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Identification and purification of cytolytic antibodies directed against O-acetylated sialic acid in childhood acute lymphoblastic leukemia

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Sialic acids typically present as terminal sugars of oligosaccharides are reported to be modified by O-acetylation at the C-9 position on lymphoblasts of childhood acute lymphoblastic leukemia (ALL) patients (Sinha *et al.*, 1999a, *Leukaemia,* 13, 119–125). We now report high titers of IgG antibodies directed against O-acetylated derivatives of sialic acids (O-AcSA) in serum of ALL patients. These antibodies were purified using bovine submaxillary mucin (BSM) and the IgG distribution was confined to IgG_1 and IgG_2 subclasses; their binding was totally abolished with de-O-acetylation confirming their specificity towards O-AcSA determinants. Flow cytometry demonstrated binding of these antibody fractions to peripheral blood mononuclear cells (PBMC) of both T- and B-ALL patients having increased cell surface 9-O-AcSA determinants. Western blotting of membranes derived from PBMC of ALL patients confirmed binding of the antibody to O-acetylated sialoglycoconjugates corresponding to 144, 135, 120, 90, and 36 kDa whereas binding to PBMC from normal individuals corresponded to 144 and 36 kDa. Specificity of the antibody fraction towards 9-O-AcSA was substantiated by hemagglutination and hemagglutination–inhibition assays. The antibody purified from ALL serum selectively mediates complement dependent cytolysis of lymphoblasts expressing O-AcSAs and thereby possibly confers passive protection. The enhanced anti O-AcSA antibody levels allowed for development of a serodiagnostic assay (BSM-ELISA) specific for ALL. Minimal crossreactivity was observed with other hematological disorders like acute myeloid leukemia (n = 16), chronic myeloid leukemia (n = 6), chronic lymphocytic leukemia (n = 7) and non-Hodgkin's lymphoma (n = 3) as well as normal healthy individuals (n = 28). The BSM-ELISA therefore provides a simple, noninvasive alternative diagnostic approach for ALL and merits clinical consideration.

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Introduction

Sialic acids, originally abbreviated as Neu5Ac, are a family of 9-carbon carboxylated monosaccharides typically found as terminal residues of vertebrate oligosaccharides. Amongst over 40 diverse structural modifications of the parent molecule, the commonest is O-acetyl substitutions at the C-7 and C-9 positions; the O-acetyl esters at the C-7 position spontaneously migrate to the C-9 position at physiological pH (Schauer, 1982; Varki, 1992; Schauer *et al.*, 1995). Although the precise function of this modification remains an open ended question, O- acetylation of sialic acids (O-AcSA) have been reported to influence enzymatic reactions regulating the catabolism of glycoconjugates, recognition of sialic acids by viral hemagglutinins and bacterial sialidases, effect tissue morphogenesis during development and can modulate the alternative pathway of complement activation (Varki, 1992; Klein and Roussel, 1998). Ligands of two important hematopoietic adhesion molecules CD22 (Siglec 2), a mature B cell surface glycoprotein and sialoadhesin (Siglec 1), a macrophage adhesion molecule can be masked by 9-O-acetylation of sialic acids (Kelm *et al.*, 1994; Sjoberg *et al.*, 1994).

Aberrations in sialylation patterns is a recurrent characteristic of malignant cells which can be detected by lectins or lectin-like proteins (Fragkiadakis and Stratakis, 1997). A lectin, *Cancer antennarius*, which recognizes sialic acids that are O-acetylated at both C4 and C9 positions, has been used to identify an O-acetylated disialoganglioside, 9-O-acetyl GD3, as a biomarker in human melanoma cells (Cheresh *et al.*, 1984; Ravindranath *et al.*, 1988). In the gastrointestinal tract, decreased O-AcSA of colonic mucin occurs in colorectal cancers and Hirschsprung's disease (Mann *et al.*, 1997; Murayama *et al.*, 1997; Aslam *et al.*, 1999). It has been proposed that this decreased O-AcSA along with increased sialylation increases susceptibility of the colonic mucosa to bacterial sialidases eventually resulting in breakdown of mucosal defenses (Corfield *et al.*, 1993). Altered O-acetylated GD3 has been found on basal and suprabasal keratinocytes of psoriasis patients (Skov *et al.*, 1997) as also in benign and atypical proliferative lesions and carcinomas of human breast (Gocht *et al.*, 1998).

Among the O-acetylated derivatives of sialic acids, 9-O acetylated sialic acids (9-OAcSA) are detectable at low levels on normal human B lymphocytes (Kamerling *et al.*, 1982) and pathological variations have been found (Holzhauser *et al.*,

1988; Stickl *et al.*, 1991). Exploiting the binding specificity of Achatinin-H, a 9-O-AcSA binding lectin, derived from the hemolymph of the African giant land snail Achatina fulica (Basu *et al.*, 1986, 1988; Mandal and Basu, 1987; Mandal *et al.*, 1989; Sen and Mandal, 1995), we have earlier reported the selective presence of 9-O-acetylated sialoglycoconjugates on erythrocytes of ALL and visceral leishmaniasis (VL) patients (Sen *et al.*, 1994; Sharma *et al.*, 1998) as also on PBMC of ALL patients (Mandal *et al.*, 1997). Subsequently, we have identified and partially characterized 9-OAcSGs specifically expressed on leukemic blasts of ALL patients (Sinha *et al.*, 1999a).

Given these observations, we wished to test whether this increased expression of 9-O-AcSGs induces B cell activation. Accordingly, we used bovine submaxillary mucin (BSM), known to contain a high percentage of O-AcSAs principally in an $\alpha 2 \rightarrow 6$ linkage (Schauer, 1982) as our analytical probe. The present study reports the identification, purification and characterization of specific O-AcSA antibodies in serum of ALL patients, the relation between isotype specific IgG_1 and IgG_2 directed against O-AcSA and blast cells having O-AcSA on their surface. This purified antibody induced the specific complement mediated lysis of leukemic blasts, which points toward its possible biological role *in vivo*. Additionally, development of a simple, serum-based BSM-ELISA assay allowed for monitoring the level of anti-O-AcSA antibodies in ALL patients as compared with normal and other hematological disorders. Given the importance of O-AcSA, we propose that this increased IgG_1 and IgG_2 O-acetylated sialic acid specific antibody levels may serve as an index to monitor the disease status of ALL patients and have therapeutic implications.

Results

Purification of antibody fractions with preferential affinity for O-acetylated sialic acids (O-AcSA) from serum of an ALL patient and a normal donor

Starting with serum from an ALL patient and a healthy normal donor (4.5 ml each), we undertook purification of O-AcSA antibody fractions. Serial purification was carried out by 33% ammonium sulfate fractionation, adsorption over an asialo-BSM Sepharose 4B column, affinity purified from BSM-Sepharose 4B and finally gradient eluted over Protein G–agarose. It is evident that the binding of ALL serum to BSM-Sepharose 4B was distinctly higher (4.1 mg) than normal human serum (NHS, 1.4 mg, Figure 1A, Table I). The proportion of serum from the ALL patient that bound to BSM-Sepharose 4B was 2.22%, of which 33.2% bound to Protein G–agarose and was therefore IgG in origin (Table I). In contrast, 0.77% of NHS bound to BSM Sepharose 4B, of which, only 16.0% bound to Protein G–agarose. A graded elution was carried out from Protein G–agarose where two major fractions were eluted at pH 6.5 and pH 3.0. This was confirmed by BSM-ELISA to belong to IgG_1 and IgG_2 subclasses, respectively (Figure 1B, Table II). Following elution from BSM, the proportion of O-AcSA binding proteins was significantly greater as compared to crude serum (Table I). Furthermore, antibody fractions were collected at pH 6.5 and 3.0, respectively, from Protein G–agarose, the proportion eluted at pH 3.0 being distinctly higher than at pH 6.5 (Figure 1B, Table I). In

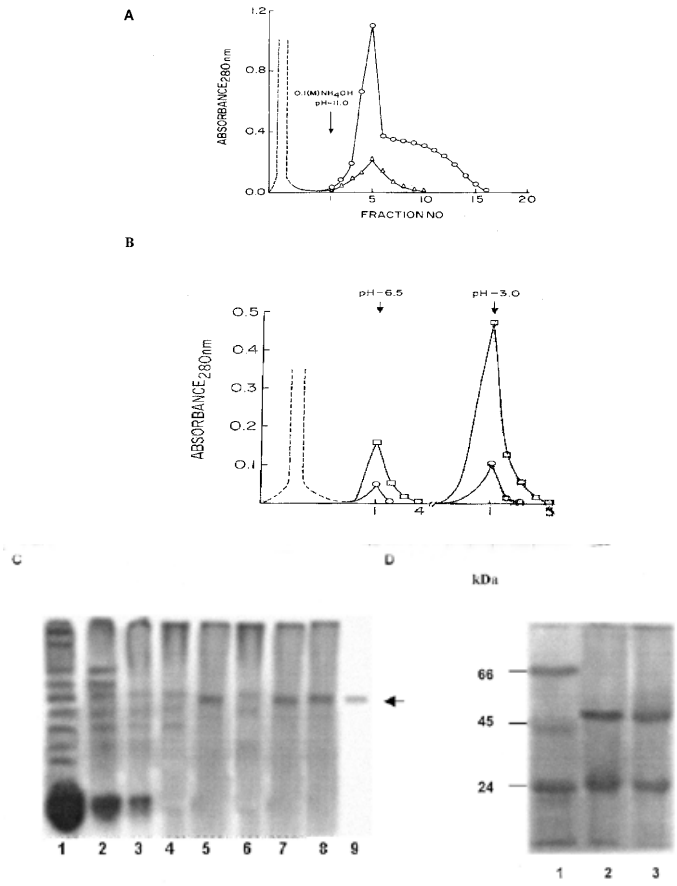

Fig. 1. (**A**) Elution profile of antibodies directed against O-acetylated sialic acids. Following a 33% ammonium sulfate fractionation, sera (4.5 ml each) of ALL patients (open circles) and healthy controls (οπεν τριανγλεσ) was passed over an asialo BSM-Sepharose 4B column (1.5 × 4 cm; 5 mg/ml). The run through was then applied to a BSM-Sepharose 4B affinity column (1.5 × 4 cm; 5 mg/ml) and bound protein was eluted by 0.1 M NH_4OH, pH 11.0. Fractions collected were analyzed for absorbance at 280 nm. (**B**) The eluate of ALL (open squares) and normal human sera (open circles) from BSM-Sepharose 4B was further purified on a protein G–agarose column (2 ml). A gradient elution was carried out with 0.1 M citric acid initially at pH 6.5 followed by pH 3.0. Fractions collected were analyzed for absorbance at 280 nm. (**C**) Native PAGE analysis (7.5%). Equal amounts (30 µg/lane) of serially purified fractions from serum of an ALL patient (lanes 1–5) and a normal human donor (lanes 6, 7) were electrophoretically analyzed on a native PAGE. Lane 1, crude ALL serum; lane 2, serum following 33% ammonium sulfate precipitation; lanes 3–5, serum following asialo-BSM adsorption, specific eluate from BSM–Sepharose column and eluate from Protein-G–agarose (pH 6.5); lanes 6 and 7, eluate of normal human serum from BSM-Sepharose column followed by Protein G–agarose (pH 3.0); lane 8, standard human IgG (150 kDa); and lane 9, Western blotting of Protein G eluted fraction (pH 6.5) from an ALL patient probed with peroxidase conjugated anti-human IgG. Gel was fixed and stained for protein with Coomassie brilliant blue R-250. (**D**) SDS-PAGE (12%) analysis of Protein G eluted fractions (30 µg/lane) from serum of an ALL patient. Lane 1, Molecular weight markers, e.g., trypsinogen (24 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa); lane 2, Protein G fraction eluted at pH 6.5; and lane 3, Protein G fraction eluted at pH 3.0.

NHS, protein yield at pH 6.5 and pH 3.0 was 0.07 mg and 0.15 mg, respectively (Figure 1A,B). These two fractions eluted at pH 6.5 and pH 3.0 were then confirmed to be IgG_1 and IgG_2, respectively, by BSM-ELISA. It may be noted that absorbances (OD_{405nm}) of the BSM-ELISA obtained from the eluates

Table I. Purification of antibodies against O-AcSA from serum of an ALL patient and a normal healthy donor

Fractions	Total volume (ml)	Total protein (mg)	Recovery (%)	O.D.$_{405\,nm}$[a]		
				IgG_1	IgG_2	IgG
Crude serum	4.50 (4.50)	184.0 (182.25)	100 (100)	0.10	0.10	0.13
33% SAS cut	1.50 (2.0)	138.0 (135.0)	75.0 (74.07)	0.18	0.22	0.28
Asialo BSM pass through	14.0 (5.0)	70.25 (65.62)	38.20 (36.00)	0.30	0.35	0.44
BSM eluted fraction	19.0 (10.0)	4.10 (1.41)	2.22 (0.77)	0.70	0.52	0.57
Protein-G eluted fraction						
(i) pH 3.0	7.0 (4.0)	0.92 (0.15)	0.50 (0.08)	0.14	0.80	1.47
(ii) pH 6.5	4.0 (2.0)	0.44 (0.07)	0.24 (0.04)	1.54	0.13	2.54

The values shown in brackets were obtained following purification of antibody against O-AcSA from serum of a normal healthy donor.
[a]Equivalent amounts of protein (10 μg) from each fraction were analyzed by BSM-ELISA (OD 405 nm).

Table II. Characterization of Protein G elution at different pH by BSM-ELISA (mean OD at 405 nm)

IgG subclasses	pH 6.5		pH 3.0	
	BSM	De-O-Ac BSM	BSM	De-O-Ac BSM
IgG_1	1.8	0.13	0.14	0.14
IgG_2	0.16	0.13	1.08	0.12
IgG_3	0.16	0.13	0.15	0.10
IgG_4	0.16	0.15	0.15	0.13

BSM, Bovine submaxillary mucin; De-O-Ac BSM, de-O-acetylated bovine submaxillary mucin.

at pH 6.5 were always higher than at pH 3.0; this possibly reflects a certain degree of loss of biological activity during purification at lower pH.

Characterization of antibodies directed against O-AcSA

To identify the O-AcSA binding proteins present in ALL serum, serially purified fractions obtained by 33% ammonium sulfate precipitation, asialo-BSM run through, specific elution from BSM-Sepharose column followed by Protein G–agarose were electrophoretically analyzed on a 7.5% native gel along with normal human IgG as standard (Figure 1C). Amongst the five non-galactose-binding proteins identified in the asialo BSM run through (lane 3), three bound to BSM (lane 4) and were therefore O-AcSA binding proteins. Among these three O-AcSA binding proteins, one was eluted from Protein G column at pH 6.5 (lane 5) and pH 3.0 (data not shown) and was accordingly considered as immunoglobulin in nature. This was confirmed by their binding to peroxidase conjugated goat anti-human IgG in Western blot (lane 9). However, in NHS, two O-AcSA binding proteins were eluted from BSM-Sepharose 4B (lane 6) and an IgG fraction from Protein G at pH 6.5 (data not shown) and pH 3.0 (lane 7). The fractions eluted from Protein G column both from ALL (lane 5) and NHS (lane 7) comigrated with standard human IgG, 150 kDa (lane 8).

SDS–PAGE (12%) analysis of the Protein G fractions eluted at pH 6.5 (lane 2) and 3.0 (lane 3) from an ALL patient reconfirmed they were IgG as they showed two characteristic bands corresponding to heavy and light chains of 50 and 25 kDa respectively (Figure 1D). A similar result was obtained with the fraction eluted at pH 6.5 and 3.0 from normal human serum (data not shown).

Table III. Correlation between haemagglutination by purified antibody directed towards O-acetylated sialic acids (O-AcSA) present on erythrocytes from different species

Erythrocytes[a]	Position of major O-acetylated group	O-AcSA content (%)[b]	HA titer of purified O-AcSA specific antibodies
Human A	—	0	0
Human B	—	0	0
Human O	—	0	0
Guinea pig	C-9	22	4
Rabbit	C-9	20	1024
Rat	C-9	25	8
Hamster	C-9	22	8

[a]Erythrocytes were collected in heparin and processed as described in *Materials and methods*
[b]Fluorimetric estimation of percentage of 9-O-acetylated sialic acids according to the method of Shukla and Schauer (1982).

Erythrocyte and sugar binding specificity of O-AcSA specific antibodies purified from ALL serum

The hemagglutination profile of erythrocytes from human and animal species by the purified anti O-AcSA fraction is shown in Table III. It is evident that the antibody agglutinated only those erythrocytes known to contain terminal 9-O-AcSA residues. Notably, human erythrocytes which contain only sialic acid and no 9-O-AcSA (Schauer, 1982) did not agglutinate.

Specificity of this antibody fraction towards 9-O-AcSA α(2→6)β-D-GalNAc was substantiated by hemagglutination inhibition assays using mono and disaccharides as well as several sialoglycoproteins. The purified antibody fraction preferentially bound to BSM having terminal 9-O-AcSA and a subterminal Gal NAc in an α2→6 linkage (Table IV). As BSM is a heterogeneously glycosylated mucin, it is possible that its terminal structure is one of many possible epitopes for this antibody. However, as no inhibition occurred with

Table IV. Sugar specificity of antibodies directed against O-AcSA purified from serum of ALL patients by hemagglutination inhibition assay

Saccharides/sialo glycoproteins	Nature of terminal linkages	Minimum concentration needed for 100% inhibition (mM)
Monosaccharide	9-O-AcSA	0.42
Disaccharide	SA α (2→6)β-D-GalNAc	NI
BSM[a]	9-O-AcSA α (2→6)β-D-GalNAc	0.00074
De-O-AcSA BSM	SA α (2→6)β-D-GalNAc	NI
Asialo BSM	GalNAc	NI
SSM	SA α (2→6) β-D-GalNAc	NI
HCG	SA α (2→3)β-D-Gal	NI
Fetuin	SA α (2→6)β-D-Gal	NI
α_1-Acid glycoprotein	SA α (2→6)β-D-Gal	NI

[a]BSM (bovine submaxillary mucin) contains 22.5% 9-O-AcSA; NI, not inhibited up to 50–70 mM; De-O-AcSA BSM, de O-acetylated sialic acid derivatives of bovine submaxillary mucin; SSM, sheep submaxillary mucin; HCG, human chorionic gonadotrophin.

de-O-acetylated BSM as well as asialo BSM, it confirmed the antibody specificity was towards O-AcSA derivatives. However, the antigen present in BSM could be present in a mono, di-, or tri-O-acetylated form, which our antibody possibly does not distinguish. Other sialoglycoproteins e.g. SSM, HCG, fetuin and α_1-acid glycoprotein having terminal sialic acid either in α2→6 or α2→3 linkage did not show any inhibition, reconfirming that presence of O-Acetylation is crucial for antibody binding (Table IV).

Selective binding of O-AcSA specific antibodies purified from ALL serum to lymphoblasts of both B and T lineage

To determine if the purified anti O-AcSA fraction selectively binds to lymphoblasts of ALL patients of both T and B lineage, flow cytometric analysis was carried out with 41 ALL patients (B-ALL, n = 37, T-ALL, n = 4). PBMC were gated for lymphocytes by staining with anti-CD 45 monoclonal antibody (MAb) and >98.0% positive cells were present (data not shown). The leukemic blast population of patients ranged from 60–90% and showed similar binding profiles with both FITC-IgG_1 and IgG_2 antibody factions directed against O-AcSA (data not shown).

To confirm, whether the purified antibody fractions against O-AcSA selectively bind to leukemic blasts of ALL patients irrespective of their lineage, double color flow cytometric analysis was carried out (B-ALL, n = 8; T-ALL, n = 2) along with normal human donors (n = 3) as controls (Figure 2).

Accordingly, the anti O-AcSA specific antibody was coincubated with PE conjugated lineage specific (B and T cells) Mabs, i.e., CD19 and CD7, respectively. Our data clearly indicates that leukemic blasts of both B and T cell lineage coexpress O-AcSA. Representative profile of a B-ALL patient having 80.0% blasts (morphologically estimated) and CD 19$^+$ (86%) is shown in Figure 2, where binding of the O-AcSA specific IgG_1 and IgG_2 antibody fractions to the CD 19$^+$ population was 98% and 99%, respectively (Figure 2). The CD7$^+$

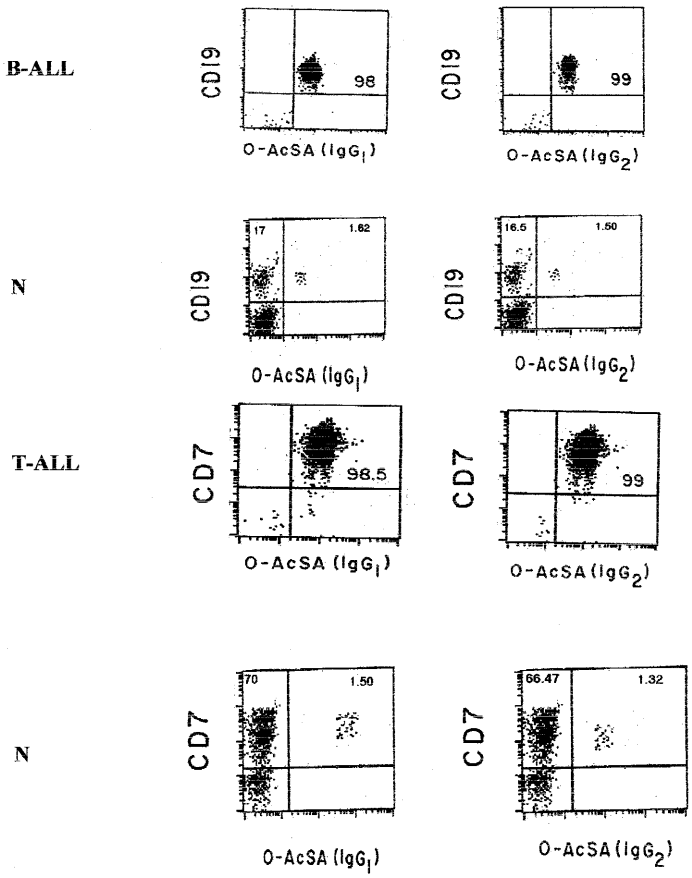

Fig. 2. Double color fluorescence dot plot to demonstrate that the purified O-acetylated sialic acid specific IgG_1 and IgG_2 fractions from an ALL patient binds to a receptor present only on CD19$^+$ cells in a B-ALL patient and CD7$^+$ cells in a T-ALL patient, minimally present in normal human PBMC. Cells (2 × 10^6) were coincubated with PE-anti–CD7/CD19 MAb and FITC-purified O-acetylated sialic acid specific IgG_1 and IgG_2 fractions and processed for flow cytometry as described in *Materials and methods*. Numbers represent the percentage of fluorescent positive cells.

population (16.0%) of this patient showed minimal binding with the O-AcSA specific IgG_1 and IgG_2 antibody fractions (data not shown). In normal donors, binding of the O-AcSA specific IgG_1 and IgG_2 antibody fractions to CD19$^+$ cells corresponded to 1.62% and 1.50%, respectively (Figure 2). Taken together, this confirmed that O-AcSA directed antibodies bound exclusively to CD19$^+$ leukemic blasts.

A representative profile of a T-ALL patient having 84% blasts (morphological estimates) and CD7$^+$ (98.0%) is shown in Figure 2. Binding of the IgG_1 and IgG_2 specific to O-AcSA to the CD7$^+$ population was 98.5% and 99.0%, respectively. Negligible binding of the O-AcSA specific IgG_1 and IgG_2 antibody fractions was observed in the CD19$^+$ population (0.24 %, data not shown). In contrast, in normal donors, binding of the O-AcSA specific IgG_1 and IgG_2 antibody fractions to the CD7$^+$ cells was only 1.50% and 1.32%, respectively. Taken together, these results indicate that antigenic epitopes specific for O-AcSA are exclusively present on leukemic blasts independent of their lineage. In parallel, PBMC from the same patients were incubated with concentration and isotype matched PE conjugated mouse IgG and FITC conjugated

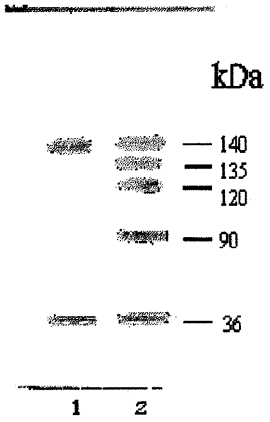

Fig. 3. Binding of the purified anti O-acetylated sialic acid specific antibody to O-acetylated sialoglycoproteins present on cell surface of an ALL patient. Total PBMC membrane proteins (30 µg/lane) from a normal donor (lane 1) and ALL patient (lane 2) were electrophoresed on a 7.5% SDS-PAGE. Following transferred onto nitrocellulose paper, it was incubated with the purified anti O-acetylated sialic acid specific antibody (50 µg) and probed with peroxidase conjugated anti-human IgG as described in *Materials and methods*.

normal human IgG, which served as the corresponding negative controls; binding was less than 2% (data not shown).

The purified anti O-AcSA antibody fraction recognizes three O-acetylated sialoglycoconjugates present on the cell surface of leukemic blasts

In a complementary study, we have previously reported that PBMC of ALL patients express cell surface 9-O AcSA determinants (Sinha *et al.*, 1999a). To further substantiate reactivity of the purified antibody fractions towards membrane O-acetylated sialoglycoproteins of PBMC of ALL patients, Western blotting was carried out. We have identified the presence of five O-acetylated sialoglycoproteins corresponding to 140, 135, 120, 90, and 36 kDa present on leukemic blasts (Figure 3, lane 2). Of these, two O-acetylated sialoglycoproteins (140 and 36 kDa) were identified on membrane fractions derived from PBMC of a healthy donor (Figure 3, lane 1). The complete abolition of staining following de-O-acetylation of the membranes with alkali treatment was evidence of its binding specificity towards O-AcSA derivatives (data not shown).

Presence of anti O-AcSA antibodies in sera of ALL patients detected by the BSM linked enzyme linked immunosorbent assay (BSM-ELISA)

The induction of anti O-AcSA antibody responses was measured in diagnostically and immunophenotypically proven B-ALL patients (n = 6) along with NHS (n = 6). The ALL patients were HLADR$^+$, CD19$^+$, CD10$^+$ and showed a high binding with Achatinin-H, a 9-O-AcSA binding lectin. BSM was used as the target antigen, as it is known to contain a high % of O-AcSA (Schauer, 1982). In comparison to NHS (open circles), the reactivity of ALL sera (solid circles) at dilutions of 1:1, 1:5, 1:10, 1:25 1:50, and 1:100 increased 3.3-, 6.3-, 7.6-, 6.8-, 5.1-, and 5.2-fold, respectively (Figure 4A), and accordingly, a dilution of 1:10 was selected.

To establish its diagnostic potential in childhood ALL, the BSM-ELISA was carried out on sera from clinically diagnosed

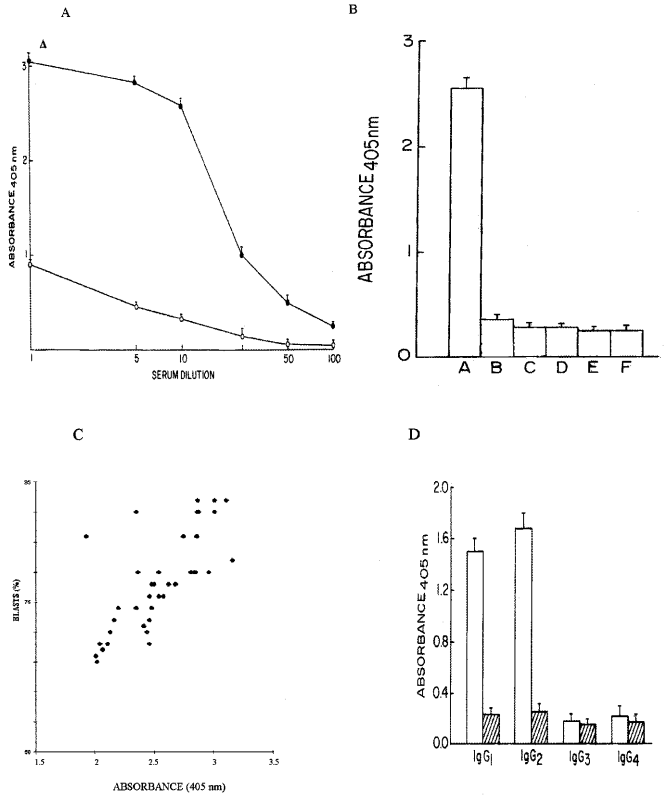

Fig. 4. (**A**) Titration of human serum binding using BSM as the coating antigen (BSM-ELISA). Sera from diagnostically and immunophenotypically proven ALL patients (n = 6) (solid circles) and normal human serum (n = 6) (open circles) were diluted 1:1, 1:5, 1:10, 1:25, 1:50, and 1:100 incubated overnight at 4°C and assayed by BSM-ELISA as described in *Materials and methods*. Each point is the average of duplicate determinations. (**B**) Diagnostic potential of the BSM-ELISA for childhood ALL. Serum (1:10 dilution) from clinically diagnosed ALL patients (n = 41) and patients with hematological disorders were assayed using BSM as the coating antigen as described in *Materials and methods*. Each point is the average of duplicate determinations. A, Acute lymphoblastic leukemia (n = 41); B, normal human serum (n = 28); C, acute myeloid leukemia (n = 16); D, chronic myeloid leukemia (n = 6); E, chronic lymphocytic leukemia (n = 7); and F, non-Hodgkin's lymphoma (n = 3). (**C**) Correlation between individual O-acetyl sialic acid specific antibody levels as measured by BSM-ELISA and percentage of leukemic blasts of 41 childhood ALL patients. (**D**) IgG subclass distribution (mean ± SE) of antibodies in ALL patients (open bars) and NHS (hatched bars) directed against O-acetylated sialic acids. Sera (diluted 1:10) was incubated overnight at 4°C, probed with mouse anti-human IgG_1, IgG_2, IgG_3, IgG_4, and binding measured by peroxidase-conjugated anti-mouse IgG. Each point is the average of duplicate determinations.

and immunophenotyped B-ALL patients (n = 37) and T-ALL (n = 4) and compared with patients having other hematological disorders such as CML (n = 6), CLL (n = 7), NHL (n = 3), AML (n =16) along with normal healthy donors (n = 28) (Figure 4B). The mean O.D. ± SE of clinically diagnosed ALL sera was 2.58 ± 0.07 and no cross reactivity was observed with sera of CML, CLL, AML, and NHL patients where antibody levels were no different from NHS. Using the mean O.D. + 3 SD of normal human serum as the cutoff value for a positive result, sera from 41/41 (100%) were positive.

To confirm antibody specificity was towards O-AcSA, we tested the reactivity of ALL sera (n = 10) replacing BSM with its de O-acetylated derivative as the target antigen. The mean

O.D. ± SD of binding was drastically reduced from 1.53 ± 0.2 to 0.2 ± 0.03, confirming that the O-AcSA derivative is critical for recognition by these antibodies.

Individual anti-OAcSA antibody levels at a serum dilution of 1:10 as determined by the BSM-ELISA showed a good correlation with the percentage of peripheral blast cells (r2 = 0.84, Figure 4C).

Characterization of the IgG subclass distribution of antibodies directed against O-AcSA in ALL patients

To determine the IgG subclass distribution of these anti O-AcSA antibodies in childhood ALL patients, levels of the four IgG subclasses were detected by anti human IgG subclass specific antibodies. Levels of IgG_1 and IgG_2 were significantly increased 6.3- and 6.6-fold respectively in ALL patients as compared to normal individuals ($p < 0.01$); their IgG_3 and IgG_4 levels were unchanged (Figure 4D).

Antibodies directed against O-AcSAs show complement dependent cytolysis of lymphoblasts of ALL patients

The specific binding of purified anti O-AcSA antibody with lymphoblasts led us to consider whether these antibodies could mediate complement dependent cytolysis of cells expressing O-AcSA determinants. The anti O-AcSA antibody fraction (0.1–1.0 µg) purified from ALL serum (Ab_{ALL}) was incubated separately with PBMC of ALL patients (n = 3) and normal donors (n = 3) in the presence or absence of 10% complement. Cytolysis was monitored by a modified MTT assay.

In PBMC of ALL patients, the Ab_{ALL} induced 21.0–28.0% cytolysis in the absence of complement. Following the addition of complement, this was significantly enhanced to 61.0–83.0% ($p < 0.1$) (Figure 5A). However, with PBMC of normal donors, cytolysis induced by Ab_{ALL} in the absence and presence of complement remained unchanged, ranging from 12.0–18.0% and 16.0–27.0%, respectively (Figure 5A).

On the other hand, using antibody purified from normal human serum (Ab_{NHS}), the degree of cytolysis of PBMC from ALL patients (n = 3) in the absence of complement was 14.0–22.0%; following the addition of complement, cytolysis increased marginally to 24.0–38.0% (Figure 5B). Similarly, the Ab_{NHS} when incubated with PBMC from normal donors in the absence and presence of complement caused minimal cytolysis, which ranged from 4.0–8.0% and 10.0–16.0%, respectively (Figure 5B).

As optimal lysis was obtained with 0.5 µg antibody (Figure 5A,B), this concentration was selected to measure the degree of spontaneous lysis of PBMC from normal donors (n = 3) and ALL patients (n = 3). The degree of spontaneous lysis was obtained by deducting the % cytolysis caused by antibody (Ab_{ALL}/Ab_{NHS}) alone. Accordingly, as shown in Figure 5C, the extent of cytolysis induced by complement alone on normal PBMC was 20.4 ± 0.5%; with the addition of Ab_{ALL}, the complement mediated lysis increased to 26.4 ± 1.0%. Accordingly, the degree of spontaneous cytolysis was 6.0 ± 1.5%. In ALL patients, the degree of spontaneous cytolysis was significantly higher being 49.8 ± 1.6%, respectively, $p < 0.01$. However, the Ab_{NHS} as seen in Figure 5D, in equivalent amounts (0.5 µg) induced minimal spontaneous cytolysis in PBMC from both normal individuals and ALL patients, the increase being marginally higher in ALL patients (2.5 ± 0.8% vs. 8.9 ± 1.7%, $p < 0.1$, Figure 5D). This clearly indicates that

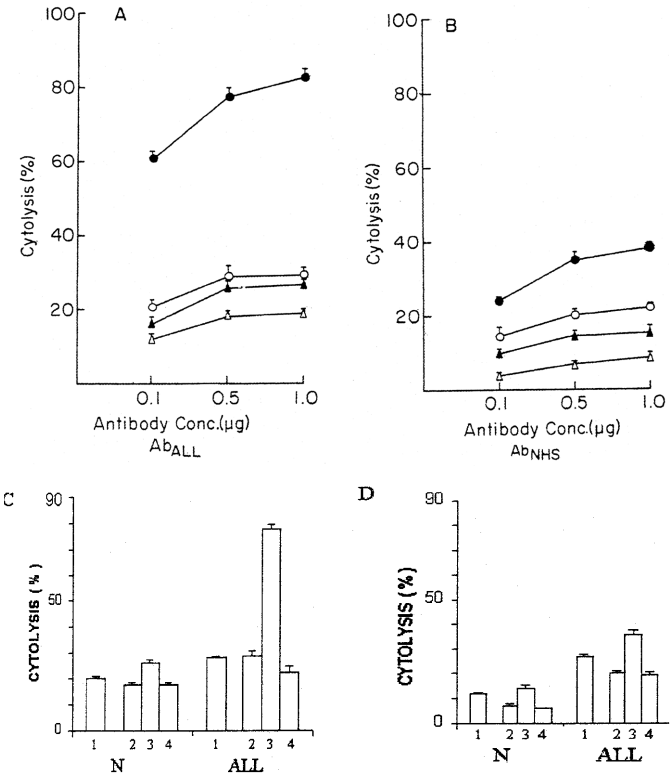

Fig. 5. Complement dependent cytolysis of lymphoblasts by purified antibody directed against O-acetylated sialic acids (O-AcSA). (**A**) Antibody fraction directed against O-AcSA purified from ALL serum (Ab_{ALL}) was incubated with PBMC of ALL patients (n = 3) in the presence (solid circles) or absence (open circles) of complement and monitored for cytolysis as measured by a modified MTT assay. Solid triangles and open triangles represent cytolysis of PBMC of normal donors (n = 3) induced by Ab_{ALL} in the presence or absence of complement. (**B**) Antibody fraction directed against O-AcSA purified from normal human serum (Ab_{NHS}) was incubated with PBMC of ALL patients (n = 3) in the presence (solid circles) or absence (open circles) of complement and monitored for cytolysis as measured by a modified MTT assay. Solid triangles and open triangles represent cytolysis of PBMC of normal donors (n = 3) induced by Ab_{NHS} in the presence or absence of complement. (**C**) Cytolysis induced by 0.5 µg of the anti O-AcSA antibody purified from ALL serum (Ab_{ALL}) of normal human PBMC (N, n = 3) and ALL patients (ALL, n = 3). The PBMC (N, ALL) was incubated with lane 1, complement; lane 2, Ab_{ALL}; lane 3, Ab_{ALL}+ complement; lane 4, Ab_{ALL}+ heat- inactivated complement. Cytolysis was measured by a modified MTT assay. (**D**) Cytolysis induced by 0.5 µg of the anti O-AcSA purified from normal human serum (Ab_{NHS}) of normal human PBMC (N, n = 3) and ALL patients (ALL, n = 3). The PBMC (N, ALL) was incubated with lane 1, complement; lane 2, Ab_{NHS}; lane 3, Ab_{NHS}+ complement; lane 4, Ab_{NHS}+ heat inactivated complement. Cytolysis was measured by a modified MTT assay.

Ab_{ALL} binds to PBMC having increased cell surface O-AcSA and thereby induces extensive cytolysis. It is essential that the complement be biologically active as spontaneous cytolysis of PBMC from normal donors and ALL patients was minimal with decomplemented, heat inactivated serum (Figure 5C,D).

Discussion

Although the presence of antibodies against O-acetylated sialic acids in normal human serum has long been identified (Ahmed and Gabius, 1989; Zeng *et al.*, 1992), little progress has been made in either identifying the presence of such an antibody

fraction in disease conditions or in assigning it a biological role. In this study, we have demonstrated the enhanced presence of IgG_1 and IgG_2 specific O-AcSA in childhood ALL and a small IgG_1 and IgG_2 fractions in normal healthy individuals (Figure 1, Tables I and II). The purified antibody fraction selectively agglutinated erythrocytes of species having terminal 9-O-AcSA and notably did not agglutinate human erythrocytes, known to be devoid of 9-O-AcSA (Schauer, 1982). Although the percentage of 9-O-AcSA in the various species had a narrow range of 20–25%, a far wider variation in the hemagglutination titer occurred (Table III). This possibly reflects variability in accessibility of the 9-O-AcSA residues (Varki and Kornfeld, 1980). A similar hemagglutination binding profile was previously reported regarding Achatinin-H, a 9-O-AcSA binding lectin (Mandal and Basu, 1987; Sen and Mandal, 1995). Our results also raise the possibility that the target antigen consists of certain O-AcSA present in proper conformation, selectively enriched in BSM and rabbit erythrocytes. The presence and absence of antibody reactivity towards BSM and its de O-AcSA derivative, respectively, clearly indicated that the purified antibody is highly specific in its antigen recognition (Table II). Taken together, antibody reactivity towards BSM indicates its specificity towards 9-O-acetylated sialyl Tn. Since normal human colonic tissue is reported to contain a mixture of O-AcSA, it may be envisaged that this purified antibody would also bind to O-AcSA present on colonic mucosa (Harms *et al.*, 1996). Ideally, to pinpoint the antibody specificity towards 9-O-acetylated sialyl Tn, one should use the disaccharide isolated from BSM. However, this is not possible, as to prepare this disaccharide the alkali treatment required would result in elimination of the O-acetyl group. Presently, no enzyme or process is available for releasing this structure from the core glycoprotein. Synthetic analogues are therefore the only alternative but are presently unavailable. In normal human serum, the presence of a small fraction of anti-O-AcSA antibodies mainly IgG_2 in nature has been purified though its role remains unanswered (Siebert *et al.*, 1996). In complementary studies, we have reported the presence of antibodies against O-AcSA, mainly of IgG_2 subtype in sera of humans and dogs infected with the *Leishmania donovani* complex responsible for VL, irrespective of their geographic origin (Chatterjee *et al.*, 1998, 1999).

In an earlier study, the selective binding affinity of Achatinin-H allowed for the detection of two membrane O-acetylated sialoglycoproteins of MW 120 and 90 kDa, both having 9-O-AcSA $\alpha2\rightarrow6$ GalNAc terminal sugar moiety as their lectinogenic epitope present specifically on PBMC of ALL patients (Sinha *et al.*, 1999a). Flow cytometry has now corroborated that the purified anti O-AcSA antibody binds selectively to PBMC of ALL patients having increased surface expression of 9-O-AcSGs (Figure 2). Subsequently, Western blotting identified three ALL specific O-acetylated sialoglycoproteins corresponding to 135, 120 and 90 kDa which reacted with the anti O-AcSA antibody fraction (Figure 3), similar to the 9-O-AcSGs recognized by Achatinin-H (Sinha *et al.*, 1999a). These are possibly the antigens responsible for the enhanced IgG_1 and IgG_2 O-AcSA fraction. Accordingly, the antigenic epitope present on lymphoblasts of ALL patients may therefore be considered to have a 9-O-AcSA $\alpha2\rightarrow6$ GalNAc as terminal sugar moiety. There were two common O-AcSGs corresponding to 140 and 36 kDa present on the cell surface of both ALL patients and normal donors. These are either less immunogenic or are inadequately exposed, accounting for the low anti O-AcSA levels present in normal individuals (Figure 4A). Taken together, these antibodies have the potential to serve as a unique probe for detailed investigation of disease specific cell surface O-AcSGs (Sinha *et al.*, 2000; Mandal *et al.*, 2000). It may be envisaged that subsequent purification of these disease specific antigens may provide clues for selective drug targeting.

We capitalized on the significantly enhanced disease specific antibody fraction to develop a noninvasive, sensitive and specific ELISA based assay using BSM as our capture antigen (Figure 4A). The absence of crossreactivity with other hematological disorders makes this assay a novel serodiagnostic approach for diagnosis of ALL (Figure 4B). The inevitable cross reactivity with VL sera and trypanosomiasis may be eliminated by developing an isotype ELISA to identify the disease specific IgG_1 subtype present in ALL (Figure 4D) but absent in VL (Chatterjee *et al.*, 1998). Antipolysaccharide responses have been identified to have a restricted heterogeneity with regard to the IgG subclasses produced, being predominantly IgM and IgG_2 in humans (Siber *et al.*, 1980). The presence of O-AcSA specific IgM cytolytic antibodies may well have a contributory role and should therefore be investigated.

O-Acetylation is one of the commonest modifications that occur in sialic acids. Initially considered to be a species specific determinant, rapid improvement of analytical techniques has demonstrated the presence of this modification in nearly all higher animals and in certain bacteria (Klein *et al.*, 1994; Klein and Roussel, 1998). The application of monoclonal antibody production for the isolation and characterization of antigens containing O-AcSA derivatives has aided significantly to pinpoint their differential expression on hematopoietic cells. The presence of O-AcSA on membrane glycoproteins and glycolipids has been demonstrated on human T and B lymphocytes using the influenza C virus (Zimmer *et al.*, 1994). On human lymphocytes, a glycolipid antigen namely 9-O-acetylated disialoganglioside GD3 (9-O-acetyl GD3) designated as Cdw60 have shown restricted surface expression (Kniep *et al.*, 1992). The highest concentration was found to be present on granulocytes and a subpopulation of T-lymphocytes (Kniep *et al.*, 1993). In the case of B-lymphocytes, the glycolipid antigen serves as a marker for activated B-cells (Vater *et al.*, 1997). Expression of this antigen has also allowed for distinguishing two functionally distinct subpopulations in human $CD8^+$ T-cells. The $CD8^+$, $CDw60^+$ T-cells are the helper cell population providing substantial help to B lymphocytes; in contrast, the $CD8^+$, $CDw60^-$ are the cytolytic T-cells which suppress B cell differentiation (Rieber and Rank, 1994). This distribution cannot be extrapolated to murine lymphocytes which have been shown to have preferential expression of 9-O-AcSA on cell surface O-linked mucin type glycoproteins on $CD4^+$ T cells (Krishna and Varki, 1997).

The physiological role assigned to this sialic acid modification in human lymphocytes has not yet been precisely defined. In murine erythroleukemia cells, Shi *et al.* (1996a) have shown a highly regulated expression of 9-OAcSA. Importantly, its presence has been shown to affect complement activation, binding to I-type lectins and tissue homing demonstrating that 9-O-acetylation can affect a variety of biological phenomena

(Shi *et al.*, 1996b). It is currently an open question as to why leukemic blasts of ALL patients synthesize these determinants and whether these anti O-AcSA antibodies contribute towards aetiopathogenesis of the disease. Rodents, humans, and primates infected with *Schistosoma mansoni* produce cytolytic IgG and IgM antibodies to the Lewis x antigen, Galβ1→4(Fuc α1→3)GlcNAcβ1→R (Nyame *et al.*, 1996, 1997). Sera from animals infected with *Schistosoma mansoni* have been reported to confer passive protection against infections in animals when administered before cercarial challenge (Sher *et al.*, 1975). Accordingly, the enhanced complement mediated cytolysis induced by the O-AcSA antibody fraction (Ab_{ALL}) selectively on PBMC of ALL patients (Figure 5A,C) may well be an attempt at active immunization and differs from the Ab_{NHS} which induces minimal cytolysis of PBMC from ALL patients and normal donors (Figure 5B,D).

Although childhood ALL is highly responsive to chemotherapy, patients in remission may harbor residual leukemic blasts, the cause of disease persistence and resurgence referred to as minimal residual disease (Pui, 1995; Coustan-Smith *et al.*, 1998). In a recent study, we have utilized the differential expression of O-AcSA α2→6 GalNAc as lectinogenic determinants detected by Achatinin-H to develop a noninvasive, blood based lymphoproliferation assay for evaluating the clinical status of B lineage ALL patients (Sinha *et al.*, 1999b–d). It was reported that ALL patients at presentation have a high expression of cell surface O-AcSA. Accordingly they need a smaller amount of Achatinin-H to induce maximal proliferation (0.25–0.25 μg) as compared to normal individuals who require 8.0 μg. With chemotherapeutic response, the maximal lymphoproliferation dose rose to 2.1 ± 0.6 μg. This increased further to 4.5 ± 1.6 μg during maintenance therapy and reached 5.5 ± 0.8 μg during follow-up. Interestingly, the maximal proliferative dose dropped sharply to 0.25 ± 0.01 μg with relapse of disease corroborating that Achatinin-H induced lymphoproliferation is indeed a measure of the cell surface O-AcSA expression. Since individual anti O-AcSA antibody titers of untreated ALL patients showed an excellent correlation with the number of circulating blasts ($r^2 = 0.84$, Figure 4C), it indicated that antibody induction is directly related to the antigenic determinant. In complementary studies, we have demonstrated the diagnostic and prognostic potential of the BSM-ELISA in longitudinal monitoring of human and canine VL (Chatterjee *et al.*, 1998, 1999). Since the presence of circulating blasts correlates with the BSM-ELISA antibody titers, this assay shows great promise in both evaluation of disease progression as also assessment of therapeutic effectiveness in ALL especially in the monitoring of minimal residual disease; such studies are underway.

Materials and methods

Study population and design

Peripheral blood (3–5 ml) was collected from 41 patients of clinically confirmed ALL, belonging to type L1 or L2 (FAB classification; Burns *et al.*, 1981), as per light microscopical appearance of bone marrow smears. Age of the patients ranged from 2.5–11 years, were of either sex, and had a leukocyte count below 7×10^{10} cells/l. Controls were age-matched normal healthy donors (n = 28) of either sex and different blood groups and patients with hematological disorders which included chronic myeloid leukemia (CML; n = 6), non-Hodgkin's lymphoma (NHL, n = 3), acute myelogenous leukemia (AML, n = 16), chronic lymphocytic leukemia (CLL, n = 7).

Blood collected at Vivekananda Institute of Medical Sciences, Calcutta, were sent as coded samples to Indian Institute of Chemical Biology, Calcutta, where serum and peripheral blood mononuclear cells (PBMC) was separated. Results were compared only on completion of the study to ensure "blindness" in the protocol. Informed consent was taken from patients and Human Ethical Clearance Committee as per the protocol of Indian Council of Medical Research.

Preparation of bovine submaxillary mucin (BSM) and estimation of 9-O-AcSA derivatives in BSM

BSM was prepared according to the method of Murphy and Gottschalk (1961) and modified as described previously (Chatterjee *et al.*, 1998). BSM and asialo-BSM were separately coupled to Sepharose 4B using the method of Kohn and Wilchek (1982). Asialo-BSM was prepared by acid hydrolysis of BSM with 0.05 M H_2SO_4 at 80°C for 1 h. De-O-acetylated BSM was prepared by incubation with 0.2N NaOH for 45 min at 4°C followed by immediate neutralization.

Estimation of percentage of 9-O-acetylated sialic acid derivatives present in BSM was measured fluorimetrically according to the method of Shukla and Schauer (1982) and modified as described by Sharma *et al.* (1998). Accordingly, the percentage of sialic acid that was O-acetylated was 22.5%.

Purification of antibody fraction directed against O-AcSA

Pooled human serum (4.5 ml) from both ALL patients and normal donors was used to purify an antibody fraction with preferential affinity for O-acetylated sialic acid derivatives using the method of Siebert *et al.* (1996). Briefly, serum following a 33% ammonium sulfate fractionation was passed over an asialo BSM-Sepharose 4B column (1.5 × 4 cms., 5.0 mg/ml) to deplete the galactose binding protein content. The run through was collected and then loaded onto a BSM-Sepharose 4B column (1.5 × 4 cm, 5.0 mg/ml) previously equilibrated with PBS, pH 7.2. Nonspecifically bound proteins were removed by extensive washings by PBS and the specific protein was eluted with 0.1 M NH_4OH, pH 11.0, followed by immediate neutralization with 0.1 M monosodium hydrogen phosphate. The total protein fraction eluted was subsequently recycled over a Protein G–agarose column (2 ml, Pierce), previously equilibrated with PBS. Gradient elution with 0.1 M citric acid from pH 6.5 to pH 3.0 were carried out and the fractions immediately neutralized with 2 M Tris followed by extensive dialysis against PBS. The biological activity and affinity of this antibody fraction towards BSM was measured by the BSM-ELISA described below. De-O-acetylated BSM replaced BSM as the coating agent to confirm the antibody affinity towards O-AcSA.

Hemagglutination activity

Hemagglutination (HA) was performed on 96 well round bottomed plates with 2% (v/v) rabbit erythrocytes incubated with the purified antibody fraction in the presence of 30 mM Ca^{2+} as previously described (Sarkar *et al.*, 1984). The HA titer was reported as reciprocal of the highest antibody dilution

giving complete agglutination. The concentration of specific sugars needed for 100% inhibition was determined by their serial addition along with 30 mM Ca^{2+} in the presence of a fixed concentration of purified antibody (which gave a minimum agglutination of 16 HA units). The sugar concentrations were based on the effective concentration of sialic acid taking into consideration that the percentage of sialic acid, which was O-acetylated in BSM, was 22.5%.

Characterization of purified O-AcSA directed antibody

(1) Polyacrylamide gel electrophoresis (PAGE, 7.5%): Individual fractions collected at every stage of purification from ALL and normal human sera were electrophoresed according to the method of Davis (1964). Gels were fixed and stained for protein with Coomassie Brilliant blue R-250. (2) SDS–PAGE (12%): Purified antibody fractions eluted from Protein G–agarose at pH 6.5 (30 µg/lane) and pH 3.0 were separated by SDS–PAGE (12%) according to the method of (Laemmli, 1970). Molecular weight markers trypsinogen (24 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa) were run simultaneously. (3) Western blot analysis of native PAGE: Purified antibody fractions eluted from Protein G-agarose at pH 6.5 (30 µg/lane) were separated by native PAGE (7.5%) and transferred to nitrocellulose. The blots were incubated overnight at 4°C with a peroxidase conjugated goat anti-human IgG and binding was colorimetrically detected. (4) Western blot analysis of membrane fractions purified from PBMC of ALL patients and normal individuals: Membrane proteins (30 µg/lane) were separated by SDS–PAGE (7.5%) according to Weissman *et al.* (1988) and transferred to nitrocellulose. The blots were incubated overnight at 4°C with the purified antibody fraction (50 µg) eluted from Protein G–agarose at pH 6.5. The O-acetylated sialoglycoproteins reacting with the anti O-AcSA antibody were colorimetrically detected with a peroxidase conjugated goat anti-human IgG.

Flow cytometric analysis of binding of purified antibody fraction to PBMC of ALL patients

The purified O-AcSA specific antibody fractions were conjugated with fluorescein isothiocyanate (FITC) as described by Coligan *et al.* (1993). PBMC from both T and B-ALL patients along with normal human donors were separated by Ficoll Hypaque density centrifugation, washed thrice in RPMI 1640, supplemented with 2 mM glutamine, gentamicin, and 10% heat inactivated human AB serum (medium A). Cells were resuspended at a final concentration of 2×10^6 cells/100 µl in medium A. After blocking the non specific binding sites with 10% goat serum for 30 min at 25°C, PBMC were individually labeled with phycoerythrin (PE) conjugated anti-CD19 (pan B-cell marker), anti-CD7 (T cell marker) and FITC labeled purified O-AcSA specific antibody fractions at 0°C for one h. Control cells were labeled with PE-conjugated murine IgG and FITC-conjugated normal human IgG. The cells were washed thrice in PBS, fixed in 1% paraformaldehyde and assessed for antibody binding analysis in a FACS Calibur Flow Cytometer. Double color flow cytometry was done with two fluorochromes by coincubating PBMC with FITC conjugated anti O-AcSA and PE-conjugated lineage specific markers and processed as described above.

Detection of antibodies against O-AcSA in ALL by the BSM-ELISA

Microtiter plates were coated with BSM prepared as described above (10 µg/ml, 100 µl/well) in 0.02 M phosphate buffer, pH 7.4 overnight at 4°C. Following three washes with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T), the wells were then blocked with 2% BSA for 2 h at 25°C. Patient sera, at different dilutions was incubated overnight at 4°C and its binding to BSM colorimetrically measured using horseradish peroxidase (HRP) conjugated goat anti-human IgG (1:5000, Sigma, St. Louis, MO) and azino-bis thio-sulfonic acid (ABTS) as the substrate on an ELISA reader at 405 nm.

For determination of the IgG subclass distribution of antibodies, a slight modification of the ELISA procedure was used. Following incubation with serum (diluted 1:10, 100 µl/well) overnight at 4°C, the wells were incubated overnight at 4°C with mouse anti-human IgG_1, IgG_2, IgG_3 and IgG_4 (diluted 1:3000, 100 µl/well, Sigma). After three washes with PBS-T, binding was measured using anti-mouse-HRP linked IgG (diluted 1:2000, Cappel) and ABTS as the colorigenic substrate.

Complement dependent lysis of PBMC of ALL patients

PBMC was harvested, washed and resuspended in medium A at a final concentration of 2×10^6 cells/100 µl. Cells (in duplicates) were incubated in PBS containing gelatin (0.1%), 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ (Buffer A) with purified antibody (0.1–1.0 µg) for 1 h at 37°C. Followed by three washes in PBS, the cells were then incubated with or without 10% complement derived from a 3-week-old rabbit for an additional hour at 37°C. Cells incubated with complement alone and antibody along with heat inactivated complement served as controls. The cells were subsequently washed with PBS and incubated with MTT (100 µl of 1 mg/ml of PBS) for 3 h at 37°C. The formazan crystals formed were dissolved in 100 µl of DMSO and absorbance was measured at 570 nm. Cell lysis (100%) was obtained by lysis of PBMC in Triton-X lysis buffer. The percentage of cytolysis was calculated as follows: $100 - [100 \times (\text{O.D. sample} - \text{OD 100\% lysis})/(\text{O.D. 0\% lysis} - \text{OD 100\% lysis})]$.

In all experimental samples, the degree of spontaneous cytotoxicity was measured by subtracting the cell lysis induced by 10% 3-week-old rabbit serum, which served as the source of complement.

Statistical analysis

The significance of differences in anti-OAcSA antibodies expression between normal donors and patients was evaluated by Student's t test. The correlation coefficient was determined between individual titers of anti-OAcSA antibodies and percentage of circulating blasts.

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