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O-acetyl sialic acid specific IgM in childhood acute lymphoblastic leukaemia

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Initial studies have revealed an enhanced surface expression of *O*-acetylated sialoglycoconjugates (*O*-AcSGs) on lymphoblasts concomitant with high titres of IgG in childhood Acute Lymphoblastic Leukaemia (ALL) (Mandal C, Chatterjee M, Sinha D, *Br J Haematol* 110, 801–12, 2000). In our efforts to identify disease specific markers for ALL, we have affinity-purified IgM directed against *O*-AcSGs that reacts with three disease specific *O*-AcSGs present on membrane proteins derived from peripheral blood mononuclear cells (PBMC) of ALL patients. Antibody specificity towards *O*-AcSGs was confirmed by selective binding to erythrocytes bearing surface *O*-AcSGs, decreased binding with de-*O*-acetylated BSM and following pretreatment with *O*-acetyl esterase. Competitive inhibition ELISA demonstrated a higher avidity of IgM for *O*-AcSG than IgG. Flow cytometry demonstrated the diagnostic potential of purified *O*-AcSA IgM as binding was specific with ALL patients and minimal with other haematological disorders and normal individuals. It therefore may be adopted as a non-invasive approach for detection of childhood ALL. Taken together, the data indicates that carbohydrate epitopes having terminal *O*-AcSA $\alpha 2 \rightarrow 6$ GalNAc determinants induce disease specific IgG and IgM, potentially useful molecular markers for childhood ALL.

Keywords: **acute lymphoblastic leukaemia (ALL), *O*-acetylated sialic acids, minimal residual disease, IgM, antibodies against *O*-acetylated sialic acids**

Abbreviations: **ALL, Acute Lymphoblastic Leukaemia; AML, Acute myelogenous Leukaemia; BSM, bovine submaxillary mucin; CHE-Fc, Influenza C haemagglutinin esterase fusion protein; CLL, Chronic Lymphocytic Leukaemia; CML, Chronic myelogenous Leukaemia; HCG, Human Chorionic Gonadotropin; MTT, (3[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue); PBMC, peripheral blood mononuclear cells; *O*-AcSA, *O*-acetylated sialic acids; *O*-AcSGs, *O*-acetylated sialoglycoconjugates.**

Introduction

Sialic acids typically present as terminal residues on glycoproteins and glycolipids are known to play a significant role in the mediation of many biological phenomena involving cell-cell and cell-matrix interactions either by reacting with specific surface receptors or via masking of carbohydrate recognition sites [1,2]. Amongst over 40 diverse structural modifications of the parent molecule, the commonest are O-acetyl substitutions at C-4, C-7 and C-9 positions [3]. As O-acetyl esters at the C-7 position migrate to the C-9 position, 9-O-acetylated sialic acids (9-O-AcSA) usually predominate on cell surface glycoconjugates generating a family of 9-O-acetylated sialoglycoconjugates or 9-O-AcSGs [4].

In haematopoietic cells, presence of 9-O-AcSGs in trace amounts has been reported on both membrane glycoproteins and glycolipids of human B and T lymphocytes [5]. A 9-O-acetylated GD3 antigen, CD60b was identified on granulocytes and a subpopulation of human T lymphocytes [6]. Exploiting the binding specificity of Achatinin-H, a 9-O-AcSA binding lectin, derived from the haemolymph of the African giant land snail *Achatina fulica* [7–9], we have reported the selective presence of 9-O-AcSGs on erythrocytes of patients with ALL [10] and Visceral Leishmaniasis [11] as also on PBMC of ALL patients [12]. Additionally, an increased presence of O-AcSG specific IgG has been reported in childhood ALL [13] as also in humans and dogs infected with Visceral Leishmaniasis [14,15].

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In light of the above observations, this study was undertaken to assess the diagnostic relevance of disease specific *O*-AcSA IgM in childhood ALL. Accordingly, the work encompasses identification, purification and characterization of *O*-AcSA IgM from serum of ALL patients. Its carbohydrate binding epitope was confirmed through (a) its selective binding to erythrocytes bearing surface *O*-acetylated sialoglycans, (b) an inhibition ELISA using affinity purified *O*-AcSGs as the coating antigen and (c) a pronounced reduction in antibody binding following removal of the *O*-AcSA determinants. Based on the enhanced expression of the binding epitope namely cell surface *O*-AcSGs, the purified antibody effectively diagnosed ALL patients of both B and T lineage by flow cytometry. Considering this purified antibody induced alternate complement pathway mediated cytolysis, it is envisaged that expression of both *O*-AcSG determinants and antibodies against these epitopes may influence the biology of childhood ALL.

Materials and methods

Study population and design

Peripheral blood was obtained from patients with childhood B or T ALL ($n = 31$), who were either $HLADR^+$, $CD3^+$, $CD7^+$ (T-ALL, $n = 8$) or were $CD10^+$, $CD19^+$ (B-ALL, $n = 23$) [16].

Patients were aged 2.5–11 years, of either sex and had a leukocyte count below 7×10^{10} cells/L. Controls included age matched normal individuals ($n = 28$) and patients with haematological disorders including chronic myelogenous leukaemia (CML, $n = 16$), acute myelogenous leukaemia (AML, $n = 19$) and chronic lymphocytic leukaemia (CLL, $n = 9$).

Venous blood was collected at Vivekananda Institute of Medical Sciences, Calcutta and coded samples were sent to the Indian Institute of Chemical Biology, Calcutta where serum and peripheral blood mononuclear cells (PBMC) were separated and stored. Results were compared only after 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.

Purification of *O*-AcSA specific IgM

An IgM fraction with affinity for *O*-AcSA was affinity purified from human serum (6.0 ml each) from an ALL patient and a normal donor using bovine submaxillary mucin (BSM) as the affinity matrix [13,17]. The percentage of sialic acid that was *O*-acetylated was fluorimetrically measured and found to be 22.5% [11]. Briefly, serum subjected to a 33% ammonium sulphate fractionation was passed over an asialo BSM-Sepharose 4B and the run through loaded onto a BSM-Sepharose4B column. After the specific protein was eluted with 0.1 M NH_4OH, pH 11.0, it was passed through a Protein G-agarose column (2 ml, Pierce); the resultant run through was subsequently loaded onto an Immunopure Column (Pierce, 2×7 cm) and IgM was eluted with TBS containing 0.1 M ethylene diamine tetraacetic acid (EDTA).

Asialo-BSM was prepared by acid hydrolysis of BSM with 0.05 M H_2SO_4 at 80°C for one hour. De-O-acetylated BSM was prepared by incubation with 0.2 N NaOH for 45 minutes at 4°C followed by immediate neutralization. This was confirmed fluorimetrically where the percentage of 9-O-acetylated sialic acid was determined by subtracting the relative unsubstituted sialic acids from that obtained after de *O*-acetylation [11].

Haemagglutination activity

Haemagglutination (HA) was performed using the purified antibody fraction as previously described [13]. The HA titre was the reciprocal of the highest antibody dilution giving complete agglutination.

Inhibition ELISA

Specificity of purified antibody towards *O*-AcSA was measured by an inhibition ELISA. *O*-AcSGs, purified by lectin affinity chromatography from PBMC of an ALL patient was used as the coating antigen (1 μg/100 μl/well in TBS, pH 7.2). Potency of mucins, carbohydrates and various sialoglycoproteins to inhibit antibody binding to immobilized *O*-AcSGs was assessed by pre-incubating them with purified *O*-AcSA specific IgM (500 ng/50 μl TBS containing 2% BSA) for 30 min at 4°C. The amount of antibody bound to 9-*O*-AcSGs was then colorimetrically detected [13]. Antibody binding to *O*-AcSGs in the absence of inhibitors represented 100% binding or 0% inhibition.

Competitive ELISA

A fixed amount of purified *O*-AcSA specific IgG was preincubated with increasing concentrations of purified *O*-AcSA IgM for 30 min at 4°C and subsequently added to a BSM coated plate and allowed to compete. Conversely, a fixed concentration of IgM with varying concentrations of IgG was similarly allowed to compete for binding to BSM and IgG or IgM bound to BSM was colorimetrically measured [13]. Binding of IgG (or IgM) to the immobilised *O*-AcSA in the absence of competitive IgG (or IgM) represented 100% binding and accordingly, percent of binding in presence of competing IgM (or IgG) was calculated for each concentration.

SDS-PAGE and Western blotting

Membrane preparations of PBMC from both ALL and normal individuals (30 μg/lane) were separated by SDS-PAGE (7.5%) and reactivity of *O*-AcSA specific purified IgM towards membrane bound 9-*O*-AcSGs detected [13]. Binding specificity towards *O*-acetyl groups was confirmed by its saponification with 0.2 M NaOH for 45 minutes at 30°C and blots were similarly probed.

Immunofluorescence staining

To establish binding of the purified antibody to cell surface *O*-AcSGs, it was conjugated with fluorescein isothiocyanate (FITC) [18]. PBMC from both T and B-ALL patients and normal human donors were stained with fluorochrome (FITC or PE) conjugated antibodies (anti-CD7, anti-CD10, anti-CD19, anti-CD20 and anti-CD45) along with appropriate isotype controls for 30 min at 4°C. In case of FITC conjugated *O*-AcSA IgM, the cells were incubated for 60 min at 4°C and after two washings, binding was measured on a flow cytometer (FACS Calibur, Becton Dickinson).

To confirm specificity of the purified antibody to cell surface *O*-acetylated sialoglycans, advantage was taken of the 9-*O*-acetyl esterase activity of influenza C virus that specifically cleaves the 9-*O*-acetyl groups of sialic acid. PBMC (5×10^6 cells in 50 μl of PBS, pH 7.2 containing 0.02% sodium azide) was incubated with Influenza C haemagglutinin esterase fusion protein (CHE-Fc, 20 μg) for 45 minutes at 37°C and similarly processed. Analysis and calculations were performed using Cell Quest software.

Detection of *O*-AcSA specific IgM in patients with childhood ALL

Microtitre plates were coated with BSM (10 μg/ml, 100 μl/well) and binding of sera was measured as previously described [13]. Quantification of *O*-AcSA IgM was done by ELISA using known concentrations of purified *O*-AcSA specific IgM as standards.

Complement dependent cytotoxicity of purified IgM

PBMC (2×10^6 cells/100 μl) were harvested and incubated with purified antibody (0.1–1.0 μg) for one hr at 37°C and percentage of complement mediated cytotoxicity was calculated [13].

Statistical analysis

Significance of differences in expression of anti-*O*-AcSA IgM between normal donors and patients was evaluated by Student's '*t*' test.

Results

Affinity purification of *O*-AcSA specific IgM

O-AcSA specific IgM was purified to apparent homogeneity from sera of ALL patients and healthy normal donors. On an average, the amount of circulating *O*-AcSA binding proteins eluted from BSM-Sepharose 4B was 3.5 fold higher in ALL patients as compared to normal human serum (NHS). Representative profile of serial purification from serum obtained from an ALL patient (6 ml) and a healthy normal donor (6 ml) is shown in Table 1. We undertook purification of *O*-AcSA antibody fractions (5.94 mg vs.1.88 mg respectively) that represented 2.48% and 0.77% of total serum proteins respectively. The Protein-G pass through (4.7 mg) was loaded onto an immunopure column and the *O*-AcSA specific IgM eluted was 0.06 mg. The homogeneity of purified IgM was confirmed by its comigration with standard human IgM heavy and light chains on SDS-PAGE under reducing conditions (data not shown).

Binding specificity of purified IgM towards *O*-Acetylated sialoglycans

The binding specificity of the purified antibody towards *O*-AcSA determinants was inferred through multiple approaches.

Purified antibody selectively agglutinated erythrocytes containing terminal O-AcSA residues

Binding of *O*-AcSA IgM with mammalian erythrocytes known to contain variable amounts of *O*-AcSA was examined. Rabbit

Table 1. Purification of antibodies against *O*-AcSA from serum of an ALL patient and a normal healthy donor

Fractions	*Serum source*	*Total volume (ml)*	*Total protein (mg)*	*Recovery (%)*	[1]*O.D. 405 nm*
Crude serum	ALL	6.0	240.00	100	0.10
	NHS	6.0	243.25	100	0.04
33% SAS cut	ALL	1.5	182.24	75.93	0.21
	NHS	2.0	180.00	74.43	0.09
Asialo BSM pass through	ALL	6.0	90.00	37.50	0.34
	NHS	6.5	87.50	35.99	0.15
BSM eluted fraction	ALL	14.0	5.94	2.48	0.48
	NHS	10.0	1.88	0.77	0.21
Protein-G pass through	ALL	14.50	4.69	1.95	0.83
	NHS	10.50	1.57	0.64	0.52
Immunopure eluted fraction (IgM)	ALL	5.0	0.06	0.205	ND
	NHS	2.0	0.01	0.004	ND

ND: not done, NHS: Normal Human Serum, BSM: Bovine Submaxillary Mucin.
[1]Equivalent amounts of protein (10 μg) from each fraction were analysed by BSM-ELISA (OD 405 nm).

erythrocytes reported to have the highest amount of *O*-AcSA [3] showed a 6.4 fold higher HU being 512 as compared to guinea pig and hamster who had a comparable HU of 8. In contrast, human erythrocytes, irrespective of their blood groups notably contain only sialic acid and are devoid of *O*-AcSA, failed to agglutinate. Taken together, these results indicate that the antibody selectively agglutinated erythrocytes that contain terminal 9-*O*-AcSA residues.

Sugar and linkage specificity of carbohydrate epitope of purified O-AcSA IgM

O-AcSGs whose specificity was confirmed by inhibition studies using 9-*O*-acetylated sialic acid as an inhibitor (C. Mandal, personal communication) was used as the coating antigen in an inhibition ELISA using several monosaccharides, disaccharides, sialoglycoproteins and mucins as inhibitors. The purified IgM showed strong binding with purified *O*-AcSGs and was best inhibited by (a) 9-*O*-AcSA as also (b) a mucin BSM having terminal *O*-AcSA and a subterminal Gal NAc in an $\alpha 2 \rightarrow 6$ linkage [19]. Minimum concentrations needed for 100% inhibition with BSM and 9-*O*-AcSA were 0.00074 and 0.42 mM respectively. Absence of inhibition with other sugars e.g. sialic acid, galactose, lactose, de-*O*-acetylated BSM (having terminal sialic acid $\alpha 2 \rightarrow 6$ Gal NAc), asialo BSM, sheep submaxillary mucin (SSM), human chorionic gonadotrophin, fetuin and α_1-acid glycoprotein having terminal sialic acid in either $\alpha 2 \rightarrow 6$ or $\alpha 2 \rightarrow 3$ linkages suggested that the antibody binding epitope was *O*-AcSA $\alpha 2 \rightarrow 6$ Gal NAc. To elucidate the antibody specificity towards this glycotope, ideally one should use the disaccharide *O*-AcSA $\alpha 2 \rightarrow 6$ Gal NAc. This however is technically not feasible, as the alkali treatment required to prepare this disaccharide, would result in elimination of the *O*-acetyl group.

O-AcSA is the critical binding determinant of the purified IgM

BSM that contained 22.5% *O*-AcSA was used as the coating antigen to examine binding of the purified IgM by BSM-ELISA. Subsequently BSM was de *O*-acetylated and the extent of de-o-acetylation was confirmed by fluorimetric estimation [11]. Replacement of BSM by its de *O*-acetylated derivative led to a drastic reduction in antibody binding as the mean OD $\pm$ S.D. decreased from 2.98 ± 0.20 to 0.42 ± 0.03, thereby reconfirming that *O*-AcSA is the critical determinant.

O-AcSA specific IgM binds selectively to cell surface O-acetylated sialoglycans

To examine the surface density of *O*-AcSGs on lymphoblasts, flow cytometric analysis was done using MOLT-4, a cell line derived from a T-ALL patient. This selection was based on our previous observation that MOLT-4 contains a high amount of surface *O*-AcSGs [12]. MOLT-4 cells showed 95% binding with FITC conjugated *O*-AcSA specific purified IgM (Figure 1A) whereas minimal binding was observed with PBMC of an AML patient (2.0%) and U937, a histiocytic lymphoma cell line (2.2%).

To further establish that *O*-AcSA is vital for recognition by the purified IgM, its binding to MOLT 4 was re-examined following selective removal of cell surface *O*-acetyl sialoglycan by CHE-Fc. With the substantial abrogation of antibody binding from 95% to 15% (Figure 1A) it may be inferred that the purified IgM binds selectively to glycotopes containing terminal *O*-AcSA.

Molecular characterization of the epitope recognized by purified *O*-AcSA IgM

Immunoblotting allowed us to further characterize the *O*-acetylated sialoglycoproteins that interact with the purified antibody. Total membrane proteins from PBMC [Figure 1B (a)] of normal donor (lane 1) and an ALL patient (lane 2) were transferred onto nitrocellulose and probed with anti *O*-AcSA IgM. Western blot analysis [Figure 1B (b)] revealed that two *O*-acetylated sialoglycoproteins (140 and 36 kDa) were common to membrane fractions derived from PBMC of a healthy donor (lane 1] and PBMC of an ALL patient (lane 2). The purified IgM bound to five *O*-acetylated sialoglycoproteins corresponding to 140, 135, 120, 90 and 36 kDa present on lymphoblasts [Figure 1B (b), lane 2). Evidence that binding was specifically with *O*-AcSA derivatives was strengthened by complete abolition of binding following de-*O*-acetylation of membranes (data not shown).

Comparison of binding avidity of purified *O*-AcSA specific IgM vs. IgG by competitive ELISA

Since the binding specificity of IgG towards common glycotopes with terminal *O*-AcSA α2-6 GalNAc has been previously suggested [13], a competitive ELISA was performed to establish the relative binding avidity of *O*-AcSA IgG and IgM. Binding of IgG decreased with increasing concentrations of competitive IgM and 50% inhibition of IgG binding was obtained with an IgM concentration of 8.8 nM (Figure 1C). On the other hand, a 13.4 fold higher amount of IgG (118 nM) was needed for 50% inhibition of IgM binding (data not shown), indicating greater functional avidity of IgM than IgG for *O*-acetylated sialoglycans.

Diagnostic potential of the purified O-AcSA specific IgM in childhood T-and B-ALL

The diagnostic potential of measuring the surface density of sialoglycoconjugates present on lymphoblasts of ALL patients through binding of *O*-AcSA IgM was examined by flow cytometric analysis. PBMC from ALL patients and normal donors were initially gated with anti-CD45 (98.0% positive cells, data not shown). By morphological estimates, the lymphoblast population of ALL patients ranged from 60–90% and showed selective binding with FITC conjugated *O*-AcSA specific purified IgM. The mean $\pm$ S.D. of binding positivity in B-ALL ($n = 5$),

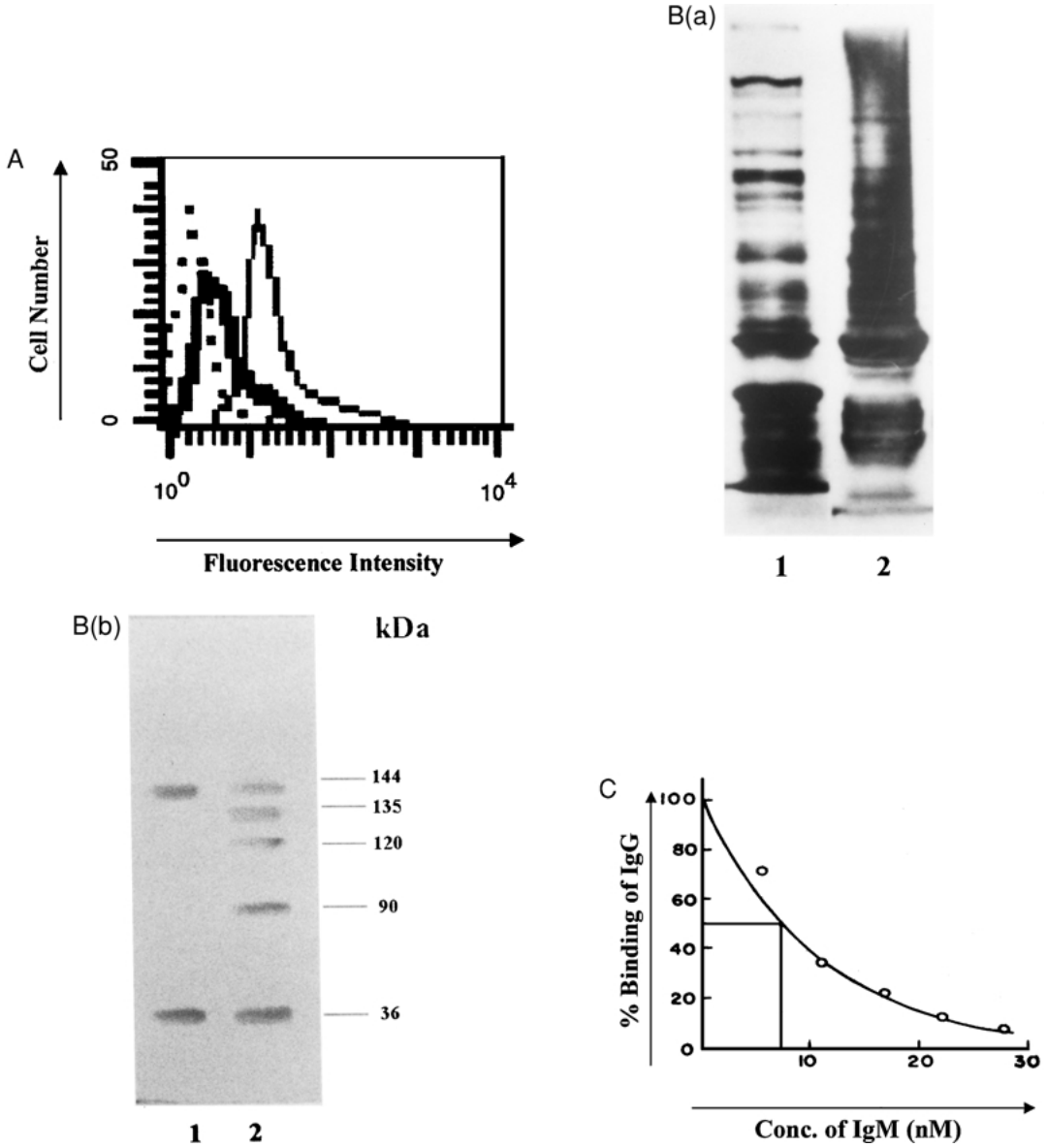

Figure 1. (A) Flow cytometric analysis to demonstrate selective binding of *O*-AcSA specific IgM with surface *O*-acetylated sialoglycans. FACS analysis showing binding of FITC conjugated purified *O*-AcSA IgM with MOLT 4 cells in the absence (thin line) or presence (thick line) of *O*-acetyl esterase as described in Materials and Methods. The dotted line represents binding with control IgM. (B) Reactivity of purified *O*-AcSA specific IgM with cell surface *O*-acetylated sialoglycoproteins by Western blot analysis. (a) Membrane proteins from PBMC of a normal donor (lane 1) and an ALL patient (lane 2) were electrophoresed (7.5% SDS PAGE) and was stained with Coomasie blue. (b) Following transfer onto nitrocellulose membranes, PBMC from normal donor (lane 1) and an ALL patient (lane 2) were incubated with purified *O*-AcSA specific IgM (50 μg) and probed with peroxidase conjugated anti-human IgM as described in Materials and methods. (C) Competitive binding of purified *O*-AcSA specific IgM and IgG by ELISA. Binding of a fixed concentration of IgG with immobilised BSM in the presence of increasing concentrations of IgM was measured by ELISA as described in Materials and methods. Total IgG binding (100%) was obtained from the incubation of IgG alone. The % of bound IgG was plotted against increasing concentrations of competing IgM.

T-ALL ($n = 3$) and normal donors ($n = 5$) was 94.8 ± 3.48, 89.56 ± 3.97 and 2.36 ± 0.72 respectively.

Representative profile of a B-ALL patient having 90.0% blasts (morphologically estimated) and 96% $CD19^+$ cells showed 98.50% positive staining (mean fluorescence intensity was 101.2) with the purified antibody (Figure 2A). Similarly, profile of a T-ALL patient having 90% blasts (morphologically estimated) and 92% $CD7^+$ cells showed 85.40% staining (mean fluorescence intensity was 86.3) with the purified antibody (Figure 2A). In contrast, binding with normal human

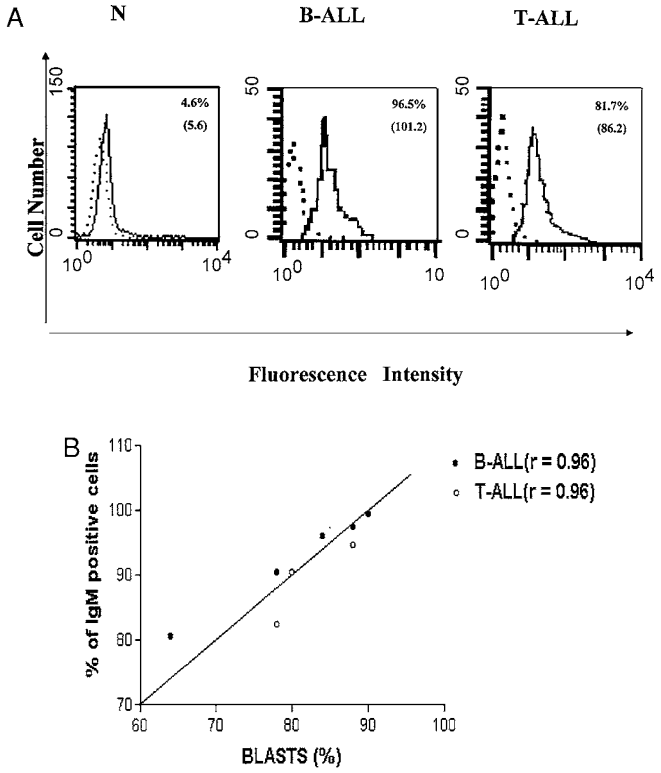

Figure 2. (A) Single color fluorescence analysis to demonstrate selective binding of anti *O*-AcSA specific IgM to PBMC of childhood ALL patients. Surface expression of *O*-AcSGs on lymphoblasts of both B and T-ALL patients at presentation along with normal human donors was determined by immunofluorescence analysis using purified *O*-AcSA specific IgM (solid line). The dotted line represents binding with control IgM. The number of positive cells are expressed as % and the numbers in parentheses correspond to mean fluorescence intensity. (B) Correlation between percentage of blast cells and *O*-AcSA specific IgM positive cells as determined by FACS analysis. Correlation between the percentage of leukaemic blasts of childhood B-ALL (-●-, $n = 5$) and T-ALL (-○-, $n = 3$) patients and their individual *O*-acetyl sialic acid specific antibody positive cells as measured by FACS analysis.

PBMC was minimal (4.6%, mean fluorescence intensity was 5.7) (Figure 2A).

A strong correlation existed between the percentage of blast cells and cells showing binding with *O*-AcSA specific IgM in both B-ALL (-●-, $r = 0.966$) and T-ALL (-○-, $r = 0.967$) (Figure 2B) thus confirming that measurement of *O*-AcSA determinants could be considered as a surrogate marker for childhood ALL irrespective of lineage.

To determine the assay sensitivity, binding of FITC-IgM was measured by admixing increasing amounts of MOLT-4 cells (1, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 respectively) with normal PBMC keeping the total cell number constant at 10^6. As the presence of one leukaemic cell was detectable in 10^3 normal PBMC, the assay sensitivity was defined as 1 in 10^3.

Increased presence of disease specific anti *O*-AcSA IgM in ALL patients

Levels of anti *O*-AcSA IgM were measured in T and B-ALL patients using BSM as the capture antigen. Initially as a prescreening procedure, sera (six each from ALL and NHS) were diluted 1:10, 1:25, 1:50 and 1:75. At all dilutions, patients with ALL showed a clear positive signal with BSM in comparison to NHS and the fold increase in binding of sera from ALL patients compared to NHS was 1.5, 2.6, 4.7 and 3.4 fold respectively; a dilution of 1:50 was accordingly selected for subsequent screening.

Subsequently, a larger sample population of ALL patients was studied ($n = 31$) and levels of anti *O*-AcSA IgM measured along with normal healthy donors and patients with other haematological disorders such as AML, CML, CLL and NHL. The mean OD $\pm$ S.D. of clinically diagnosed ALL sera was 0.43 ± 0.06. Notably, absence of detectable levels of *O*-AcSA IgM in sera from AML, CML, CLL and NHL patients suggested that this humoral response was directed specifically towards disease specific *O*-AcSGs (Figure 3A). Using the mean OD + 3 S.D. of normal human serum as the cut off value for a positive result, sera from 31/31 (100%) were positive.

Quantitatively, the concentration of *O*-AcSA specific IgM at presentation was significantly higher in ALL patients 10,310 ng/ml (SD 1,585 median; 10,57 ng/ml) as compared to normal individuals 1,880 ng/ml (SD 316, median; 1,867 ng/ml) $p < 0.01$, Figure 3B).

Antibody mediated complement dependent cytolysis of lymphoblasts bearing *O*-AcSA determinants

The specific binding of antibody purified from an ALL patient (IgM_{ALL}) with lymphoblasts led us to consider whether these antibodies could mediate complement dependent cytolysis of cells expressing *O*-AcSA determinants. In ALL patients, the IgM_{ALL} induced 10.2–17.4% cytolysis in the absence of complement which significantly increased to 51.0–77.0% ($p < 0.01$) following addition of complement (Figure 4A), optimal lysis being obtained at 5.5 nM. However, cytolysis of PBMC of normal donors by IgM_{ALL} was marginal both in the absence (2.8–9.0%) and presence (13.1–22.1%) of complement (Figure 4A). Conversely, with antibody purified from NHS (IgM_{NHS}), cytolysis of PBMC from ALL patients in the absence and presence of complement ranged from 7.4–16.6% to 18.6–32.0% (Figure 4B). Similarly, the IgM_{NHS} when incubated with PBMC from normal donors in the absence and presence of complement caused minimal cytolysis that ranged from 7.1–12.8% and 12.5–20.7% respectively (Figure 4B).

Discussion

A striking feature of childhood ALL is the high expression of a unique disease specific antigen having 9-*O*-AcSA α2-6 Gal NAc as its terminal carbohydrate epitope [12]. Their enhanced

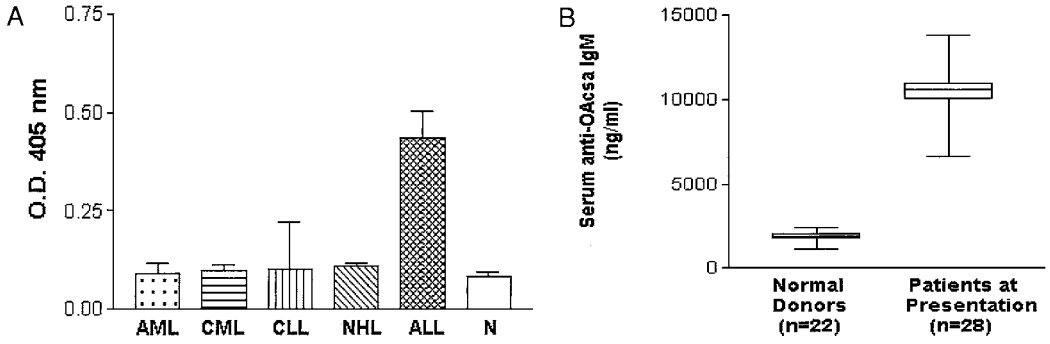

Figure 3. (A) Detection of elevated anti-*O*-AcSA IgM levels in childhood ALL. Sera (diluted 1:50, 100 μl/well) from clinically diagnosed ALL patients ($n = 31$), patients with other haematological disorders e.g. AML, CML, CLL, NHL and normal human donor (N) were assayed for *O*-AcSA specific IgM using BSM as the coating antigen as described in Materials and methods. Each point is the average of duplicate determinations. (B) Quantitative analysis of *O*-AcSA specific IgM. Median values of IgM in normal human donors (1,887 ng/ml, $n = 22$) and ALL patients at presentation (10,570 ng/ml, $n = 28$) is indicated with horizontal bars. The vertical bars indicate the range and the horizontal upper and lower boundaries of the boxes represent the first and third quartiles respectively.

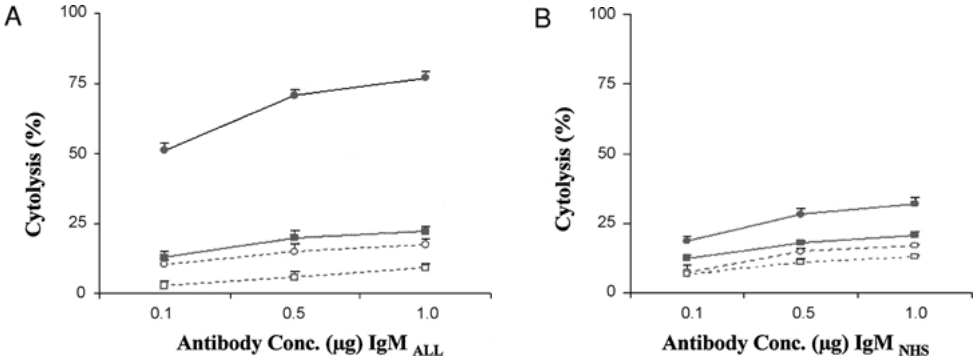

Figure 4. Complement dependent cytolysis of lymphoblasts by *O*-AcSA specific IgM purified from an ALL patient (IgM_{ALL}) and a normal donor (IgM_{NHS}). (A) IgM_{ALL} was incubated with PBMC of ALL patients ($n = 4$) in the presence (-●-) or absence (-○-) of complement as also PBMC from normal donors were similarly incubated in the presence (-■-) or absence (-□-) of complement. Cytolysis was monitored by MTT assay. (B) IgM_{NHS} was incubated with PBMC of ALL patients ($n = 4$) in the presence (-●-) or absence (-○-) as also PBMC from normal donors were similarly incubated in the presence (-■-) or absence (-□-) of complement. Cytolysis was monitored by MTT assay.

immunogenicity was reflected in an increased presence of *O*-AcSA directed IgG1 and IgG2 isotypes [13]. We now report that in childhood ALL, these disease specific glycotopes with terminal *O*-AcSGs induce an IgM response directed specifically towards *O*-AcSA determinants as demonstrated through multiple evidences namely (i) the substitution of BSM with de-*O*-acetylated BSM caused a drastic 7.1 fold reduction in antibody binding, (ii) antibody binding with mammalian erythrocytes was proportional to their expression of surface *O*-AcSA, (iii) Inhibition ELISA showed highest inhibition potency with 9-*O*-AcSA and BSM known to have 9-*O*-AcSA $\alpha 2 \rightarrow 6$ GalNAc as its terminal glycotope [19] and (iv) the pronounced decline in antibody binding to leukaemic blasts following *O*-acetylesterase treatment known to selectively cause cleavage of the *O*-acetylated sialoglycans [20]. Taken together, the data corroborated that cell surface *O*-acetylation was vital for antibody binding to leukaemic blasts (Figure 1A) and its binding domain has 9-*O*-AcSA $\alpha 2 \rightarrow 6$ GalNAc as the terminal glycotope.

Molecular characterization by immunoblotting with the purified *O*-AcSA specific IgM revealed three disease-specific surface *O*-AcSGs corresponding to 135, 120 and 90 kDa [Figure 1B, (b) lane 2] and was analogous to similar binding observed with both purified *O*-AcSA specific IgG from patients with childhood ALL [13] and Achatinin-H, a 9-*O*-AcSA binding lectin [12]. As the competitive ELISA showed both *O*-AcSA specific IgG and IgM compete for binding to common carbohydrate epitopes, it further supported our findings that the same glycan epitopes contribute towards induction of elevated levels of *O*-AcSA specific IgM and IgG. As concentrations needed for 50% inhibition of IgM binding by IgG was 13.4 fold higher than the concentration of IgM needed for IgG inhibition (118 nM vs 8.8 nM respectively), we concluded that the binding avidity of IgM for *O*-AcSG is far higher than that of IgG

(Figure 1C). Since IgM induced complement activation is known to trigger the complement cascade, differences in avidity of IgM vs. IgG may account for the observed variations in the degree of complement dependent cytolysis. Lymphoblasts expressing high *O*-AcSA determinants needed 6.0 fold less IgM than IgG for a comparable degree of complement mediated spontaneous cell lysis (5.5 nM vs. 33.0 nM respectively, Figure 4A and B).

Normal sera known to contain natural IgM to the ganglioside GM2 cause complement mediated cytolysis of HIV infected cells and have been postulated as an effective adjuvant to chemotherapy [21]. Similarly, one could envisage that enhanced complement mediated cytolysis of leukaemic blasts of ALL patients induced by the *O*-AcSA specific IgM (IgM_{ALL}) (Figure 4A) may well be an attempt to clear the leukaemic blasts. As immunotherapy of tumour cells is based on cancer-specific expression of antigens, use of this antibody as an adjuvant to chemotherapy could be considered. However, the presence of *O*-AcSG dependent ligand-receptor interactions in normal human tissues should first be addressed. To date, knowledge regarding the tissue distribution of *O*-AcSGs remains an intractable problem as potential pitfalls during analysis include the incomplete release of sialic acids, de *O*-acetylation and spontaneous migration of *O*-acetyl groups and accordingly indirect methods for analysis are applied [15].

Antibodies that react with tumour but not with normal tissue also offer great potential for diagnosis. As high levels of IgG specific antibodies directed against *O*-AcSGs were previously identified in children with ALL, we wanted to test the potential of this immunoreactive *O*-AcSG as a surrogate marker using flow cytometric analysis. The high percentage of antibody binding to PBMC of ALL patients as observed by flow cytometric analysis corroborated that purified *O*-AcSA specific IgM binds selectively with PBMC of ALL patients in contrast to that of normal donors (Figure 2A). Minimal antibody binding with PBMC from patients with other cross-reactive haematological disorders highlighted the specificity of the test and merited its application in clinical practice. As we found a strong positive correlation between antibody binding to lymphoblasts and percentage of circulating blasts we conclude that this approach is of immense diagnostic value (Figure 2B). As the presence of one leukaemic cell was detectable in 10^3 normal PBMC by flow cytometry, it sugests that this assay could be utilised in longitudinal follow up studies for monitoring of residual leukaemic cells; such studies are presently ongoing. Ideally to increase the assay sensitivity further, acquisition should be continued when over 2×10^5 cells or more have passed through the flow cytometer or when 10,000 events are recorded [22] which was not achievable due to technical constraints.

The potential of the circulating *O*-AcSA specific IgM as a clinical guide for patients with ALL looks promising as a higher level was consistently measured in ALL patients as compared to normal individuals and patients with other haematological disorders (Figure 3A). However, for optimisation of an ELISA based assay, one should aim for estimation of total *O*-AcSA specific immunoglobulin i.e. IgG1, IgG2 [13] and IgM. This is achievable by using HRP conjugated Protein A and such studies are ongoing. Quantitatively, the titer of *O*-AcSA specific IgM was considerably higher in ALL at presentation (10,310 ng/ml) (Figire 3B) as compared to normal (1,880 ng/ml) sera. Although two common *O*-AcSGs are present on the cell surface of both ALL patients and normal donors corresponding to 140 and 36 kDa, their relatively low immunogenicity could be attributed to their being constitutively present [23] or are inadequate exposed. This quantification of *O*-AcSA IgM may be eventually helpful in predicting the outcome of patients with childhood ALL. As augmentation of both antibody classes IgG [13] and IgM against this glycan epitope occurs in childhood ALL, it can be harnessed to develop a simple, ELISA based sensitive and specific diagnostic assay; such studies are underway.

Although childhood ALL is highly responsive to chemotherapy, patients in remission may harbor morphologically undetectable, self renewing residual leukaemic blasts, considered the principal cause of disease persistence and resurgence and referred to as minimal residual disease (MRD) [24]. Correlative studies have demonstrated that MRD detection by flow cytometric or polymerase chain reaction analysis of leukaemia-specific markers have a strong correlation with subsequent relapse [25]. Unfortunately, the stumbling block impeding introduction of these methods for MRD detection is that none of these techniques are universally applicable in all ALL patients [26]. Exploiting the specificity of *O*-AcSA IgM and IgG to detect patients with childhood ALL irrespective of their lineage, we propose that future avenues of research should be directed towards their application in monitoring assessment of chemotherapeutic effectiveness [27]. This may eventually lead to the establishment of an alternative approach for universal monitoring of MRD.

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