increased presence of linkage-specific 9-O-AcSGPs on erythrocytes was quantified by an erythrocyte binding assay [9] and anti-9-O-AcSGP antibodies in serum or plasma were detected by ELISA [18,29]. The serum was also checked for the level of parasite-specific antibodies by ELISA with parasite lysates as coating antigen [22]. The hematological parameters of the patients were indicative of anemia but no other blood cell disorder. Controls included normal healthy individuals from endemic (n = 20) and non-endemic areas (n = 20) of the median age 28 for age matched study. The Institutional Human Ethical Committee had approved the study and samples were taken with the consent of donors, patients.

Anti-9-O-AcSGP IgG and sensitization of erythrocytes

Antibodies (IgG) specific for 9-O-AcSGPs were affinity purified from pooled sera from three patients (anti-9-O-AcSGP IgG<sub>VL</sub>) and normal healthy (NHS) individuals (anti-9-O-AcSGP IgG<sub>NHS</sub>), respectively, as described by Pal et al. [24]. Briefly, the succession of purification steps was 33% ammonium sulfate fractionation, removal of galactose-binding antibodies by passage through asialo-BSM Sepharose 4B, purification of anti-9-O-AcSGP by BSM (bovine submandibular mucin) Sepharose 4B affinity chromatography, and isolation of IgG by Protein G affinity chromatography. The yield of anti-9-O-AcSGP IgG purified from 12 ml VL sera was 588±25 µg (49 µg/ml serum) and from 20 ml normal control sera 240±19 µg (12 µg/ml serum). For sensitization with anti-9-O-AcSGP IgG, RBC (1×10<sup>7</sup>) were suspended in Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 32 mM HEPES, 5 mM glucose, 5 mM CaCl<sub>2</sub>, pH 7.4) and incubated with the antibodies at 37°C for 30 min. As controls, RBC were incubated with calcium ionophore (A23187, 1.0 µM, Sigma) in Ringer solution.

Osmofragility of erythrocytes

RBC (1×10<sup>7</sup>) were incubated in NaCl concentrations from 0.1 to 0.9 g % as indicated for 1 h at 37°C and extent of hemolysis measured spectrophotometrically at 412 nm. Likewise, erythrocytes in Ringer solution were incubated with anti-9-O-AcSGP IgG antibodies (6 µg/ml) or A23187 (1 µM) or buffer only for 30 min at 37°C. After centrifugation at for 3 min 4000 rpm, the extent of hemolysis in supernatant was determined spectrophotometrically at 412 nm. Hemolysis in distilled water was taken as 100% lysis. Percent hemolysis (%) was calculated as OD<sub>412</sub> mm at the given condition/OD<sub>412</sub> mm with 100% lysis×100 [20].

Hydrophobicity measurements

Hydrophobicity was detected before and after sensitization of RBC (1×10<sup>7</sup>) with anti-9-O-AcSGP IgG<sub>VL</sub> antibodies (6 µg/ml) in phosphate buffered saline (PBS) at 37°C for 30 min. Sensitized erythrocytes were washed with PBS, suspended in PBS and loaded with ANS in PBS (5 µl, 1 mM) for 1 h at 37°C. The binding of ANS to hydrophobic sites on erythrocyte membrane was measured with a spectrophoelumeter (Perkin-Elmer, LS55, E<sub>λmax</sub> = 365 nm) as described elsewhere [20,30]. Fluorescence emission spectra were recorded from 400 to 800 nm with excitation and emission band pass of 5 nm.

Scanning electron microscopy (SEM)

The morphology of RBC before and after sensitization with anti-9-O-AcSGP IgG<sub>VL</sub> antibodies (6 µg/ml) at 37°C for 30 min was done by SEM. Cells were fixed overnight with 2.5% glutaraldehyde in PBS followed by an overnight incubation with osmium tetroxide (1%), dehydration in an ethanol series, carbon dioxide by the critical point method, sputter coating with gold and examined with a SEM (Vegiiu Lsu, Tescan, Czech Republic) [31]. Micrographs were taken at magnification of 18,000 and about 200 erythrocytes were counted to calculate the percentage of deformed cells.

Measurement of reactive oxygen species

RBC were incubated with 2′,7′-dichlorofluorescein diacetate (H<sub>2</sub>DCF-DA,100 µM) in PBS for 30 min at 37°C and sensitized with anti-9-O-AcSGP IgG. ROS generation was detected and quantified by measuring the fluorescence intensity at λ<sub>ex</sub> = 485 nm and λ<sub>em</sub> = 530 nm. As controls, ROS generation was determined after treatment of the RBC with N-acetyl cysteine (10 mM), a scavenger of ROS. The results are expressed as fold increase in comparison to untreated erythrocytes [32].

Lipid peroxidation

Lipid peroxidation of erythrocyte membrane was measured by the thiobarbituric acid (TBA) method in which malondialdehyde (MDA); a product of peroxidation reaction of polyunsaturated fatty acids and thiobarbituric acid-reactive species (TBA-RS) is used as indicator [33]. In brief, RBC membrane proteins were precipitated in twice volume of trichloro acetic acid (10%) for 30 min at 37°C and centrifuged at 1000× g for 10 min at 4°C. The cleared supernatant was incubated with thiobarbituric acid (0.67% in 7.1% sodium sulphate) at 100°C for 25 min in a water bath. After centrifugation at 1000× g for 10 min the absorbance of the pink color reaction product of MDA with TBA was measured at 535 nm. Calibration standards were generated with 1,1,3,3-tetramethoxypropane. Results are expressed as fold increase in comparison with unsensitized RBC.

Phosphatidyl serine (PS) externalization

RBC (1×10<sup>6</sup>) in annexin V-binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) were incubated with FITC-labeled annexin V in the dark for 15 min at room temperature and analyzed by flow cytometry. A23187 (1.0 µM) treated and unsensitized RBC served as positive and negative controls, respectively.
Table 1. Clinical and laboratory features of patients with visceral leishmaniasis (VL).

| Parameters                  | PatientVL | Normal |
|-----------------------------|-----------|--------|
| Number                      | Male (n = 20), Female (n = 20) | endemic area (n = 20), non-endemic area (n = 20) |
| Age (yr)                    | 30±2.25   | 28±4.32 |
| Height (m)                  | 1.55±0.72 | 1.65±0.52 |
| Weight (kg)                 | 38.50±4.25| 60±5.25 |
| Body Mass Index (BMI)a      | 16±0.55   | 22±1.25 |
| Duration of illness (month) | 4.02±0.25 | Not applicable |
| RBC count                   | 0.92±2.5×10^12/μl | 4.5–6.2×10^12/μl |
| Leukocyte count (/mm³)       | 3.48–3.55×10^9 | 5–11×10^9 |
| Hemoglobin concn. (g/dl)    | 5.32±0.32 | 11.5±2.2 |
| Mean cell hemoglobin concn. (g/dl) | 30–31 | 30–36 g/dl |
| Hematocrit (%)              | 35        | 41–53  |
| Reticulocyte count (%)      | 4.25–5.05 | 0.5–2.5 |
| Spleen size (cm)            | 10.72±1.06| Not palpable |
| Splenic aspirate scoreb     | 4.05±0.17 | Negative |
| Bilirubin (mg/dl)           | 1.5–2.5   | 0.9–1   |
| Albumin (g/dl)              | 3.0–3.5   | 3.5–5.5 |
| Aminotransferase            | Normal    | Normal  |
| Alkalinephosphatase         | Normal    | Normal  |
| RBC-ELISAc                  | 0.77–1.1  | 0.16–0.23 |
| BSM-ELISAd                  | 1.1–1.7   | 0.22–0.32 |
| Parasite-ELISAe             | 1.06 Not palpable |

aBMI is weight in kilograms divided by height square in meter, normal BMI = 18.5–24.9.
bSplenetic aspirate score is taken to be 4 when the number of parasites per microscopic field ranges from 1–10.
cRBC-ELISA refers to the antigen-ELISA as described elsewhere [9]. The presence of 9-O-AcSGP on RBCVL was determined by exploiting the binding specificity of a lectin, Achatinin-H, which has a restricted specificity towards 9-O-acetylated sialic acids [62] and therefore used as coating antigen. Briefly, Achatinin-H was immobilized in a 96-well plate and allowed to bind with RBC in 4°C for overnight. After washing, bound RBC was lysed by double distilled water. The extent of binding was determined by using a chromogenic substrate 2,7-diamino fluorine dihydrochloride and measuring absorbance values at 620 nm.
dAnti-9-O-AcSGP antibody was detected by using BSM as coating antigen as described elsewhere [22].
eParasite specific antibody was detected by using parasite lysate as coating antigen as described elsewhere [8].
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Erythrophagocytosis assay

RBC (2×10^6) with or without sensitization with 6.0 μg/ml anti-O-AcSGP IgG for 30 min at 37°C, were layered over macrophages adhered on cover slips and incubated at 37°C for 1 h. Non-adherent erythrocytes were removed by gentle washing with PBS and cell surface-bound erythrocytes lysed by treatment with Tris-NH₄Cl (140 mM NH₄Cl, 17 mM Tris-HCl, pH 7.6) for 5 min. The slides were stained with dianinobenzidine for erythrocytes and counterstained with Giemsa stain for macrophages. Phagocytosis was calculated as the percentage of macrophages that had ingested one or more erythrocytes [34].

Measurement of cytoplasmic Ca²⁺

Erythrocytes (2×10⁷) were washed in Ringer solution, loaded with Fluo-3/AM (2 μM, Calbiochem, Germany) in Ringer solution for 10 min at 37°C in dark under gentle shaking and washed twice with the same buffer [35]. Fluo 3-loaded erythrocytes were sensitization with buffer only or varying concentrations of anti-9-O-AcSGP IgG (0–40 μg/ml) in Ringer solution for 30 min at 37°C washed twice and resuspended in same buffer and analyzed by spectrophuorometry (λex 506 nm, λem 530 nm) and flow cytometry. Time dependent increase of intracellular calcium ion was also carried out by incubating RBC with anti-9-O-AcSGP IgG (2.5 μg/ml) for different time point at 37°C followed by flow cytometry. Calibration was done at the end of each experiment.

To determine the Ca²⁺ channel type, Fluo 3-loaded RBCVL (2×10⁶) in Ringer solution containing Ca²⁺ (5 mM) were preincubated without or with the indicated doses of ø-agatoxin TK, a spider venom peptide (P/Q-type Ca²⁺ channel blocker, Calbiochem) or nifedipine (L-type Ca²⁺ channel blocker, Sigma) for 20 min at 37°C, washed, sensitized with anti-9-O-AcSGP IgG (2.5 μg/ml) as above and analyzed by flow cytometry [35]. The Ca²⁺ concentrations were calculated as [Ca²⁺] = K₄ [(F–Fₘₐₓ)/(Fₘₐₓ–F)] where K₄ is the dissociation constant of the Ca²⁺ Fluo-3 complex (864 nM at 37°C), F fluorescence of anti-9-O-AcSGP IgG-treated RBC, Fₘₐₓ the maximal fluorescence intensity with A23187 treated, Fₘᵢₙₐᵢₙ corresponds to minimum fluorescence intensity with A23187 and EGTA [36].

Electrophoresis and Western Blotting

RBC (2×10⁶) were incubated or not with anti-9-O-AcSGP IgGVL (10 μg/ml) for 60 min at 37°C in Ringer solution, washed and lysed by sonication on ice. Cell lysates were centrifuged to remove debris, the proteins separated in SDS-PAGE (10% or 7.5%) and blotted onto nitrocellulose. The Western blots were developed separately with horse reddish-labeled anti-calpain I antibodies (1:1000, Cell Signaling, USA) and rabbit anti-spectrin antibodies (1:1000, Sigma, USA). Positive and negative controls were RBC treated with A23187 (1 μM) in Ringer solution for 30 min at 37°C in the absence or presence of EGTA (25 mM),
respectively. For confirmation of specificity, RBC were preincubated (15 min, 37°C) or coincubated during sensitization with the calpain inhibitor I N-acetyl-leucyl-leucyl-norleucinal (ALLN; 200 μM, Sigma) in Ringer solution before sensitization or A23187 treatment. The blots were developed with dianibenzidine and peroxide, scanned densitometrically and analyzed with the Quantity One software (Bio-Rad, USA).

Calpain I assays
Spectrin was purified separately from RBC as described by Ungewickell et al. [37] and confirmed by SDS-PAGE and Western blot analysis as above. Spectrin (3.0 μg) was digested with the indicated doses of active calpain I (Sigma) for 60 min at 24°C in reaction buffer (50 mM HEPES, pH 7.0, 50 mM NaCl, 1 mM NaN₃, 1 mM CaCl₂, 1 mM DTT), the reactions stopped with EGTA (15 mM final concentration) [38] and the reaction product analyzed by SDS-PAGE. For specificity control calpain I (10 μg/ml) was pre-incubated with ALLN (150 μM) for 15 min on ice prior to addition of reaction buffer containing purified spectrin. Gels were stained by Coomassie brilliant blue and band densities were compared by densitometric analysis.

Statistical analysis
Results are reported as mean ± SD. All statistical analyses were done using Excel software (Microsoft Co.). The one or two-tailed t test for significance was performed, P<0.05 was considered significant.

Results
Alterations in the membrane characteristics of RBC in VL
To test the stability of RBCVL in comparison to RBCN, the cells were incubated in NaCl solutions with at concentrations ranging from 0 to isoosmotic 0.9 g%, and the degree of hemolysis was determined spectrophotometrically. The osmofragility thus determined is an indicator of cell stability and alterations in membrane properties. RBCVL were osmotically more fragile than RBCN, as indicated by an approximately 3-fold enhancement of lysis at 0.5 g% NaCl (Fig. 1A).

Both RBCVL and RBCN were sensitized for 30 min, at 37°C with increasing concentrations of anti-9-O-AcSGP IgGVL and anti-9-O-AcSGP IgGNS, respectively. Sensitization of RBCVL with 6.0 or 10.0 μg/ml of anti-9-O-AcSGP IgGVL resulted in a high percentage of lysis, i.e. 50.2±2.0% and 61±3.0%, respectively (Fig. 1B). An increase in the lysis of sensitized RBCVL with 6.0 μg/ml of anti-9-O-AcSGP IgGVL was observed over time compared to sensitized RBCVL (Fig. 1C). Sensitization of RBCVL with 6.0 μg/ml of anti-9-O-AcSGP IgGVL was observed over time compared to sensitized RBCVL (Fig. 1C). Sensitization of RBCVL with 6.0 μg/ml of anti-9-O-AcSGP IgGVL, for 30 min at 37°C resulted in a 4.5-fold higher lysis than un-sensitized RBCVL i.e. 63±4.0% vs. 14±2.0% (Fig. 1D). Sensitized RBCVL displayed a 5.7-fold higher lysis compared to sensitized RBCN, A23187 in the presence of Ca²⁺ induced the maximum possible hemolysis of the erythrocytes.

RBCVL exhibited higher membrane hydrophobicity than RBCN, as indicated by increased fluorescence due to enhanced ANS binding. Sensitized RBCVL demonstrated a further increase in ANS binding and enhanced hydrophobicity compared to un-sensitized erythrocytes (Fig. 1E). A blue shift of the emission maxima from 544 to 500 nm in sensitized RBCVL suggested that sensitization increased the membrane hydrophobicity resulting in a greater number of accessible sites for the binding of ANS. Unsensitized RBCVL exhibited a higher hydrophobicity than sensitized RBCN (emission maxima at 560 nm). Unsensitized RBCN displayed only a negligible reading.

SEM revealed sensitized RBCVL to have a greater number of ultra structural morphological changes compared to un-sensitized RBCVL, suggesting a stressed condition in these erythrocytes (Fig. 1F). The presence of shrunken RBCVL, as reflected by morphometric analyses indicating membrane alterations due to a sensitization of the 9-O-AcSGPs. The sensitized RBCN did not exhibit any noteworthy alterations of the membrane, retaining a normal discoid shape.

Altered cell morphology in RBCVL
For further demonstration of changes in cell size, we used flow cytometry to monitor the forward light scattering of RBCVL before and after sensitization. The forward scattering data (FSC) showed there were only 27% un-sensitized RBCVL in M1 as compared to 73% cells in M2 (Fig. 1G). In contrast, sensitized RBCVL exhibited a higher percentage (59%) of cells in M1. RBCN exhibited only 17% cells in M1. A considerably enhanced (80%) percentage of cells were observed in the A23187-treated RBCVL.

Sensitized RBCVL exhibit enhanced ROS generation, lipid peroxidation and externalization of PS
The generation of ROS is usually linked with membrane damage, specifically lipid-peroxidation, which may lead to membrane alterations such as PS externalization which are associated with cell death. As mature RBC is devoid of intracellular components such as nuclei and mitochondria, alterations may also result in these cells in a stressed condition. To address the question whether anti-9-O-AcSGP IgGVL is capable of inducing stress, we measured ROS generation, lipid peroxidation and PS-externalization in sensitized RBCVL. 5.7 and 3-fold higher levels of ROS and TBA-RS were observed in sensitized RBCVL compared to un-sensitized erythrocytes (Fig. 2A–B). RBCN displayed negligible ROS generation and insignificant lipid peroxidation. Sensitized RBCVL also showed enhanced annexin-V binding (32±5%) compared to un-sensitized (0.2±0.01%) erythrocytes, indicating a rapid externalization of PS, whereas sensitized and unsensitized RBCC demonstrated only a negligible percentage of annexin-V positive cells (Fig. 2C–D). A23187 treated RBCVL exhibited an increase in annexin-V positivity and served as a positive control.

Sensitized RBCVL exhibit increased erythrophagocytosis
The exposure of PS on the outer leaflet of the plasma membrane is one of the signals that induce macrophages to bind and ingest apoptotic cells [39]. Sensitization of 9-O-AcSGPs on RBCVL by anti-9-O-AcSGP IgGVL antibodies resulted in an increase in phagocytosis of RBCVL compared to unsensitized, as evidenced by the increase in the percentage of positive macrophages that ingested one or more erythrocytes, from 3.0±1.0 to 52.0±5.0 (Table 2). The extent of phagocytosis may be dependent on the externalization of PS, as suggested by the good correlation (r = 0.92) with annexin-V positivity. In contrast, RBCN demonstrated negligible uptake by macrophages under identical conditions.

Intracellular accumulation of Ca²⁺ in sensitized RBCVL
Spectrofluorimetric data suggested that the cytosolic Ca²⁺ ion content of un-sensitized RBCVL is slightly higher than RBCN, suggesting a stressed condition in VL (Fig. 3A). However, the sensitization of 9-O-AcSGPs on Fluo-3-loaded RBCVL showed a significant increase in fluorescence with increasing concentrations of anti-9-O-AcSGP IgGVL antibodies, indicating an enhanced Ca²⁺ influx as compared to the un-sensitized erythrocytes (Fig. 3B).
Spectrin Proteolysis and Hemolysis of RBC

A. Graph showing hemolysis (% vs. saline concentration (g%) for RBC and RBC_VL.

B. Graph showing hemolysis (%) vs. antibody concentration (μg/ml) for sensitized RBC_VL and RBC_N.

C. Graph showing hemolysis (%) vs. time (min) for sensitized RBC_VL and RBC_N.

D. Bar graph showing hemolysis (%) for untreated, antibody, and A23187 treated RBC_VL and RBC_N.

E. Graph showing fluorescence intensity (arbitrary units) vs. wavelength (nm) for sensitized and unsensitized RBC_VL.

F. Images showing unsensitized and sensitized RBC_N and RBC_VL.

G. Flow cytometry histograms showing FSC-H vs. counts for RBC_N in Ringer solution only, RBC_VL in Ringer solution only, RBC_N + Anti-β+D-Ac:SGP IgG, and RBC_VL + A23187.
Figure 1. Altered membrane properties and reduced stability of RBCVL after sensitization with anti-9-O-AcSGP IgGVL antibodies. A. The osmotic fragility of RBCVL (open square) compared to RBCN (filled square) was measured by degree of hemolysis with increasing osmotic stress by incubation in NaCl (0–0.9%) determined by spectrophotometry. Data are represented as % hemolysis, are means ± SD and representative of three independent experiments. B. Sensitization of RBCVL for hemolysis with anti-9-O-AcSGP IgGVL antibodies. RBCVL (open bars) and RBCN (filled bars) were incubated for 30 min at 37°C with increasing concentration (0.5 μg/ml-12.0 μg/ml) of anti-9-O-AcSGP IgGVL and anti-9-O-AcSGP IgGNHS, respectively. The extent of hemolysis was then determined by spectrophotometry. The Spectrophotometric readings of RBCVL or RBCN in buffer under similar condition were taken as base values and subtracted from the experimental values. Data are expressed as means ± SD of three independent experiments. C. Kinetics of the hemolysis of RBCVL and RBCN after sensitization with anti-9-O-AcSGP IgGVL antibodies. RBCVL (open squares) and RBCN (filled squares) were incubated with equal (6.0 μg/ml) amounts of anti-9-O-AcSGP IgGVL and anti-9-O-AcSGP IgGNHS, respectively, for different time points (0–120 min). Base value was routinely subtracted from each experimental value as in Fig. 1B. Data are means ± SD of three independent experiments. D. Hemolysis of RBCVL (filled bars) and RBCN (open bars) after sensitization of RBCVL with anti-9-O-AcSGP IgGVL or anti-9-O-AcSGP IgGNHS. The results are expressed as means ± SD of three independent experiments. doi:10.1371/journal.pone.0042361.g001

Figure 2. Enhanced ROS, lipid peroxidation and externalization of phosphatidylserine (PS) in RBCVL after sensitization with anti-9-O-AcSGP IgGVL antibodies. A. Induction of ROS in sensitized RBCVL. Prior to sensitization with anti-9-O-AcSGP IgGVL or anti-9-O-AcSGP IgGNHS, RBCVL and RBCN were incubated with the hydroperoxide indicator H2DCF-DA in PBS for 30 min at 37°C. Then the antibodies were added, the cell incubated as before and ROS generation determined by fluorimetry. As controls, ROS generation was determined after prior treatment of the RBC with N-acetyl cysteine, a quencher of hydroperoxides. The results are expressed as means ± S.D. (n = 5) of fold increases in comparison to the fluorescence levels detected with untreated erythrocytes. B. Increased lipid peroxidation in sensitized RBCVL. Lipid peroxidation of erythrocyte membrane was detected and quantified with thiobarbituric acid (TBA) and the TBA-reactive species (TBA-RS) measured with a spectrophotometer at 532 nm. The results are mean ± S.D of fold increase in comparison with unsensitized RBC of five independent measurements. C-D. Enhanced externalization of phosphatidylserine on sensitized RBCVL. Sensitized or unsensitized erythrocytes were incubated in annexin-V binding buffer with FITC-annexin-V and analyzed by flow cytometry. A23187-treated RBC in the presence of Ca²⁺ served as positive control. The results are shown as representative histogram of three independent experiments (C) and as comparative flow-cytometric analysis (D). doi:10.1371/journal.pone.0042361.g002
Table 2. Erythrophagocytosis Assay.

| Group          | RBC<sub>N</sub> | RBC<sub>VL</sub> |
|----------------|-----------------|-----------------|
| RBC           | 2 ± 1           | 3 ± 1           |
| Sensitized RBC| 3 ± 2           | 52 ± 5          |

*RBC<sub>N</sub> and RBC<sub>VL</sub> (2×10<sup>6</sup>) without or with sensitization by anti-O-AcSGP IgG<sub>N</sub> and anti-O-AcSGP IgG<sub>VL</sub> antibodies and processed for erythrophagocytosis assay as described in Materials and Methods. Results represent the mean ± S.D. of five separate determinations.
*Positive macrophages (%) are the percentage of macrophages that ingested one or more erythrocytes and were used as the index of phagocytosis. Results represent the mean ± S.D. of five separate determinations.

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Sensitized Fluo-3-loaded RBC<sub>VL</sub> also displayed enhanced fluorescence with an increasing concentration of anti-9-O-AcSGP IgG<sub>N</sub> antibodies as compared to un-sensitized erythrocytes, as determined by flow cytometry (Fig. 3C). It is noteworthy that not all of the cells responded equally to stimulation at the lower doses of the antibody (1.0 μg/ml) compared to 2.5 μg/ml, at which almost all of the cells showed higher fluorescence. In contrast, Fluo-3-loaded sensitized RBC<sub>N</sub> displayed only a minimal increase in fluorescence, even at higher doses, as compared to un-sensitized RBC<sub>N</sub>, suggesting the absence of 9-O-AcSGPs (Fig. 3B, 3D). However, a lack of signaling for other, undetermined reasons cannot be ruled out.

A time dependent increase in intracellular Ca<sup>2+</sup> was observed in sensitized RBC<sub>VL</sub> (Fig. 3E–F). Almost all of the cells exhibited higher Fluo-3 fluorescence after 30 min of stimulation. As expected, Fluo-3-loaded RBC<sub>VL</sub> and RBC<sub>N</sub> incubated with A23187 exhibited maximum fluorescence and were used as positive control, which effect was decreased in the presence of EGTA, confirming the assay specificity.

P/Q-type channel mediated influx of Ca<sup>2+</sup> ions

In order to understand the Ca<sup>2+</sup> influx pathway of sensitized RBC<sub>VL</sub>, we used different concentrations (0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM, 1.0 μM and 2.0 μM) of α-agonist toxin, a P/Q-type Ca<sup>2+</sup>-ion channel blocker (Fig. 3G). At a concentration of 1 μM, α-agonist toxin strongly inhibited the influx of Ca<sup>2+</sup> ions by reducing the MFI from 210 arbitrary units to the background value of 43 arbitrary units, suggesting the involvement of P/Q-type calcium channels in the Ca<sup>2+</sup>-ion influx in sensitized RBC<sub>VL</sub>. In contrast, nifedipine, an L-type channel blocker, even at a higher concentration of 10 μM, did not inhibit the influx of Ca<sup>2+</sup> ions (Fig. 3H). No inhibition could be detected at up to a 50 μM concentration of nifedipine.

Enhanced cytoplasmic Ca<sup>2+</sup> activated calpain I in sensitized RBC<sub>VL</sub>

Increased intracellular Ca<sup>2+</sup> activates the Ca<sup>2+</sup>-dependent protease calpain I [40]. Therefore, we investigated the involvement of enhanced Ca<sup>2+</sup> in activating calpain I in sensitized/unsensitized RBC<sub>VL</sub> (Fig. 4A). Sensitized RBC<sub>VL</sub> displayed an approximate 2-fold increase of the 75 kDa active form of calpain I as a result of Ca<sup>2+</sup>-dependent autoproteolysis of the inactive membrane localized 80 kDa native form, indicating that an increased cytosolic Ca<sup>2+</sup> level was sufficient for protease activation. It also suggested that the activation of calpain I was dependent on Ca<sup>2+</sup> through sensitized 9-O-AcSGPs. A23187-treated RBC<sub>VL</sub> or RBC<sub>N</sub> produced an intense 75 kDa band. Unsensitized RBC<sub>VL</sub> inherently contain a less intense 75 kDa active form of calpain I which was completely absent in RBC<sub>N</sub>. Sensitization of RBC<sub>N</sub> with A23187 in the presence of EGTA resulted in only the native 80 kDa form.

Activated calpain I induced proteolysis of spectrin<sub>VL</sub> in sensitized RBC<sub>VL</sub>

Activated calpain I cleaved purified spectrin

Purified spectrin<sub>VL</sub> showed bands of 280, 246 and 60 kDa (Fig. 5A). SGP-60 was not present in spectrin<sub>N</sub>. Purified spectrin<sub>VL</sub> digested with active calpain I displayed a similar enhancement of fragmented spectrin<sub>VL</sub>, as evidenced by the increased presence of the SGP-60 band and reduced intensity of α-spectrin compared to undigested spectrin<sub>VL</sub>, suggesting the presence of active calpain in the patient’s erythrocytes may be responsible for such proteolysis (Fig. 5B). Similar treatment in the presence of ALLN exhibited a reduced intensity of the SGP-60 band, thus showing the specificity of the reaction (Fig. 5C). Spectrin<sub>N</sub> digested with active calpain I also displayed an increase in the intensity of SGP-60 and corresponding decrease in α-spectrin in a calpain I dose-dependent manner (Fig. 5B). The SGP-60 band was absent in both undigested spectrin<sub>N</sub> and in the case of treatment with active calpain I in the presence of ALLN (Fig. 5C).

Activated calpain I cleaved purified spectrin

Purified spectrin<sub>VL</sub> showed bands of 280, 246 and 60 kDa (Fig. 5A). SGP-60 was not present in spectrin<sub>N</sub>. Purified spectrin<sub>VL</sub> digested with active calpain I displayed a similar enhancement of fragmented spectrin<sub>VL</sub>, as evidenced by the increased presence of the SGP-60 band and reduced intensity of α-spectrin compared to undigested spectrin<sub>VL</sub>, suggesting the presence of active calpain in the patient’s erythrocytes may be responsible for such proteolysis (Fig. 5B). Similar treatment in the presence of ALLN exhibited a reduced intensity of the SGP-60 band, thus showing the specificity of the reaction (Fig. 5C). Spectrin<sub>N</sub> digested with active calpain I also displayed an increase in the intensity of SGP-60 and corresponding decrease in α-spectrin in a calpain I dose-dependent manner (Fig. 5B). The SGP-60 band was absent in both undigested spectrin<sub>N</sub> and in the case of treatment with active calpain I in the presence of ALLN (Fig. 5C).

Discussion

Studies reported by our group initially demonstrated the presence of 9-O-AcSGPs and anti-9-O-AcSGP antibodies in VL [22,29]. Earlier we have reported the presence of only two 9-O-AcSGPs of molecular weight 36 kDa and 144 kDa on PBMC<sub>N</sub> respectively [41–43]. In contrast, several other distinct VL-associated newly induced 9-O-AcSGPs (19, 56, 65 kDa) were demonstrated on PBMC<sub>VL</sub> [21,41,44]. Interestingly, almost 40% of the membrane proteins present on the RBC<sub>VL</sub> were 9-O-acetylated, that were totally absent on RBC<sub>N</sub> which further
Figure 3. Calcium influx into RBC upon sensitization with anti-9-O-AcSGP IgGVL antibodies. For all experiments, erythrocytes were washed and loaded with the Ca\(^{2+}\)-indicator fluorochrome Fluo-3/AM in Ringer solution. A23187-treated Fluo-3/AM-loaded RBC\(_{VL}\) served as positive controls (black line) and A23187-treated cells in the presence of EGTA as negative controls (gray background).

A. Fluo-3/AM loaded RBC\(_{VL}\) and RBC\(_{NL}\) \((2 \times 10^7)\) were left unsensitized or sensitized with varying concentrations of anti-9-O-AcSGP IgG\(_{VL}\) or anti-9-O-AcSGP IgG\(_{NHS}\), respectively, \((0–40 \mu\text{g/ml})\) in Ringer solution for 30 min at 37°C. After washing the cells twice with same solution the fluorescence intensities of the Ca\(^{2+}\)-Fluo-3 complexes was determined by spectrofluorimetry and the levels of intracellular calcium calculated as described in Materials and Methods.

B. Cells treated as in A with 0.5 \(\mu\text{g/ml}\) (red line), 1.0 \(\mu\text{g/ml}\) (green line) and 2.5 \(\mu\text{g/ml}\) (pink line) anti-9-O-AcSGP IgG\(_{VL}\) were analyzed by flow cytometry to determine the fraction of responding RBC. C. Similarly, RBC\(_{NL}\) were analyzed without and with sensitization with 5 \(\mu\text{g/ml}\) anti-9-O-AcSGP IgG\(_{NHS}\) (blue line).

D–E. Time dependent increase of intracellular Ca\(^{2+}\) in RBC\(_{VL}\) after sensitization with anti-9-O-AcSGP IgG\(_{VL}\) \((2.5 \mu\text{g/ml})\) for 5 min (red line), 10 min (green line), 20 min (pink line) and 30 min (blue line) at 37°C. Cells were washed and analyzed by flow cytometry.

F–G. Inhibition of the Ca\(^{2+}\) influx into RBC sensitized with anti-9-O-AcSGP IgG\(_{VL}\). Prior to sensitization with anti-9-O-AcSGP-IgG\(_{VL}\) \((2.5 \mu\text{g/ml})\), the cells were incubated without (green line) or with the P/Q-type channel blocker ω-agatoxin TK (1 \(\mu\text{M}\), pink line, F), or without (green line) or with the L-type channel blocker nifedipine (10 \(\mu\text{M}\), blue line, G) and analyzed by flow cytometry.

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signified their link with disease pathogenesis [8–11]. However, little progress has been made determining the extent of the contribution of 9-O-AcSGPs to the enhanced hemolysis of RBC in VL in the active disease state. Accordingly, our aim was to investigate the specific role of 9-O-AcSGPs in RBCVL hemolysis and their ligand-specific interaction with anti-9-O-AcSGP IgGVL.

The major achievement of the study was to demonstrate the involvement of VL-associated 9-O-AcSGPs in triggering the altered cellular and membrane biochemical characteristics leading to the phagocytosis of these altered erythrocytes.

Sensitization of 9-O-AcSGP using anti-9-O-AcSGP IgGVL antibodies led to an alteration of the membrane characteristics, as evidenced by enhanced osmotic fragility and hydrophobicity, suggesting a mechanism for the membrane damage which developed in RBCVL in contrast with RBCN. Moreover, profound ultrastructural changes in morphology from the normal discoid shape and oxidative stress induced in the sensitized RBCVL indicate definite alterations in their membranes, suggesting a key role for 9-O-AcSGPs. Similar alterations in erythrocyte membrane organization have been documented in Fanconi’s anemia [45] and acute childhood lymphoblastic leukemia [33].

The physiological concentration of anti-9-O-AcSGPs antibodies in normal serum against two 9-O-AcSGPs (36 kDa and 144 kDa) present on PBMCN is only 11–13 μg/ml [13,18,22,46]. However,
the total anti-9-O-AcSGPs (54 μg/ml) in VL-serum are developed against several 9-O-AcSGPs (112,107,103,57,51, and 48 kDa) newly envisaged both on PBMCVL and RBCVL. Therefore, it may be envisaged that the enhanced anti-9-O-AcSGP antibody found in the VL serum is definitely different from the antibody present in normal human serum. Hence, affinity purified anti-9-O-AcSGPs antibody used to sensitize 9-O-AcSGPs on RBCVL certainly has a distinct identity, specific and active. Accordingly, even a lower concentration (6 μg/ml) of anti-9-O-AcSGP IgGVL antibody used for sensitization is capable of inducing 5.7-fold higher degree of RBCVL hemolysis compared to sensitized RBCN. This observation signifies that even lower doses of the anti-9-O-AcSGP IgGVL antibody through the ligand-specific interaction could play an important role in hemolysis of RBCVL leading to anemia.

In contrast, no significant increase in the hemolysis (%) of RBCN was observed even at higher concentration of the anti-9-O-AcSGP IgGVL antibody because of the negligible presence of 9-O-AcSGPs on normal cells. This further indicated that this change in red cell morphology due to this specific ligand-mediated interaction was VL-associated.

ROS are linked to cell death signaling in a variety of cell types. Loss of membrane PS asymmetry has been reported in human erythrocytes, sickle cell disease, thalassemia and diabetes [39,47–48]. Increased oxidative stress in erythrocytes of Leishmania-infected hamsters has been reported [49]. A 6-fold higher ROS generation and a 4-fold increased lipid peroxidation in sensitized RBCVL suggested that the signaling through 9-O-AcSGPs was indeed VL-associated.

PS exposure on the outer leaflet of the membrane serves as a signal for the removal of apoptotic cells from the circulation [50]. Sensitized RBCVL demonstrated display an enhanced externalization of PS, an event which is reported to be correlated with membrane damage in other diseases [46,47–48,50–51]. A 17-fold higher erythropagocytosis of sensitized RBCVL was demonstrated which indicated their efficient removal from the circulation, suggesting a probable cause for the anemia-associated VL patients.

Sensitization of RBCVL with anti-9-O-AcSGP IgGVL resulted in an increase in the cytosolic Ca2+ level. However, the uptake of Ca2+ was not equal in all of the cells, suggesting that the 9-O-AcSGP content of the cells is also not equal, with possibly a few cells having a higher number of 9-O-AcSGPs stimulated earlier at a lower dose of antibody and earlier time point. The increased cytosolic Ca2+ causes the activation of calpain I, which in turn mediated an enhanced proteolysis of spectrinVL. Hence, the possible mechanism of a destabilization of RBC by damaging cytoskeleton proteins which in turn leads to hemolysis has been established in VL, with a cell-specific role for 9-O-AcSGPs.

Calcium must be taken up from the extracellular compartment into the inside of the cell, as erythrocytes are devoid of any Ca2+ storage organelles such as the endoplasmic reticulum and mitochondria. We performed a channel-inhibition experiment in order to characterize the specific type of channel utilized in the course of ion influx. Inhibition of Ca2+ influx by ω-agatoxin TK confirmed the involvement of the P/Q-type channel. In contrast, even at higher doses, an L-type channel blocker (nifedipine) was unable to block the influx of Ca2+ during the sensitization process, clearly showing that this influx was not through the L-type channel.

The sensitization of RBCVL caused activation of the Ca2+ channel and along with an enhanced influx of the Ca2+ ion, which may have further caused the activation of the Na+/K+ ion channel [52]. Activation of the Na+/K+ ion channel opens up the Gardos channel followed by an efflux of water from the cells, as reflected by the shift of a significant population of sensitized cells towards a lower FSC. The RBCVL cell size was typically lower than RBCN, possibly due to the higher level of intracellular Ca2+ in the VL condition.

Spectrin, a cytoskeleton membrane protein which is crucial for the maintenance of the structural integrity of the cell, is thought to be a target of calpain-mediated proteolysis [53]. Supporting this idea, we observed increased cytosolic Ca2+ activated calpain I in sensitized RBCVL, as evidenced by the appearance of the active form mediating the proteolysis of spectrinVL. Calpain is activated by autophosphorylation and calpain I relocates from the cytoplasm to the inner surface of the plasma membrane, where it may cause damage to the cytoskeleton structure [40]. It degrades cytoskeletal proteins in neuronal cells during cerebral malaria, traumatic/post-traumatic neurodegeneration [54–57], aneurysmal subarachnoid hemorrhage [58] and spinal cord ischemia [59]. Calcium and phenylhydrazine-induced proteolysis of spectrin in rat and human erythrocytes has been documented [60–61].

The enhanced presence of SGP-60 in sensitized RBCVL, which indicates the enhanced degradation of spectrin may be due to Ca2+-mediated proteolysis of cytoskeleton proteins destabilizing RBCVL. However, SGP-60 is already present in unsensitized RBCVL and its lack in RBCN suggested an association of degraded spectrin with the active disease state. The identification of SGP-60 as a fragment of erythrocytic 9-AI spectrin was confirmed by sequencing [11]. The fragmentation of purified spectrinVL/ spectrinN by active calpain I displayed a similar pattern of spectrin-proteolysis as in RBCVL, suggesting an enhanced activation of calpain in RBCVL in vivo. Erythrocytes may have lost their membrane integrity as a consequence of spectrin degradation, as reflected in the enhanced hemolysis of the A23187-sensitized RBC. A higher degree of RBCN hemolysis may occur through the activation of calpain I, as chelating the cellular Ca2+ by EGTA suppressed the hemolysis. This was corroborated when a specific inhibitor of calpain I which blocks spectrin proteolysis (ALLN) was used, resulting in a decrease in the degree of hemolysis.

Taken together, the evidence suggests that 9-O-acetylated sialoglycoproteins have an important role in Ca2+- influx, activating calpain-I, which in turn cleaves spectrin, causing destabilization of RBCVL and ultimately their removal through phagocytosis by macrophages. Hence the study findings have yielded important insight into the pathophysiological role of 9-O-AcSGPs on RBCVL, including potential cell-biological mechanisms which result in anemia (Fig. S1).

Supporting Information

Figure S1 Overview of the proposed mechanism. A hypothetical model has been shown in describing the role of 9-O-AcSGPs for the hemolysis of erythrocytes in VL. The model highlights the possible events inside the RBCVL, including activation of calpain I followed by spectrin degradation and phosphatidylserine exposure after sensitization of 9-O-AcSGPs by anti-9-O-AcSGP IgGVL.

(TIF)

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Author Contributions
Conceived and designed the experiments: SS AG KB CM. Performed the experiments: SS AG KB. Analyzed the data: SS AG KB PW CM.

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