Antibodies against 9-O-acetylated sialic acids in childhood acute lymphoblastic leukemia: A two-year study with 186 samples following protocol MCP 943

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Initial studies have revealed an enhanced surface expression of 9-O-acetylated sialoglycoconjugates (9-OAcSGs) on lymphoblasts concomitant with high titers of antibodies (anti-9-OAcSGs) in childhood acute lymphoblastic leukemia (ALL)1-4. This study was undertaken in 186 coded samples from 69 ALL patients to evaluate if antibodies against these sialoglycans could monitor response to the treatment. An ELISA was developed using bovine submaxillary mucin (BSM) containing high % of 9-O-acetylated sialic acids (9-OAcSA) as the capture antigen, to investigate serum levels of anti-9-OAcSGs in a single-center series of pediatric, clinically-diagnosed and immunophenotypically confirmed ALL patients, as compared to 130 healthy controls. At presentation, a 3.8-fold increase in anti-9-OAcSGs levels was detected in 63/69 ALL patients (mean ± SEM was 102.8 ± 6.3 µg/ml) as compared to normal controls (27.17 ± 0.76 µg/ml), assay sensitivity being 91.3%. On an individual basis (n = 25) in patients who were longitudinally monitored for two years, a significant decline in their mean ± SEM of OD was observed from 0.85 ± 0.06 to 0.28 ± 0.03. Additionally, a dot-blot was developed to evaluate the proportion of immune-complexed 9-OAcSGs in these patients employing achatinin-H, a 9-OAcSA-binding lectin. Our data indicate that these economically viable ELISA-based approaches allow for reliable, sensitive and rapid diagnosis of ALL. We contend that these disease-specific antibodies could be considered as potential markers both for the initial diagnosis of ALL and possibly for longitudinal monitoring of the disease.

Keywords: Acute lymphoblastic leukemia, 9-O-acetylated sialoglycoconjugates (9-OAcSGs), bovine submaxillary mucin, achatinin-H, BSM-ELISA, Dot-blot

Although childhood acute lymphoblastic leukemia (ALL) is highly responsive to chemotherapy, relapse occurs in at least 20-25% of children who are treated in contemporary therapeutic regimens5,6. This occurs primarily as patients in remission may continue to harbor submicroscopic levels of residual leukemic blasts (<5%), thus contributing to disease persistence and resurgence. The detection of minimal residual disease (MRD), therefore, depends on improved assays that can evaluate individual chemotherapeutic response and predict impending relapse prior to its clinical manifestations. In this regard, identification of disease-specific molecular marker(s) whose expression is altered with treatment would be of utmost biomedical importance7-10.
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-9 position\(^\text{11}\). Owing to the strategic terminal location of sialoglycoconjugates on vertebrate oligosaccharides, they are involved in a multitude of biological processes and, therefore, their identification and characterization is currently a subject of intense study\(^\text{12}\). With regard to its O-acetylated derivative, an O-acetylated disialoganglioside 9-O-acetyl GD3 has been reported as a biomarker in human melanomas\(^\text{13,14}\). The presence of O-AcGD3 has also been detected in breast tumours and other tumours of neuroectodermal origin that has provided a basis for their application in diagnosis and vaccine therapy\(^\text{15,16}\). The origin of breast adenocarcinomas traced by these biomarkers suggests immense clinical relevance, as the tumour origin is often difficult to ascertain, because the primary sites are destroyed, prior to diagnosis\(^\text{17}\). The ligands of two important hematopoietic adhesion molecules CD22 (Siglec 2), a mature B cell surface glycoprotein and sialoadhesin (Siglec 1), a macrophage adhesion molecule could be masked by 9-O-acetylation of sialic acids\(^\text{18,19}\).

Exploiting the binding specificity of achatinin-H, a 9-OAcSA\(_2\)-6GalNAc-binding lectin, derived from the hemolymph of the African giant land snail Achatina fulica\(^\text{20-23}\), we have earlier reported the enhanced expression of 9-OAcSGs on peripheral blood mononuclear cells (PBMC) of ALL patients with a basal level of expression in normal PBMC\(^\text{1,2,8,24}\). Immunogenicity of these 9-OAcSG determinants was demonstrated by increased antibody production principally of IgM, IgG\(_1\) and IgG\(_2\) subtypes in ALL patients\(^\text{3,4}\). Importantly, no cross-reactivity occurred with patients having other hematological disorders i.e., chronic myeloid leukemia (CML), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), aplastic anemia, thalassemia and NHL (non Hodgkin’s lymphoma)\(^\text{7}\), thus making the assay specificity 100%. Therefore, we contend that these disease-specific immunoglobulins could be used for evaluating the chemotherapeutic response of ALL patients.

In view of the importance of 9-OAcSGs\(^\text{1,2,7,9,10}\), the present investigation has focussed on (i) establishment of an effective serodiagnostic approach in 69 ALL patients by measuring anti 9-OAcSGs titers using a user-friendly ELISA, (ii) quantitation of anti 9-OAcSGs in these ALL patients along with patients with other hematological disorders and normal individuals, (iii) determination of the utility of sequential measurement of levels of anti-9-OAcSGs in a prospective study of 100 coded blood samples from 25 ALL patients at four time-points, and (iv) addressing the presence of 8.7% false negative cases at presentation by measuring the presence of diseasespecific antibodies in a complexed form with shedded antigen using a dot-ELISA. We propose that evaluation of the increased 9-OAcSA-specific antibody levels may serve as an economically effective index for diagnosis of ALL and may have therapeutic implications in monitoring response to treatment.

**Patients and Methods**

**Study population and design**

The study was conducted on children suffering from ALL, who were diagnosed and treated at the Tata Memorial Hospital, Mumbai, India. The diagnosis of ALL was confirmed by examination of bone marrow aspirates. At diagnosis, cytochemistry (myeloperoxidase) and immuno-phenotyping was performed on all samples by flow cytometry using a panel of monoclonal antibodies which included CD2, 3, 4, 7, 8, 10, 19, 20, 36, 45, 13, 33, 34, HLA-DR and surface immunoglobulin (SIg). Common ALL was defined as CD10 positive with other B cell markers positive and CD3 negative. T-cell ALL was defined as CD3 positive with either CD5 or CD7 positive, with or without additional T-cell markers. Morphological study of peripheral blood smears was also carried out. Patients with L3 (according to French-American-British criteria) morphology and/or B-ALL (SIg positive) were excluded from the study.

All patients were treated uniformly on the institutional protocol for ALL (MCP 943). Patients received 2 induction cycles, repeat induction, consolidation and 6 maintenance cycles. This protocol did not include any cranial irradiation. Central nervous system (CNS) prophylaxis, however, included multiple triple intrathecal injections as well as high dose cytosine arabinoside infusions. The total duration of therapy was approximately two years.

Peripheral blood was collected at four time points...
(i) Phase A: at disease presentation, n = 69, (ii) phase B: after completion of induction chemotherapy and recovery of counts at the time of post-induction work-up (4 to 7 weeks after starting chemotherapy, n = 67), (iii) phase C: on completion of consolidation therapy; prior to starting of maintenance therapy (22 to 33 weeks after starting chemotherapy, n = 25), and (iv) phase D: on completion of maintenance therapy (100 to 110 weeks after starting chemotherapy, n = 25). Coded samples (n = 186) were sent to Indian Institute of Chemical Biology, Kolkata and decoded on completion of the study to ensure ‘blindness’ in the protocol. These studies received approval from the institutional review board.

Preparation of bovine submaxillary mucin (BSM) and desialylated bovine submaxillary mucin (Asialo-BSM)

BSM was prepared according to the earlier described method. Briefly, tissues were homogenized and extracted with water by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was collected, pH adjusted to 4.5 and the resulting precipitate removed by centrifugation at 5000 g for 20 min. The supernatant was neutralized (pH 6.0) and dialyzed against water. Barium acetate was slowly added to the dialyze to make it 0.1 M. Pre-cooled methanol was then slowly added to give an alcohol concentration of 64% (v/v) and incubated overnight at 4°C. The precipitate formed was retrieved by centrifugation, dissolved in 0.1 M ammonium hydroxide, pH adjusted to 4.5 and the resulting solution neutralized with 0.2 M sodium di-hydrogen phosphate, pH 11.0, followed by immediate neutralization with 0.2 M sodium di-hydrogen phosphate, pH 4.0 and extensively dialyzed against Tris-buffered saline (TBS). The precipitate was slowly added to the dialyzate to make it 0.1 M. Pre-cooled methanol was then slowly added to give an alcohol concentration of 64% (v/v) and incubated overnight at 4°C. The precipitate formed was retrieved by centrifugation, dissolved in 0.1 M EDTA, dialyzed extensively against water and stored at -20°C until use.

Protein content was measured by the method of Lowry using bovine serum albumin (BSA) as the standard. The percentage of 9-OAcSA derivatives present in BSM was quantified fluorimetrically and by fluorimetric high performance liquid chromatography (HPLC) as previously described. BSM was de-sialylated by incubation with 10% H3SO4 (0.05 M) for 1 hr at 80°C.

Detection of antibodies against 9-OAcSGs by an ELISA using BSM as the coating antigen (BSM-ELISA)

Microtitre 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 blocked with 2% BSA for 2 hr at 25°C. Sera, at a fixed dilution of 1:10 was incubated overnight at 4°C and its binding to BSM was colorimetrically measured using horseradish peroxidase (HRP) conjugated protein A (1:10,000; Zymed) and azinobis thiosulfonic acid (ABTS, Roche) as the substrate on an ELISA reader at 405 nm.

Quantitation of total anti 9-OAcSGs

Preparation of affinity matrix using purified BSM and asialo-BSM

Activation of Sepharose 4B (Pharmacia) was brought about as previously described using cyanogen bromide. Activated beads were allowed to couple separately with both BSM and asialo-BSM (5 mg/ml gel) in 0.1 M sodium bicarbonate, 0.5 M NaCl, pH 7.4.

Purification of anti 9-OAcSGs

Human sera (6.0 ml) from an ALL-patient, at presentation, was used to purify anti-9-OAcSGs fraction with affinity for 9-OAcSGs derivatives using the method of Siebert et al. and modified by Pal et al. Briefly, serum was subjected to a 33% ammonium sulfate fractionation, passed over an asialo BSM-Sepharose 4B column to remove any galactose binding fractions and the run through was loaded onto a BSM-Sepharose 4B column. The specific protein was eluted with 0.1 M ammonium hydroxide, pH 11.0, followed by immediate neutralization with 0.2 M sodium di-hydrogen phosphate, pH 4.0 and extensively dialyzed against Tris-buffered saline (TBS). To determine the proportion of immunoglobulin present in this fraction, it was passed through protein A-Sepharose 4B. Subsequently, protein content of the bound fraction was measured by the method of Lowry.

Generation of standard curve using purified anti 9-OAcSGs

Increasing amounts of purified anti 9-OAcSGs (0-1.0 µg/well, 20 µg/ml) were added to BSM-coated plates and binding was measured using HRP-conjugated protein A. This allowed us to generate a standard curve for quantitation of the total anti-9-OAcSGs present in sera of ALL patients at various stages of the disease.

Detection of immune-complexed 9-OAcSGs by Dot-blot

A Dot-blot was developed to detect immune-complexed 9-OAcSGs as previously described. Briefly, protein A-Sepharose beads (50 µl in TBS, pH 7.2, 1:1 v/v) (Sigma) were incubated with individual sera (40 µl) for 15 min at 25°C. The beads were washed thrice with TBS and an aliquot (5 µl) was
applied to nitrocellulose membranes (Bio-Rad). This was followed by addition of glycine-HCl buffer (0.1 M, 5 µl), pH 2.5 for 5 min at 25°C. After washing the membranes with TBS and blocking the non-specific binding sites with 2% BSA in TBS containing 0.03 M CaCl$_2$, the membranes were incubated for 2 hr with achatinin-H at 4°C. The binding of achatinin-H was a measure of the amount of shedded 9-OAcSGs present as an immune-complex in ALL serum. This was detected by incubating the membrane with rabbit anti-achatinin-H (diluted 1:100 in TBS) for 2 hr at 4°C. The antigen-antibody complex was colorimetrically detected using biotinylated goat anti-rabbit IgG (Cappel), followed by avidin-peroxidase (Pharmingen) and diamino-benzidine (Sigma) as the substrate. The developed Dot-blots were subsequently quantified in arbitrary units using Image Master Totallab Software, version 1.11 (Amersham Pharmacia Biotech).

Statistical analysis
The significance of differences in anti-9-OAcSG expressions between normal donors and patients at different time points of treatment was evaluated by Student’s ‘t’ test.

Results
Patients characteristics
Out of 186 coded samples, 69 were at presentation (53 males and 16 females giving an M: F ratio of 3.3: 1). The median age was 8 years with a range of 2 to 18 years. Of these, 55 (80%) had a precursor B phenotype and 14 (20%) were classified as T-ALL. These 69 patients were enrolled on the MCP 943 protocol.

Optimization of BSM-ELISA
Previous studies$^{3,4}$ from our group have demonstrated a significant increase in antibodies against 9-OAcSA, IgG of mainly IgG$_1$ and IgG$_2$ subtypes along with IgM. Attempts were taken to standardize the assay using peroxidase conjugated anti human IgG, IgG$_1$, IgG$_2$ and IgM. However, to ensure measurement of total 9-OAcSG specific immunoglobulins, in the present study, we used HRP-conjugated protein A as the detecting reagent. Once the assay conditions were established, we evaluated levels of anti-9-OAcSGs in the sera of 130 normal healthy individuals whose mean absorbance ± SD was 0.229 ± 0.057. To minimize the false positivity, the cut off value was selected as 0.4, based on the mean O.D. ± 3 SD obtained from normal controls.

Diagnosis of ALL
Amongst 186 coded samples at different stages of the disease, patients at presentation (n = 69) had an enhanced expression of bone marrow blasts ranging from 24 to 100% (Fig. 1). The peripheral blasts ranged from 1-98% and total WBC counts from 961 to 529000/ mm$^3$. We identified a concomitant increase in the expression of anti-9-OAcSGs in 63/69 cases that corresponded to a 3.8-fold increase in absorbances compared to controls (Fig. 1). All patients produced significant levels of anti-9-OacSGs, irrespective of the number of blasts present in the bone marrow or peripheral blood and their total WBC counts. This possibly suggests an early humoral response against these newly induced O-acetylated glycotopes and was corroborated by the poor correlation between the initial antibody titers with per cent blasts in the bone marrow ($r = -0.028$). Therefore, these disease-specific antibodies can be considered as potential biomarkers for childhood ALL, irrespective of their lineage. Assay specificity and sensitivity was 100% and 91.3% as false positivity and false negativity was 0/130 and 6/69 respectively. Patients having CML, AML, CLL, aplastic anemia, thalassemia and NHL
also showed minimal presence of anti-9-OAcSGs, further establishing the assay specificity to pediatric ALL (Fig. 2A). Thus, the assay holds promise as an alternative serum-based diagnostic tool in ALL.

Quantitation of disease-specific antibodies

For quantification of circulating anti-9-OAcSGs, binding of purified anti-9-OAcSGs ranging from 0 to 1.0 µg/ml was measured by the BSM-ELISA and a standard curve was generated. At presentation, the level of anti-9-OAcSGs was high, the mean ± SEM of total anti-9-OAcSGs being 102.8 ± 6.3 µg/ml (n = 69). Following 4-7 weeks of chemotherapy, circulating anti-9-OAcSGs significantly decreased to 65.7 ± 6.3 µg/ml (n = 67), which further decreased to 43.01 ± 3.2 µg/ml (n = 25) after six months of chemotherapy. After two years of treatment i.e., on completion of maintenance therapy disease specific antibody level was 33.79 ± 3.0 µg/ml (n = 25), much lower than the cut-off value of the test (Fig. 2B). In contrast, normal sera consistently showed minimal antibody concentrations (27.17 ± 2.0 µg/ml).

Detection of immune-complexed 9-OAcSGs

As only free anti-9-OAcSGs in circulation could be measured by the BSM-ELISA, the proportion that is immune-complexed remains undetected, possibly accounting for the observed 8.7% false negativity. Accordingly, the status of immune-complexed 9-OAcSG was evaluated by a Dot-blot using achatinin-H, a lectin that binds selectively to 9-O-acetylated sialic acids. Normal individuals showed consistently low antibody values in the BSM-ELISA (0.229 ± 0.019), which was reflected in their low densitometric score, mean ± SEM being 8079 ± 541. However, in the 6/69 ‘blast-positive’ yet ‘BSM-ELISA negative’ ALL patients, the mean ± SEM of the densitometric score increased to 15,521 ± 525, their OD_{405} nm remaining low at 0.34 ± 0.005. In the remaining 63/69 ALL patients, the densitometric score was 15,368 ± 439 concomitant with high absorbances in BSM-ELISA (0.909 ± 0.053) (Table 1).

Longitudinal monitoring

Patients (n = 25) were longitudinally monitored for two years and their anti-9-OAcSG levels measured. At presentation, the mean ± SEM of OD_{405} was 0.85 ± 0.06, which decreased slightly to 0.72 ± 0.09 after 4-7 weeks (phase B). After 22-33 weeks (phase C), the OD_{405} decreased significantly to 0.36 ± 0.03 (p<0.01) and this trend was retained after two years of treatment, OD_{405} being 0.28 ± 0.03 (p<0.01). Amongst 25 patients, who were closely monitored for two years, all of them showed normal or low levels of anti-9-OAcSGs as measured by the BSM-ELISA and were in clinical remission in phase D. Thus, the BSM-ELISA proved to be an economical, yet effective tool for monitoring the clinical status of ALL patients (Fig. 3A).

On closer examination of antibody levels at phases A and B, there appeared to be three distinct antibody distribution patterns (a) OD_{405} phase
A > phase B, n = 15, mean ± SEM being 0.88 ± 0.07 and 0.48 ± 0.06, respectively, (b) OD\textsubscript{405}, phase A < phase B, n = 4, mean ± SEM being 0.95 ± 0.06 and 1.61 ± 0.19, respectively, or (c) OD\textsubscript{405} phase A = phase B, n = 6, mean ± SEM being 0.72 ± 0.15 and 0.76 ± 0.15, respectively. Samples in phase B were collected 4-7 weeks after phase A and this variation in antibody profiles in phase B may be attributed to individual humoral response kinetics (Fig. 3B).

**Discussion**

Although childhood ALL is highly responsive to chemotherapy, patients in continual clinical remission may continue to harbor <5% blasts, resulting in disease persistence and resurgence. Detection of these residual blasts is defined as minimal residual disease (MRD), currently a subject of intense study\textsuperscript{5,32}. Modern treatment protocols of childhood ALL utilize the biological and clinical features of the disease to tailor the intensity of therapy to the risk of relapse by evaluation of MRD using several molecular techniques\textsuperscript{33}. The mandatory technical expertise required for presently available methods (FACS and PCR) for detection of MRD limits their widespread clinical acceptability, mainly in developing countries. An urgent need currently exists to identify cellular and biochemical markers, specific to lymphoblasts whose altered expression could be used for comprehensive and reliable monitoring of MRD in childhood ALL\textsuperscript{34,35}. To minimize the likelihood of false negative results, tandem application of at least two methods for detection of MRD is currently recommended.

The selective presence of 9-OAcSGs in α 2,6-linkage to sub terminal GalNAc have been demonstrated both on B and T-lymphoblasts\textsuperscript{1,2,7,9,10,24}. More importantly, disease-specific antibodies are also induced against these common glycotopes on the lymphoblasts. The present investigation indicates that anti-9-OAcSGs, induced in patients’ sera irrespective of their lineage, are an effective tool for diagnosis of both B- and T-ALL and accordingly may be considered as potential marker for monitoring all types of pediatric ALL. The utility of such purified antibodies in diagnosis of childhood ALL may be envisaged for detection of cell surface 9-OAcSGs by flow cytometric analysis.

Considering the enormous cost of existing protocols for monitoring these children, there is a certain degree of reluctance amongst parents in developing countries to cooperate with long-term monitoring especially as the child appears apparently well. In such a socio-economic scenario, we envisage that application of these simple ELISA-based methods using only a few micro-liters of blood will definitely improve compliance, thus minimizing the occurrence of late relapses.

In the ‘BSM-ELISA negative’ group (6/69, Table 1), we propose that non-availability of antibodies for binding with glycotopes of BSM could be due to their being immune-complexed with free-
shedded antigen, a common phenomenon in tumor biology. This was validated by the Dot-blot that indicated the presence of immune-complexed 9-OAcSGs. We, therefore, contend that the ‘BSM-ELISA negative, Dot-blot positive’ phenotype be considered as positive for ALL. An increased presence of disease-specific immune-complexed antigen in circulation has been reported in leukemic patients that may be attributed to defective phagocytic activity. In this situation, these cells are unable to keep pace with the increased rate of immune-complex formation. This may account for the ~2-fold increase in densitometric scoring, as compared to normal individuals at the initial phase of diagnosis. Accordingly, monitoring such immune-complexes may provide valuable information regarding the disease status of these patients.

During two years of chemotherapeutic regimen, 57/69 presentation patients showed the pattern of continual clinical remission that corroborated with low antibody titer in sera. However, 12 patients came to the clinic with relapse, mostly during and after phase D; unfortunately, these patients were not included in this study. However, it is worthwhile to point out that patients who relapsed clinically, showed enhanced level of antibody, suggesting the prognostic potential of these disease-specific antibodies. However, extensive studies in this regard with strategically placed investigative time-points are needed; studies are currently underway.

Methods for detection of MRD are currently evolving and there is still no clear-cut consistency with regard to the detection method and source of sample for analysis i.e. bone marrow or peripheral blood for proper assessment of the disease status. The detection of enhanced expression of antibody levels against 9-OAcSGs from only a few microliters of serum using the BSM-ELISA, represents a convenient tool for potential evaluation of the chemotherapeutic response of ALL patients, irrespective of lineage and chemotherapeutic regimen.

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