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Identification of antibodies directed against O-acetylated sialic acids in visceral leishmaniasis: its diagnostic and prognostic role

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A significantly increased O-acetylated sialic acid (O-AcSA) binding fraction was purified from serum of visceral leishmaniasis (VL) patients by affinity chromatography on immobilized bovine submaxillary mucin (BSM) and found to be immunoglobulin in origin. The serodiagnostic and prognostic potential of BSM as a capture antigen was established by ELISA with no cross reactivity with coendemic diseases like malaria, tuberculosis, leprosy, chagas disease and cutaneous leishmaniasis; however, a strong cross reactivity was present with trypanosomiasis patients. In 56 clinically diagnosed VL patients, the BSM-ELISA was compared with diagnosis by microscopy using Giemsa stained tissue smears and direct ELISA using crude parasite antigen (parasite-ELISA); 49/56(87.5%) and 5/56(9.0%) were positive and negative respectively by all 3 methods. The BSM-ELISA failed to diagnose 2/56(3.5%) patients which were biopsy and parasite-ELISA positive. The prognostic potential of the BSM-ELISA in 18 longitudinally monitored VL patients before and after conventional antimonial treatment showed a significant decrease in anti O-AcSA titres in drug responsive patients whereas anti O-AcSA levels persisted in drug unresponsive patients. The IgG subclass distribution of antibodies directed against O-AcSA showed increased IgG2 levels in VL patients as compared to healthy controls. The BSM-based ELISA holds great promise as a serodiagnostic and prognostic assay for VL.

Introduction

Visceral leishmaniasis is classically caused by the digenetic protozoan parasite of the *Leishmania donovani* complex. The complex comprises of *Leishmania chagasi* in Latin America, *Leishmania donovani* in Africa and Asia and *Leishmania infantum* in the Mediterranean region. The presenting clinical features are usually a chronic febrile illness with emaciation, marked hepatosplenomegaly, pancytopenia and hypergammaglobulinemia. These features often resemble other infectious diseases including malaria, tuberculosis, chagas disease and trypanosomiasis which have overlapping geographical distributions. These factors added to serological cross reactivity often poses a diagnostic dilemna.

Definitive diagnosis of VL still relies on visualization of the intracellular, aflagellated amastigote stage of the parasite in host tissue or of the extracellular, flagellated promastigote stage of the parasite in culture form by aspiration from the spleen, bone marrow or lymph node. Owing to the invasiveness and relative insensitivity of the procedure [1] it is gradually being replaced by serological and DNA based diagnostic methods. However, due to the predominantly rural nature of the disease and the profound humoral response induced, serodiagnosis is being accepted as the most convenient method for diagnosing VL in India [2,3] and attention is now focused on minimizing cross reactions yet retaining the sensitivity.

Employing a 9-O-acetylated sialic acid binding lectin Achatinin-H derived from the hemolymph of the African giant land sail *Achatina fulica* [4–9] we have earlier reported the selective presence of 9-O-acetylated sialic acid (9-O-AcSA) derivatives on erythrocytes of VL patients [10] and peripheral blood mononuclear cells of acute lymphoblastic leukaemia patients [11,12]. These 9-O-AcSA are absent on normal erythrocytes and present in negligible amounts on lymphocytes [13]. This modification of sialic acids at the C_9 position by an O-acetyl ester is expressed on the outer most part of the carbohydrate moiety of membrane and secreted glycoconjugates and possibly accounts

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for its involvement in cell-cell recognition phenomena [14,15]. Ligands of two important hematopoietic adhesion molecules CD22 (Siglec 2), a mature B cell surface glycoprotein and sialoadhesin, a macrophage adhesion molecule can be masked by 9-O-acetylation of sialic acids [16,17]. Accordingly, inhibition of cell-cell adhesion may well be an effective approach adopted by the *Leishmania* parasite to circumvent immune surveillance and the presence of antibodies against O-acetylated sialic acids could be an attempt at active immunisation.

In this study we report a selective increase in antibodies against O-acetylated sialic acids (O-AcSA) in VL patients utilising the presence of O-AcSA on bovine submaxillary mucin (BSM), principally 9,7 and 8-O-acetylated derivatives of sialic acid [18]. We report that individuals infected by the *Leishmania* parasite belonging to the *Leishmania donovani* complex from India, South America and the Mediterranean region have increased antibody titres against O-AcSA. This allowed for the development of an ELISA-based method for diagnosis and monitoring of VL patients irrespective of their geographical origin.

Materials and Methods

Preparation of bovine submaxillary mucin (BSM)

BSM was prepared according to the method of Murphy & Gottschalk [19]. Briefly, tissues were homogenized and extracted thrice with an equal amount of water by centrifugation at 10,000 g for 15 minutes at 4 °C. The supernatant was collected, pH adjusted to 4.5 and the resulting precipitate removed by centrifugation at 5000g for 20 minutes. The supernatant was then neutralized (pH 6.0) and dialyzed against water. Barium acetate was slowly added to the dialysate to make it 0.1 M followed by precooled methanol to give an alcohol concentration of 64% (v/v) and incubated overnight at 4°C. The precipitate formed was retrieved by centrifugation, dissolved in 0.1M EDTA, dialyzed extensively against water and stored at −20°C until use. Protein content was measured by the method of Lowry [20] using bovine serum albumin (BSA) as the standard. BSM and asialo-BSM were separately coupled to sepharose 4B using the method of Kohn and Wilchek [21]. Asialo-BSM was prepared by acid hydrolysis of BSM with 0.05M H_2SO_4 at 80°C for one hour. De-O-acetylated BSM was prepared by incubation with 0.2N NaOH for 45 minutes at 4°C followed by immediate neutralization.

Estimation of 9-O-acetylated sialic acid derivatives in BSM

The % of 9-O-acetylated sialic acid derivatives present in BSM was measured fluorimetrically as described in Sharma *et al.* [10].

Purification of O-acetylated sialic acid antibody fraction

Human serum (5 ml) from VL patients and normal donors was used to purify the polyclonal antibody fraction with preferential affinity for O-AcSA using the method of Siebert *et al.* [22]. Briefly, serum following a 33% ammonium sulphate fractionation was passed over an asialo-BSM-Sepharose 4B (3.6 mg/ml) column to remove galactose binding proteins. The resulting eluate was then loaded onto a BSM-Sepharose 4B column (5.7 mg/ml) which had been previously equilibrated with Phosphate buffered saline (PBS, pH 7.2). Following removal of non-specifically bound proteins by extensive washings in PBS, specific protein was eluted with 0.1M NH_4OH, PH 11.0 and immediately neutralized with 0.2 N acetic acid. The eluted protein fraction was then passed over a Protein G-agarose column (2 ml, Pierce), previously equilibrated with PBS, eluted with 0.1M citric acid, pH 2.5 followed by immediate neutralization with 2M Tris and extensively dialysed against PBS. The biological activity and affinity of this antibody fraction for BSM was confirmed by the BSM-ELISA and its specificity for O-acetylated sialic acids validated using de-O-acetylated BSM as the coating agent.

Study population and design

Peripheral blood (0.5 ml) was taken from patients residing in or having visited kala-azar endemic areas of Bihar and Bengal. Patients with cross reactive diseases were malaria (n = 11), tuberculosis (n = 9) and leprosy (n = 9). Controls were normal human serum (NHS) from VL endemic areas (n = 10) and non endemic areas (n = 10).

Initially, for standardization of the BSM-ELISA, biopsy proven VL patients (n = 20) were tested. Subsequently, for validation of the assay, coded blood samples of patients (n = 56) clinically diagnosed as kala-azar were sent to the Indian Institute of Chemical Biology, Calcutta where the BSM-ELISA and parasite-ELISA were carried out whereas Giemsa stained smears of tissue aspirates were examined by S. Sundar in Muzaffarpur and S. Sen in Patna.

A longitudinal study was carried out wherein pretreatment serum was collected on admission and later on completion of a single course of sodium antimony gluconate (SAG) treatment (20 mg/kg body weight for 4–6 weeks). Based on their clinical and parasitological response to treatment, they were grouped as either drug responsive (remission of fever, regression of liver and spleen and absence of parasites in Giemsa stained tissue smears) or drug unresponsive (persistence of fever and hepatosplenomegaly as also persistence of parasites in Giemsa stained tissue smears). Results were compared only on completion of all assays to ensure 'blindness' in the protocol.

BSM-linked enzyme-linked immunosorbent assay (BSM-ELISA)

BSM served as the coating antigen (5 µg/ml, 100 µl/well) in 0.02M Phosphate buffer, pH 7.4. The wells were washed thrice with PBS containing 0.1% Tween-20 (PBS-T) and blocked with 2% BSA for 2 hrs. at 25°C. Sera (diluted 1:10, 1:25, 1:50 and 1:100, 200 µl/well) of biopsy confirmed VL patients (n = 20) along with endemic controls and coendemic diseases was incubated overnight at 4°C and binding measured colorimetrically using horse radish peroxidase (HRP) conjugated goat anti-human IgG (1:5000, Sigma) and 2,2′- Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ammonium salt (ABTS) as the substrate at 405 nm. Absorbances greater than mean plus three times the standard deviation (S.D.) of endemic controls were considered positive.

To determine the IgG subclass distribution, BSM coated wells were incubated overnight at 4°C with serum of VL patients (n = 10) and NHS (n = 10), dilution being 1:50, 100 µl/well. The plates were washed thrice with PBS-T and mouse anti-human IgG1, IgG2, IgG3 and IgG4 (1:1000, 100 µl/well, Sigma) added for 2 hr. at 25°C. Binding was measured using peroxidase conjugated anti-mouse (1:5000, Cappel) and ABTS as the colorigenic substrate.

Parasite-ELISA

Immobilized crude antigen prepared from *Leishmania donovani* strain MHOM/IN/83/AG83 [23] was used as the coating antigen (5 mg/ml, 50 µl/well in 0.02M phosphate buffer, pH 7.8). Following an overnight incubation, the wells were washed thrice with washing buffer PBS-T. The wells were then blocked with 2% fetal calf serum in PBS for 1 hour at room temperature. Patient sera (n = 56), diluted 1:500 was incubated overnight at 4°C and its binding assayed colorimetrically using peroxidase conjugated goat anti-human IgG (1:5000) and ABTS as the substrate. Optical density was measured at 405 nm along with negative controls which were asymptomatic individuals from endemic areas. Absorbances greater than mean plus three times S.D. of endemic controls were considered positive.

Statistical analyses

Statistical significance of results was compared by the Student's t-test (paired and independent) and p values < 0.05 were considered significant.

Results

Fluorimetric estimation of 9-O-acetylated sialic acid (9-O-AcSA) in BSM

The % of sialic acid present in BSM was 27.0% of which 53.0% was 9-O-acetylated. Accordingly, the % of 9-O-AcSA in BSM was 14.35%.

Purification of O-acetylated sialic acid antibodies

A representative elution profile of pooled serum (5 ml) of VL patients and NHS from BSM-Sepharose4B is shown (Figure 1). The binding of VL sera to BSM-Sepharose4B is distinctly higher than NHS whose binding is negligible. The % of serum that bound to BSM-Sepharose4B was 0.74% of which 18.2% is IgG in origin as confirmed by its binding to Protein G-agarose (Table 1). Elution of this antibody fraction from a Protein G-agarose column was initially attempted at pH 4.0 and 3.0, but could only be eluted at pH 2.5. VL sera eluted from BSM-Sepharose4B showed a 40 fold higher binding to BSM than crude sera and was enhanced an additional 2.5 fold after purification on a Protein G-agarose column. The BSM-ELISA confirmed the biological activity of this affinity purified antibody fraction from crude VL sera and the IgG subclass was restricted to IgG2. This antibody fraction was confirmed to be directed selectively towards O-acetylated sialic acids as its binding was decreased when de O-acetylated BSM was used as the coating agent as compared to BSM.

Binding of VL sera to bovine submaxillary mucin (BSM)

The binding of sera of biopsy proven VL patients (n = 20) to BSM was examined by ELISA and compared with non VL control sera. The control sera included normal human sera from both endemic (n = 10) and nonendemic areas (n = 10), patients with malaria (n = 11), tuberculosis (n = 9) and leprosy (n = 10) (Fig. 2). The reactivity of VL sera as compared to NHS was 2.2, 2.6, 2.7 and 3.1 fold higher at dilution's of 1:10, 1:25, 1:50 and 1:100 respectively. Accord-

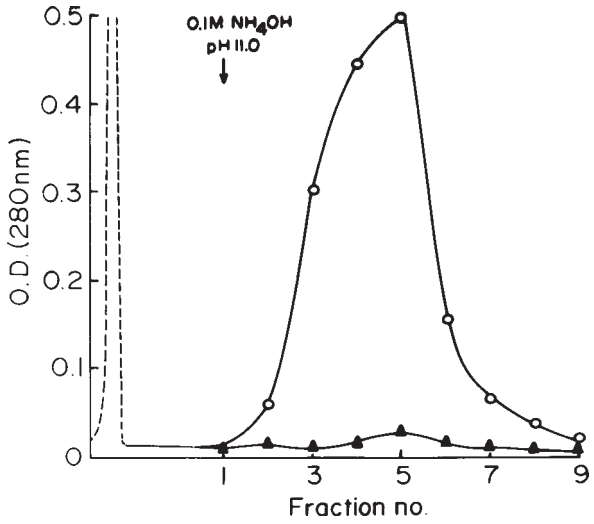

Figure 1. Elution profile of antibodies directed against O-acetylated sialic acids purified from BSM. Pooled sera (5 ml) of VL patients (○) and healthy controls (▲) was applied to a BSM-sepharose 4B affinity column and eluted by NH_4OH, pH 11.0. Fractions collected were analyzed for absorbance at 280 nm.

Table 1. Purification of antibodies directed against O-acetylated sialic acids from pooled sera of visceral leishmaniasis patients.

	Crude serum	*Ammonium sulphate (33%) fractionation*	*Run through of Asialo-BSM Sepharose4B*	*Eluate of BSM-Sepharose 4B*	*Eluate of Protein G agarose*
Volume (ml)	5.00	2.50	5.20	9.00	2.45
Total protein (mg)	200.0	130.0	62.40	1.48	0.27
BSM-ELISA OD_{405}/10 μg protein	0.01	0.02	0.05	0.40	0.90

ingly, a dilution of 1:100 was selected for subsequent screening of clinically suspicious cases. No cross reactivity was observed with malaria, tuberculosis and leprosy patients.

Assessment of BSM-ELISA for Indian Leishmaniasis

The assay was extended to testing sera of clinically diagnosed VL patients (n = 56) by the BSM-ELISA (Fig. 3b) and compared with (i) parasite specific ELISA (Fig. 3a) and (ii) microscopic examination of Giemsa stained smears of bone marrow or splenic aspirates as shown in Table 2. The mean O.D. ± S.E. of clinically diagnosed VL sera by BSM-ELISA and parasite-ELISA was 0.6 ± 0.07 and 0.9 ± 0.05 respectively (Fig. 3). Comparison of individual antibody titres obtained by the two ELISAs did not show any correlation.

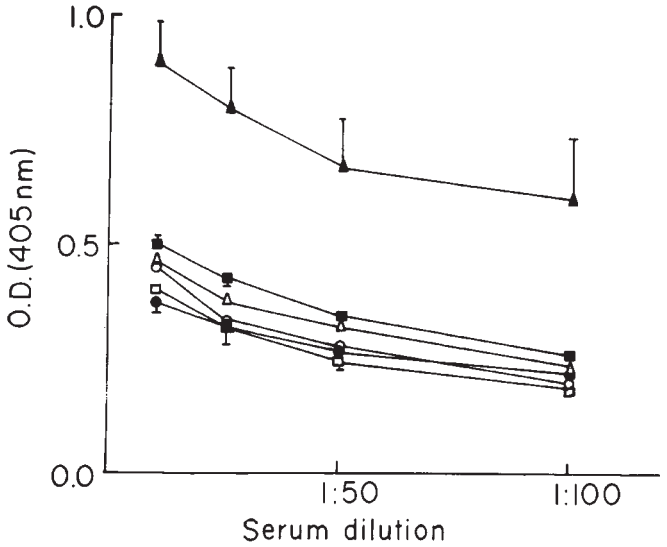

Figure 2. Titration of human serum binding using BSM as the coating antigen; VL (▲), NHS-non endemic (△), NHS-endemic (○), malaria (●), tuberculosis (□) and leprosy (■). Serum (diluted 1:10, 1:25, 1:50 and 1:100) was incubated overnight at 4°C and assayed by BSM-ELISA as described in Materials and Methods.

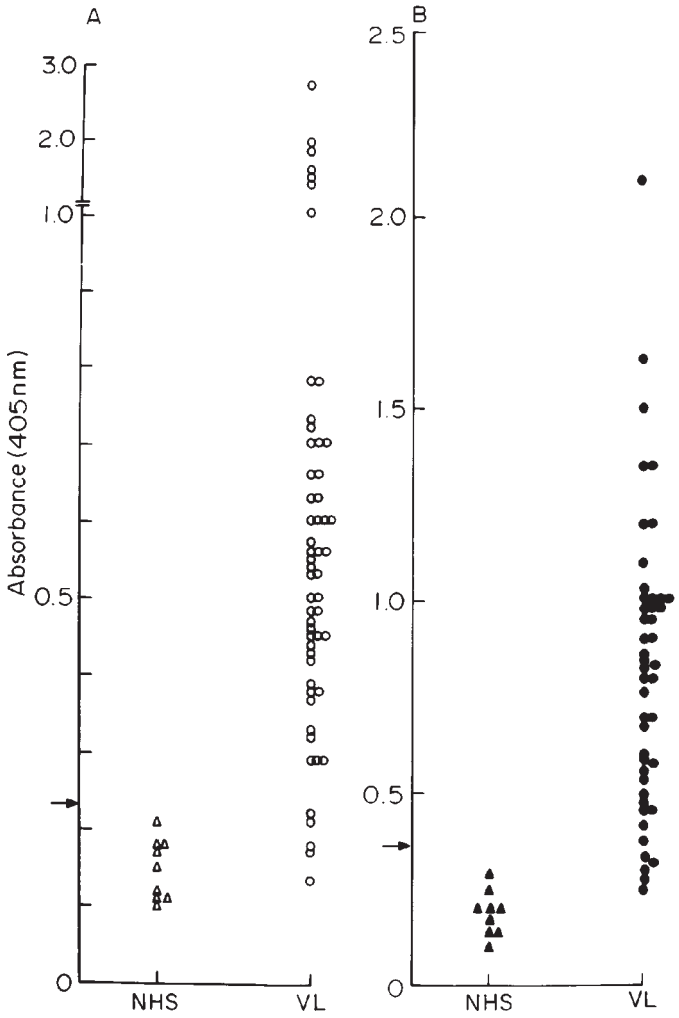

Figure 3. Comparison of parasite-ELISA (A) with BSM-ELISA (B) in 56 clinically diagnosed VL patients. **A:** Binding to crude parasite lysate of serum (diluted 1:500) of VL patients (○) and NHS (△) was assayed by parasite ELISA as described in Materials and Methods. **B:** Binding to BSM of serum (diluted 1:100) from VL patients (●) and NHS (▲) was assayed by BSM-ELISA as described in Materials and Methods. Each point is the average of duplicate determinations. In both groups, mean O.D. + 3 S.D. of NHS was taken as the cutoff value for a positive result as indicated (➔) on the y axis.

Table 2. Performance of BSM-ELISA, parasite-ELISA and Giemsa staining for detecting visceral leishmaniasis in 56 patients with a clinical diagnosis of visceral leishmaniasis.

Assay	*Positive*	*Negative*
Giemsa-staining	51	5
Parasite-ELISA	51	5
BSM-ELISA	49	7

Anti-O-AcSA antibody levels of 18 paired pre and post-treatment sera of VL patients was measured by BSM-ELISA. A significant increase was observed in mean O.D. ± S.D. of pre-treatment sera in both drug responsive and unresponsive patients (2.24 ± 0.47 and 1.25 ± 0.44 respectively). Post-treatment, the mean O.D. ± S.D showed a significant decrease in patients who responded (1.4 ± 0.58, $p < 0.0005$) whereas in drug unresponsive patients, the mean O.D. ± S.D. was marginally increased (1.67 ± 0.6). On an individual basis, 9/10 drug responsive patients had decreased antibody levels (Fig. 4a) and 7/8 drug unresponsive patients showed increased anti O-AcSA levels (Fig. 4b). The mean protein content of serum samples pre and post-treatment was 46.75 ± 11.5 mg/ml and 50.5 ± 11.0 mg/ml respectively.

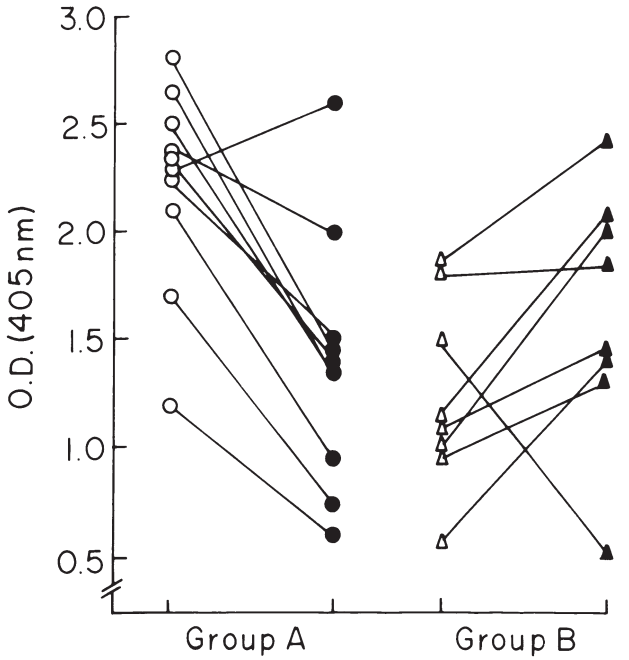

Figure 4. Levels of antibodies directed against O-acetylated sialic acids in VL patients before and after a single course of pentavalent antimonial treatment. **Group A:** drug responsive patients before (○) and after (●) treatment and **Group B:** drug unresponsive patients before (△) and after (▲) treatment. Sera was diluted 1:25 and assayed by BSM-ELISA as described in Materials and Methods.

Identification of O-acetylated sialic acid antibodies in other Leishmanial species

The BSM-ELISA was used to assess the status of antibodies against O-AcSA in other leishmanial species along with their coendemic diseases. This included sera of VL patients from India (n = 12), South America (n = 4), Mediterranean (n = 2), chagas disease (n = 5), trypanosomiasis (n = 2) and cutaneous leishmaniasis patients from South America (n = 4) and the Mediterranean region (n = 3) along with their respective endemic controls.

The mean O.D. ± S.E. of all VL sera irrespective of Indian, South American or Mediterranean origin showed significantly increased antibody titres against O-AcSA (Fig. 5a) as compared to NHS; however a strong cross reactivity was also observed with Trypanosomiasis patients. Sera from cutaneous leishmaniasis along with their endemic controls were all negative (Fig. 5b).

IgG subclass distribution of antibodies against O-acetylated sialic acid

The IgG subclass distribution of antibodies against O-AcSA in Indian VL patients (n = 20) in comparison to normal healthy individuals (n = 10) was measured by the BSM-ELISA. The mean O.D. ± S.E. of BSM bound anti O-AcSA levels was significantly increased in the IgG2 subclass in VL patients as compared to normal individuals (0.78 ± 0.02 vs. 0.41 ± 0.01; $p < 0.01$); the levels of IgG1, IgG3 and IgG4 were marginally but not significantly increased (Fig. 6.).

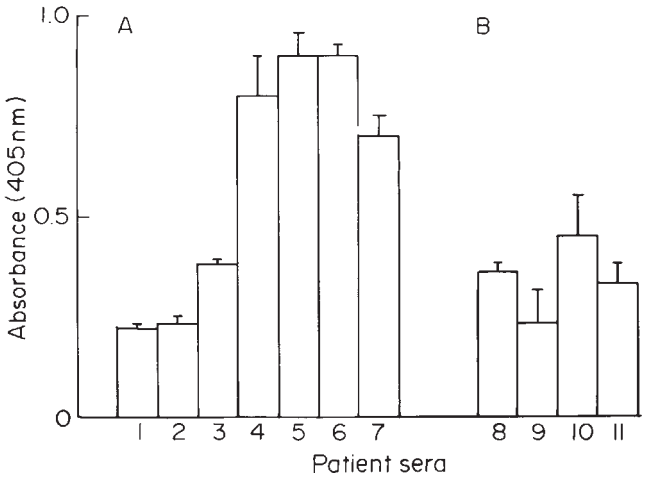

Figure 5. Diagnostic potential of BSM-ELISA for serodiagnosis of visceral leishmaniasis and cutaneous leishmaniasis patients. **A:** VL sera alongwith endemic controls and cross reactive diseases **B:** Cutaneous leishmaniasis and endemic controls. 1 = NHS (India); 2 = malaria; 3 = Chagas disease; 4 = VL (India); 5 = VL (S.America); 6 = Trypanosomiasis; 7 = VL (Mediterranean); 8 = NHS (S. America); 9 = Cut. Leish. (S. America); 10 = NHS (Mediterranean); 11 = Cut. Leish. (Mediterranean). Serum (diluted 1:100) was incubated overnight at 4°C and assayed by BSM-ELISA as described in Materials and Methods

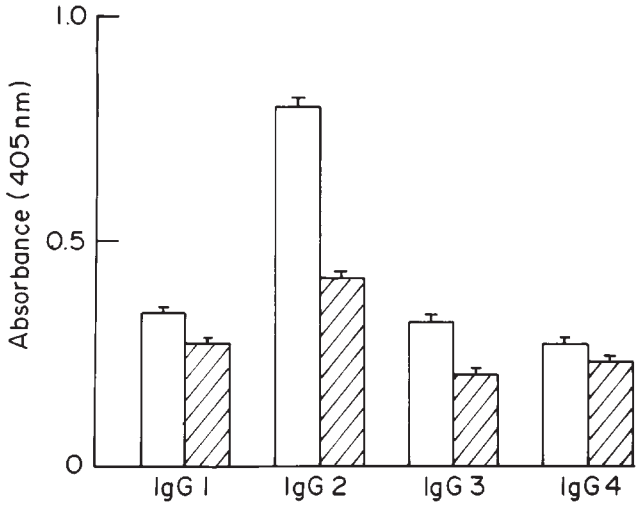

Figure 6. Mean O.D. ± S.E. of IgG subclass distribution of antibodies directed against O-acetylated sialic acids in VL patients (□) and NHS (▨). Sera (diluted 1:50) was incubated overnight at 4°C and assayed by BSM-ELISA as described in Materials and Methods.

Discussion

Lectins or lectin like proteins which bind to sialoglycoconjugates serve as recognition molecules to study alterations in sialylation patterns. Achatinin-H is a sialic acid binding lectin with a narrow binding spectrum to 9-O-AcSA in the α2,6 linkage [4–9]. This binding specificity of Achatinin-H allowed us to identify the presence of 9-O-AcSA on both erythrocytes of VL patients [10] and in the peripheral blood mononuclear cells of patients with acute lymphoblastic leukaemia [11,12].

In biological systems, one of the commonest modifications of sialic acids is the addition of O- acetyl esters to the hydroxyl groups at C_4, C_7, C_8 and C_9 positions [24]. These 9-O-AcSA have been implicated in modulating a multitude of cellular and molecular interactions [25]. Its functions range from its being a differentiation marker in developmental processes [26], as a tumour associated marker in melanomas [27,28], protection against sialidases [24], regulation of the alternative pathway of complement activation [29], inhibition of binding and invasion of malarial parasite to murine erythrocytes [30] and modulation of binding of cell adhesion molecules such as sialoadhesin and CD 22β (Siglec-2) [16,17]. Molecular dynamic studies have confirmed that linkage of 9-O-AcSA to the penultimate galactose residue provides flexibility to the oligosaccharide chain [22] which is reflected in its influence on a diverse range of cellular and molecular interactions. The presence of an immunoglobulin fraction directed against O-AcSA of IgG2 subclass in normal serum has been previously reported [31] though its biological role is not yet understood.

We therefore felt it relevant to identify whether the increased presence of 9-OAcSA in erythrocytes of VL patients [10] can initiate B-cell activation resulting in increased antibody titres against O-AcSA. The high % of O-AcSA in BSM has been earlier used as an affinity matrix to purify a 9-O-AcSA binding lectin Achatinin-H [9] as also for purification of an immunogloblin fraction directed against 9-O-AcSA from normal human serum [31].

We report the purification of an IgG fraction from VL sera directed specifically against O-AcSA (Fig. 1, Table 1) as proven by its binding to BSM alone and not to de-O-acetylated BSM. Accordingly, we then utilised this antibody fraction to develop a serodiagnostic assay for diagnosis of VL patients. No cross reactivity occurred with controls from endemic and nonedemic areas and patients with cross reactive diseases such as malaria, tuberculosis and chagas disease [Fig. 2,3 & 5]. Amongst the 51 samples positive by parasite ELISA and microscopy, 49 were detected by BSM-ELISA and only 2 were false negative. These 2 patients had a short duration of illness which may account for the inability of the BSM-ELISA to detect anti-OAcSA antibodies. No correlation could be derived between antibody titres obtained by the two ELISA's as the BSM-ELISA measured antibodies directed against O-AcSA's which were IgG2 specific; in contrast, the parasite-ELISA measured total antileishmanial antibodies which were principally IgG1 and IgG2 in nature [32].

A longitudinal study revealed that anti O-AcSA levels decreased with effective elimination of the parasite but persisted in patients who were drug unresponsive (Fig. 4) confirming its prognostic relevance. To avoid misleading interpretations that normalization of hypergammaglobulinemia in VL patients following antimonial treatment is responsible for the decrease in O.D. post-treatment, we measured the total protein content of these patients and found no significant difference before and after therapy. This increased antibody fraction is confined to the visceral form of the disease irrespective of its geographic distribution with no cross reactivity with NHS and coendemic diseases (Fig. 5). The mean O.D. ± S.E. of NHS from South America and the Mediterranean region was slightly higher than the mean O.D. ± S.E. of NHS from India. This is possibly due to a smaller sample pool of NHS from S. America (n = 3) and the Mediterranean region (n = 5). There was strong cross reactivity with *Trypanosoma brucei* infection causing African sleeping sickness or Trypanosomiasis clearly disallowing this assay to be effective in places where both diseases coexist; however such foci are limited to certain areas in Africa. A similar pattern of cross reactivity occurs using native gp63 as the coating antigen in an ELISA based method for diagnosis of VL [33]. Trypanosomal species *T. brucei* and *T. cruzi* have been identified to possess trans-sialidases which have been implicated in playing a critical role in host cell invasion [34]. As this protozoan parasite is devoid of the sialic acid biosynthesis machinery it relies on these trans sialidases to cleave sialic acids from host glycoconjugates and transfer them onto

acceptors on the parasite cell surface. Furthermore these sialidases can transfer hydroxylated and 9-O-acetylated sialic acids onto trypanosomal and synthetic acceptor compounds [35].

The IgG subclass distribution of antibodies against O-AcSA in VL is strictly IgG2 in nature (Fig. 6). Antipolysaccharide responses have been identified to have a restricted heterogeneity with regard to the IgG subclasses produced, being predominantly IgM and IgG2 in humans [36]. Our current results indicate that monitoring of anti O-AcSA levels in VL have both a diagnostic and prognostic relevance and may be applied for evaluation of disease progression as also assessment of therapeutic effectiveness.

Acknowledgments

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