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BIO-TECHNICAL METHODS SECTION (BTS)

BTS Leukemia

Identification of 9-O acetyl sialoglycoconjugates (9-OAcSGs) as biomarkers in childhood acute lymphoblastic leukemia using a lectin, Achatinin$_H$, as a probe

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Neoplastic transformation causes changes in cell surface architecture, most notably, aberrant sialylation. Exploiting the restricted specificity of a 9-O acetyl sialic acid (9-OAcSA) binding lectin, Achatinin$_H$ (ATN$_H$), we have identified two 9-O acetyl sialoglyconjugates (9-OAcSGs) on lymphoblasts of 87 children suffering from acute lymphoblastic leukemia (ALL). The preferential binding of ATN$_H$ to lymphoblasts induces their 11-fold increased agglutination (81 ± 7.8%) compared to peripheral blood mononuclear cells (PBMC) of normal donors (8 ± 4.3%) which corroborates with flow cytometry studies. Agglutination of MOLT-4 (87 ± 4.8%), a lymphoblastoid cell line and MDCK (91.25 ± 0.01%), a cell line expressing surface 9-OAcSA, confirms the preferential binding of ATN$_H$ to lymphoblasts through their surface 9-OAcSGs. Furthermore, fluorometric quantitation reveals a 4.6-fold increase in % of 9-OAcSA on lymphoblasts of ALL patients (42.1 ± 4.1%) compared to normal donors (9.2. ± 3.4%). Western blotting confirms that ATN$_H$ recognizes two membrane sialoglycoconjugates, of MW 120 kDa and 90 kDa, both having 9-OAcSA $\alpha 2 \rightarrow 6$ GalNAc terminal sugar moiety as their lectinogenic epitope. We propose that these 9-OAcSGs may serve as biomarkers for detection and monitoring of lymphoblasts in ALL and accordingly merit therapeutic considerations.

Keywords: 9-O acetyl sialic acids binding lectin (Achatinin$_H$); 9-O acetylated sialoglycoconjugates; biomarkers; ALL

Introduction

Neoplastic transformation is often associated with a variety of structural changes on the cell surface, most notably, changes in agglutinin receptor sites favoring cell agglutination by lectins[1,2] and increased sialic acid densities.[1] Sialic acids (SA), originally abbreviated as Neu5Ac, are a family of 9-carbon carboxylated monosaccharides often found as terminal residues of vertebrate oligosaccharides.[3] They are modified in various ways giving rise to a family of more than 30 different structures which in turn can be presented in a variety of linkages to the underlying sugar chains.[4,5] These modifications significantly effect the physicochemical properties of parent molecule (Neu5Ac/SA) and can thereby modify and/or create new biological functions.[6,7] In mammals, one of the most common modification of sialic acids is the addition of O-acetyl esters to hydroxyl groups at C_4, C_7, C_8 and C_9 positions. The O-acetylation of sialic acids influences enzymatic reactions in the catabolism of glycoconjugates,[7] effects recognition of sialic acids by viral hemagglutinins and bacterial sialidases,[4,5] may effect tissue morphogenesis during development[8] and can modulate the alternative pathway of complement activation.[9]

Lectins or lectin-like proteins endowed with the ability to bind sialoglycoconjugates have been used as recognition molecules to predict changes in the pattern of sialylation and the degree of O-acetylation during malignant transformation.[10–13] Binding studies with *Sambuccus nigra* agglutinin (SNA) state *de novo* expression of specific sialic acids on selected glycoproteins in colon carcinoma.[14] Employing *Ricinus cummunis* agglutinin, the granulocytes of patients with chronic myelogenous leukemia (CML) are reported to contain more sialylated glycopeptides than normal granulocytes.[15,16] Lymphoblasts of children with acute lymphoblastic leukemia (ALL) are also reported to be electrophoretically distinct from normal lymphocytes due to their increased concentration of sialic acids which causes charge differences.[17] A lectin, *Cancer antennarius*, which recognizes sialic acids O-acetylated both at C_4 and C_9 positions, has been used to identify an O-acetyl ganglioside, GD_3, as a biomarker in human melanoma cells.[18] Although the major sialic acid in human is Neu5Ac, 9-O acetylated sialic acids (9-OAcSA) have been detected at a low level in human saliva, colonic mucins, serum and B lymphocytes[19] and are markedly elevated in melanomas[18] and ALL.[20,21] However, the detailed biochemical characterization and biological significance of 9-OAcSA on lymphoblasts of ALL patients remains obscure.

Childhood ALL, a clonal lymphoproliferative disorder, is characterized by marked overproduction of lymphoblasts[22,23] morphologically different from mature functional lymphocytes.[24] Analysis of cell surface structures specific to lymphoblasts of ALL patients may provide biomarkers useful for its diagnosis and prognosis. In a pilot study, exploiting the selective affinity of a lectin, Achatinin$_H$ (ATN$_H$) towards 9-OAcSA,[12,25–30] we have detected subtle changes on the surface of erythrocytes[20] and lymphocytes[21] in ALL patients. Employing ATN$_H$ as an analytical probe, the present study reports (1) the identification of specific biomarkers, ie 9-O acetyl sialoglycoconjugates (9-OAcSGs) on the surface of peripheral blood mononuclear cells (PBMC) of ALL patients by leukoagglutination, flow cytometry and Western blotting; (2) their quantitation; (3) determination of their molecular weight (MW); and (4) identifying their terminal oligosaccharide structure which serves as the binding epitope for ATN$_H$.

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Materials and methods

Study subjects

Blood samples from 87 patients of clinically confirmed ALL, belonging to type L_1 or L_2 (FAB classification),[24] as per the light microscopical appearance of bone marrow smears, were taken. The patients were between 2.5–11 years age, of either sex and having a leukocyte count below 7×10^{10} cells/l. Their salient presenting features were progressive pallor, anemia with occasional hemorrhagic spots, fever and bone pain.

Controls were normal healthy donors ($n = 30$) of either sex and different blood groups. Patients diagnosed for other hematological disorders ($n = 26$), ie CML ($n = 7$), non-Hodgkin's lymphoma (NHL, $n = 4$), acute myelogenous leukemia (AML, $n = 5$), thalassemia ($n = 5$) and aplastic anemia ($n = 5$) were also included in the study.

Peripheral blood (2–3 ml) was collected at Vivekananda Institute of Medical Science, Calcutta and coded samples sent to Indian Institute of Chemical Biology, Calcutta. The results were compared only on completion of assays to ensure 'blindness' in the protocol. Informed consent was taken from patients and the Human Ethical Clearance Committee as per the protocol of Indian Council of Medical Research.

Cell lines, probes and reagents

A lymphoblastoid cell line, MOLT-4 (ATCC No. 1582), derived from an ALL patient and MDCK (ATCC No. CCL 34), a cell line expressing surface 9-OAcSA,[31] were included in the study.

The lectin, ATN_H, with preferential affinity for sialoglycoconjugates having terminal 9-OAcSA in $\alpha 2 \rightarrow 6$ linkage to the underlying structure,[25–30] was purified from *Achatina fulica* snail.[12] Influenza C hemagglutinin esterase fusion protein (CHE-Fc) which recognizes sialoglycoconjugates with terminal 9-OAcSA, irrespective of their underlying structure,[32,33] was a generous gift from Prof A Varki (Division of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, USA).

Di-isopropyl flurophosphate (DFP) was obtained from Aldrich (1 M) and stored in isopropyl alcohol at –20°C. All other chemicals and biological reagents were from Sigma (St Louis, MO, USA), unless otherwise stated.

Leukoagglutination assay[34]

PBMC were separated by Ficoll-Hypaque density centrifugation[35] and suspended in RPMI 1640, supplemented with 2 mM glutamine, gentamycin and 10% heat-inactivated human AB serum (medium A). PBMC (1×10^5 cells/100 μl of medium A) of patients with ALL ($n = 87$), CML ($n = 7$), NHL ($n = 4$), AML ($n = 5$), thalassemia ($n = 5$) and aplastic anemia ($n = 5$) and normal donors ($n = 30$) were incubated with ATN_H (2 μg) for 1 h at 37°C. PBMC of patients with no ATN_H served as cell control. The % of leukoagglutination was calculated as follows:

$$\text{Leukoagglutination \% in a field} = \frac{\text{Agglutinated cells}}{\text{Total cells}} \times 100.$$

In parallel, to confirm the preferential binding of ATN_H to leukemic blasts was through 9-OAcSA, agglutination of MOLT-4 and MDCK cell lines was also determined.

Quantitation of 9-OAcSA

The % of 9-OAcSA on PBMC surface of ALL patients ($n = 87$)/normal donors ($n = 30$) and MOLT-4 and MDCK cell lines was determined as described by Shukla and Schauer.[36,37] Briefly, PBMC (1×10^6 cells/100 μl) were washed and suspended in buffer A (sodium chloride (3 g), potassium chloride (0.2 g), disodium hydrogen phosphate dihydrate (1 g), sodium dihydrogen phosphate monohydrate (0.15 g), potassium dihydrogen phosphate (0.2 g) per liter, pH 7.2). In one series, PBMC were hydrolyzed to saponify O-acetyl groups. In other series no de-O-acetylation was performed.

All samples were oxidized with sodium metaperiodate (200 μl, 2.5 mM in buffer A) at 4°C for 15 min in the dark. After centrifuging at 12000 *g* for 5 min, supernatant (400 μl) was mixed with sodium arsenite (100 μl, 2% in 0.5 M HCl) and 300 μl acetylacetone solution (0.075 M glacial acetic acid, 0.05 ml of acetylacetone and 3.75 g of ammonium acetate in 25 ml double-distilled water). After incubating at 60°C for 10 min samples (0.8 ml) were diluted to a final volume of 2.5 ml with double-distilled water. The relative fluorescence intensity ($\lambda_{max(exitation=410nm)}$, $\lambda_{max(emmision=510nm)}$) of each sample was measured against the reagent blanks. The % of 9-OAcSA was determined on a Hitachi F-4010 spectrofluorimeter (Hitachi, Tokyo, Japan) by subtracting the relative unsubstituted sialic acids from that obtained after de-0-acetylation.

Flow cytometry studies

ATN_H was conjugated with fluorescein isothiocyanate (FITC) as described by Coligan *et al*.[38] Cells (1×10^6 cells/100 μl) were suspended in medium A and their non-specific binding sites blocked for 30 min with 10% normal goat serum in the same medium. Following two washes, the cells were labelled with FITC-ATN_H (2 μg) at 0°C for 1 h. Cells labelled with FITC conjugated lipopolysaccharide (FITC-LPS), diluted to the same protein concentration as ATN_H, served as a negative control.[39] The cells were washed, suspended in phosphate buffered saline (PBS, pH 7.2) and sorted using a FACS Calibur flow cytometer.

To determine the percentage of cells expressing common acute lymphoblastic leukemia antigen (CALLA), cells were incubated with anti-CALLA monoclonal antibody (mAb), of IgM class (2.5 μg/50 μl, Boehringer Mannheim, Mannheim, Germany), at 0°C for 1 h. A mouse IgM, diluted to the same protein concentration as anti-CALLA mAb, served as a negative control. Following three washes with medium A, the cells were labelled with 1:40 dilution of FITC-conjugated anti-mouse IgM at 0°C for 30 min. Cells were washed and sorted as previously described.

Double color flow cytometry studies

The binding of ATN_H to surface 9-OAcSGs and not CALLA antigen was confirmed by flow cytometry studies employing two different fluorochromes for ATN_H and anti-CALLA mAb. Initially, PBMC of ALL patients were incubated with anti-CALLA mAb (2.5 μg/1×10^6 cells) for 1 h at 0°C. Following three washes in medium A, the binding of anti-CALLA mAb was detected by labeling the cells with 1:40 dilution of phycoerythrin (PE) conjugated goat anti-mouse IgM (Becton Dickinson, San José, CA, USA) for 30 min at 0°C. Subsequently, the

cells were incubated with FITC–ATN_H (2 μg) for 1 h at 0°C, washed and sorted, as previously described.

In parallel, PBMC incubated with mouse IgM (concentration matched with anti-CALLA mAb), followed by PE-conjugated goat anti-mouse IgM (1:40 dilution) and finally FITC-LPS (diluted to the same concentration as FITC-ATN_H) served as the corresponding negative control.

SDS PAGE and Western blot analysis

For lectin overlay experiments, PBMC membranes were prepared from ALL patients and normal donors according to Weissman *et al*.[40] The purity of membrane fractions was confirmed by measuring 5′ nucleotidase activity[41] and their protein concentration determined using bovine serum albumin (BSA) as the standard.[42]

The membrane fractions (30 μg) were reduced by boiling with β-mercaptoethanol for 10 min, electrophoresced on a 7.5% SDS-PAGE at 60 V for 1.5 h (Bio-Rad Minigel apparatus; Bio-Rad, Hercules, CA, USA) and transblotted on a nitrocellulose paper at 100 V for 2 h. The membranes were blocked overnight at 4°C in Tris-buffered saline (0.05 M TBS, pH 7.4), containing 10% desialylated BSA (ds-BSA) and probed with ^{125}I-ATN_H (10^6 c.p.m./ml in TBS-dsBSA containing 0.03 M calcium chloride) for 1 h at 25°C. After extensive washing with TBS having 0.2% noniodate 40 (NP40) and 0.2% Tween 20, the paper was dried and loaded on to X-ray film for autoradiography. For de-O-acetylation of membrane fractions, the blots were incubated with 0.1 N sodium hydroxide (NaOH) for 45 min at 4°C, neutralized by extensive washing with TBS and similarly processed.

Membrane sialoglycoproteins with terminal 9-OAcSA were also detected using CHE-Fc as a probe. CHE-Fc is a soluble and versatile chimeric protein that retains both the hemagglutinin and esterase activity of influenza C virus as well as those of the Fc portion of human IgG_1.[5,31–33] At ambient temperature and neutral pH, the esterase activity is dominant and it cleaves of the 9-O-acetyl groups of sialic acid. The use of CHE-FcD as a probe to detect sialoglycoconjugates having terminal 9-OAcSA has been validated by several groups.[5,31–33] CHE-Fc (10 μg/ml) was treated with 1 mM DFP to irreversibly block the esterase activity and stabilize the hemagglutinin activity to give a probe (CHE-FcD) that specifically detects sialoglycoproteins with terminal 9-OAcSA at ambient temperature.[33] Sialoglycoproteins reacting with CHE-FcD were colorimetrically detected with goat anti-human IgG antibody-conjugated horse-radish peroxidase.

Results

Characterization of ATN_H

Hemagglutination inhibition assays using mono-, di- and trisaccharides containing 9-OAcSA and various sialoglycoproteins, mainly bovine submaxillary mucin (BSM) having 20% 9-OAcSA, have confirmed that ATN_H selectively binds to sialoglycoconjugates having terminal 9-OAcSA in $\alpha 2 \rightarrow 6$ linkage to a subterminal GalNAc of the underlying structure (Table 1). Sialoglycoproteins, ie sheep submaxillary mucin (SSM), having only sialic acid, is 350-fold less inhibitory to ATN_H in comparison to BSM.[25,28–30] The preferential affinity of this lectin towards 9-OAcSA ($K_a = 1.20 \times 10^3 \pm 0.07 \times 10^3\ M^{-1}$) as compared to other O-acetylated derivatives has been confirmed by physicochemical techniques.[26,27]

Table 1 Binding specificity of ATN_H by hemagglutination inhibition assay

Saccharides/ Sialoglycoproteins	*Types/Nature of terminal linkages*	*I_{50}*
(1) Monosaccharides	Neu 5 Ac	30.48
	Neu 5,9 Ac_2	1.30
	Neu 4,5 Ac_2	NI[a]
(2) Di/tri-saccharides	α-Neu 5,9 Ac_2-(2 → 6)-GalNAc-ol	0.41
	α-Neu 5 Ac-(2 → 3)-βGal-(1 → 3)-GalNAc-ol	25.00
(3) Sialoglycoproteins		
BSM	α-Neu5,9 Ac_2-(2 → 6)-β-DGalNAc-	0.0002
SSM	α-Neu5Ac-(2 → 6)-β-DGalNAc-	NI[b]

Data collected from Refs 25–30.
[a]NI, not inhibited up to a concentration of 100 mM.
[b]NI, 350-fold less inhibitory than BSM on the basis of 9-OAcSA.
I_{50}, the minimal concentration of the monosaccharide required for 50% inhibition of 16 hemagglutination units of $ATNo_H$; BSM, bovine submaxillary mucin; SSM, sheep submaxillary mucin.

ATN_H preferentially binds to and agglutinates leukemic blasts

PBMC of ALL patients showed an 11-fold increased agglutination (81 ± 7.8%), in response to ATN_H (Table 2), as compared to normal donors (8 ± 4.3%). No inherent leukoagglutination was observed for ALL patients in the absence of ATN_H. The patients of other hematological disorders showed a distinctly low, ie NHL (2.35 ± 0.4) thalassemia (2.82 ± 0.8) or no leukoagglutination, ie CML, AML and aplastic anemia, in response to ATN_H. The observation that ATN_H agglutinates the cells through binding to surface 9-OAcSA is evident from a 91.25 ± 0.01% agglutination of MDCK cell line. An 87 ± 4.8% agglutination of MOLT-4 cell line reflects the preferential binding of ATN_H to leukemic blasts. Furthermore, the pattern

Table 2 Leukoagglutination in response to ATN_H

Types of cell	*Leukoagglutination titer*							
	Number of cells agglutinated							*Agglutination (%)*
	2	*3*	*4*	*5*	*6*	*9*	*16 or more*	*(X ± s.d.%)*
ALL Patient	18	9	14	9	6	3	8	81.0 ± 7.8
MOLT-4	13	3	—	3	4	7	10	87.0 ± 4.8
MDCK	5	4	6	8	1	9	24	91.25 ± 0.01
Normal donor	4	1	—	—	—	—	—	8.0 ± 4.3
Thalassemia	2	1	—	—	—	—	—	2.82 ± 0.8
NHL	—	3	—	—	—	—	—	2.35 ± 0.4
CML	—	—	—	—	—	—	—	—
AML	—	—	—	—	—	—	—	—
Aplastic anemia	—	—	—	—	—	—	—	—

Cells (1×10^5 cells in medium A) were incubated with ATN_H (2 μg) at 37°C for 1 h. The % of agglutinated cells was determined under phase contrast microscope as described in Materials and methods.
—, Denotes no agglutination.

of agglutination is different in that the total number of cells per clump is distinctly higher in ALL patients and MOLT-4 and MDCK cell lines, as compared to normal donors, where clumps of maximum two or three cells are observed.

Leukemic blasts show an increased percentage of surface 9-OAcSA

The increase in % of 9-OAcSA on PBMC surface of ALL patients, MOLT-4 and MDCK cell lines in comparison to normal donors was confirmed by its fluorimetric quantitation (Figure 1). The mean ($X \pm$ s.d.%) of % 9-OAcSA in ALL patients was 4.6 folds higher ($42.1 \pm 4.1\%$) than normal donors ($9.2 \pm 3.4\%$). The % 9-OAcSA of $49.2 \pm 0.01\%$ for MOLT-4 and $71.6 \pm 0.01\%$ for MDCK cell lines reasons for their higher agglutination in the presence of ATN_H.

Flow cytometric analysis demonstrates the preferential binding of ATN_H to leukemic blasts

Figure 2a gives a representative profile of the flow cytometry studies of an individual ALL patient having 90% leukemic blasts (morphological estimates) as also corroborated by their binding to anti-CALLA mAb (92.80%, Figure 2b, panel B). In comparison to normal donor (9.15%, Figure 2a, panel B), 93.81% of patient PBMC (Figure 2a, panel B) showed a positive binding to FITC-ATN_H. A 10-fold increased binding of FITC-ATN_H to MOLT-4 cell line (98%, Figure 2a, panel B) as compared to normal donors (9.15%, Figure 2a, panel B) reflects its preferential affinity for leukemic blasts. FITC-LPS having a significantly low percentage binding to PBMC of normal donors (2.58%, Figure 2a, panel A), ALL patients (0.58%, Figure 2a, panel A), and MOLT-4 cell line (0.54%, Figure 2a, panel A) served as a negative control.

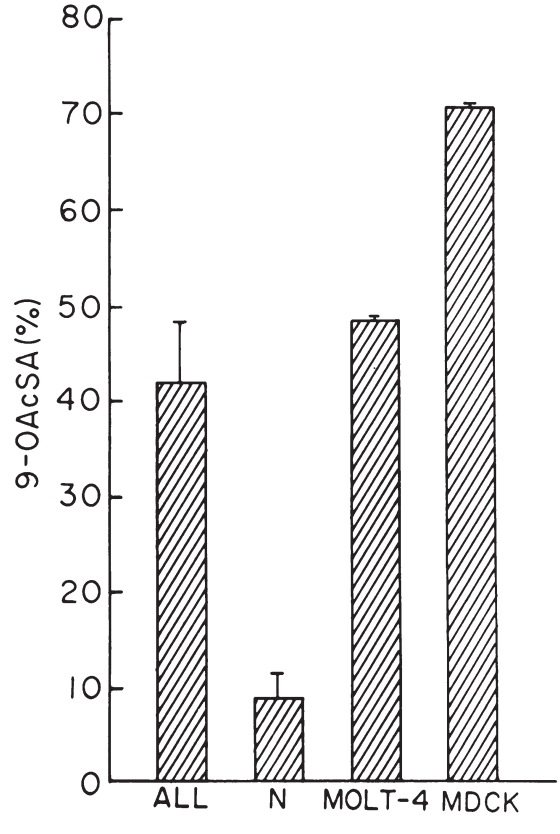

Figure 1 Fluorometric quantitation of % 9-OAcSA. PBMC of ALL patients and normal donors (N) and the two cell lines, namely, MOLT-4 and MDCK were oxidized by sodium metaperiodate and processed as per the method of Shukla and Schauer,.[36,37] The % 9-OAcSA ($X \pm$ s.d.%) was determined by subtracting the relative unsubstituted sialic acids from that obtained after de-O-acetylation.

ATN_H recognizes 9-OAcSGs and not CALLA antigen on the surface of leukemic blasts

Figure 3 gives a representative profile of double-color flow cytometry studies to identify both CALLA antigen and 9-OAcSGs on leukemic blasts of an ALL patient. Of the total cell population, 78.27% were $CALLA^+9\text{-}OAcSGs^+$ in comparison to a very low percentage of $CALLA^+9\text{-}OAcSGs^-$ (1.02%) and $CALLA^-9\text{-}OAcSGs^+$ cells (2.20%, Figure 3a). The data

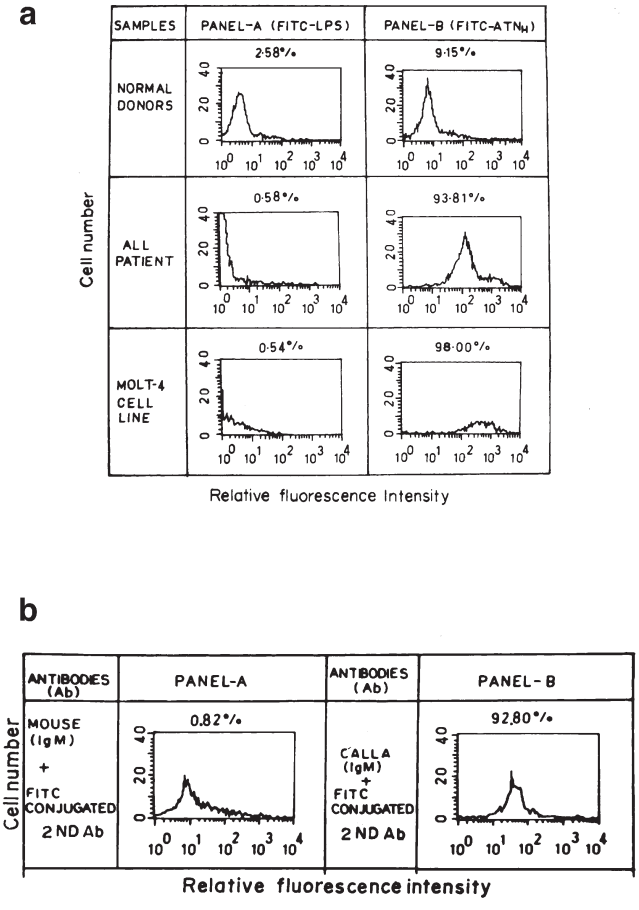

Figure 2 (a) A representative profile of flow cytometric analysis to determine the percentage binding of PBMC of ALL patients, normal donors and MOLT-4 cell line to FITC-ATN_H. Cells (2×10^6) were incubated with FITC-ATN_H and processed for flow cytometry as described in Materials and methods. Panels A and B indicate binding of the cells to FITC-LPS (negative control) and FITC-ATN_H. Numbers represent the percentage of fluorescent positive cells. (b) A representative profile of percentage binding of PBMC of ALL patient to anti-CALLA mAb by flow cytometry studies. Cells (2×10^6) were incubated with anti-CALLA mAb and processed for flow cytometry as described in Materials and methods. Panel B represents treatment with anti-CALLA mAb while panel A represents the corresponding negative control. Numbers represent the percentage of fluorescent-positive cells.

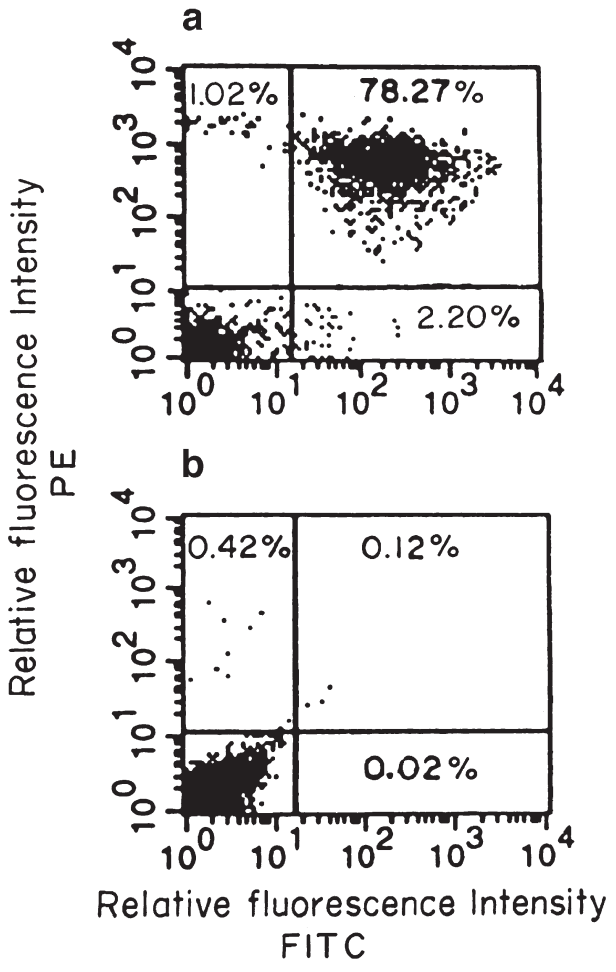

Figure 3 Double-color fluorescence dot plot to demonstrate that ATN_H binds to a receptor/epitope different from CALLA antigen. (a) The PBMC incubated with anti-CALLA mAb and FITC-ATN_H and processed for flow cytometry as described in Materials and methods. (b) The corresponding negative control. Numbers state the percentage of fluorescent positive cells.

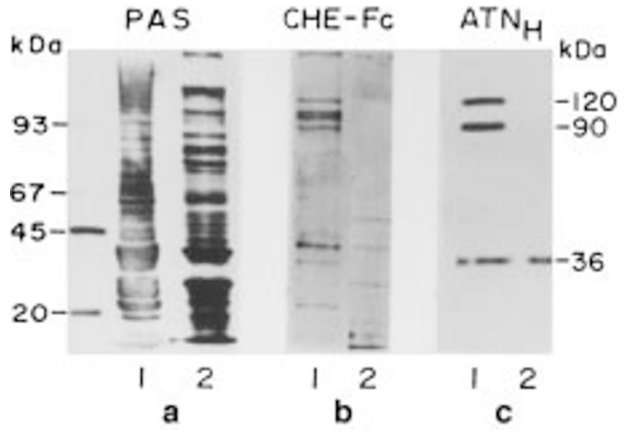

Figure 4 Western blot analysis of membrane 9-OAcSGs. PBMC membrane fractions (25 μg) of a representative ALL patient (lane 1) and normal donor (lane 2) were electrophoresced on a 7.5% SDS-PAGE and stained with (a) periodic acid Schiff's base. SDS-PAGE separated membrane glycoproteins were transferred to nitrocellulose paper and stained with (b) CHE-FcD and (c) ^{125}I-ATN_H.

Table 3 Molecular weight of the 9-OAcSGs of PBMC membranes reacting with CHE-FcD and ^{125}I-ATN_H

PBMC membrane	*Probes*	*No. of bands*	*MW (kDa) of the glycoproteins reacted*
(1) ALL	CHE-FcD	7	120, 101, 90, 34, 36, 31, 25
	^{125}I-ATN_H	3	120, 90, 36
(2) Normal donor	CHE-FcD	3	40, 36, 29
	^{125}I-ATN_H	1	36

therefore reflect the binding of both ATN_H and anti-CALLA mAb to leukemic blasts but through two different receptors, namely, 9-OAcSGs and CALLA antigen. In parallel, PBMC incubated with concentration matched mouse IgM and FITC-LPS, served as the corresponding negative control (Figure 3b).

Detection of sialoglycoconjugates with terminal 9-OAcSA

A representative profile of PBMC membrane glycoproteins, both from ALL patients (lane 1) and normal donors (lane 2) as observed by periodic acid Schiff's base staining is shown in Figure 4a. Amongst the membrane glycoproteins, sialoglycoproteins with terminal 9-OAcSA (9-OAcSGs) were directly detected by immunoblotting employing CHE-FcD as a probe (Figure 4b). Blots of PBMC membrane proteins showed selective expression of 9-OAcSA on specific membrane glycoproteins of MW 120 kDa, 101 kDa, 90 kDa, 34 kDa, 36 kDa, 31 kDa and 25 kDa in ALL patient (Figure 4b, lane 1, Table 3) as compared to 40 kDa, 36 kDa and 29 kDa in normal donors (Figure 4b, lane 2, Table 3). The staining was completely abolished after alkali treatment (data not shown) owing to de-O-acetylation of 9-OAcSGs.

ATN_H recognizes two specific 9-OAcSGs on leukemic blasts

Of the 9-OAcSGs being recognized by CHE-FcD, only two sialoglycoproteins of MW 120 kDa and 90 kDa, in ALL patients (Figure 4c, lane 1), showed strong reactivity with ^{125}-I-ATN_H in addition to a 9-OAcSG (MW 36 kDa), common to ALL patients and normal donors (Figure 4c, lane 2, Table 3). After de-O-acetylation, these bands disappeared (data not shown) confirming that they are O-acetylated sialoglycoproteins presumably containing terminal 9-OAcSAα2 $\rightarrow$ 6GalNAc which serves as the binding epitope for ATN_H.

Discussion

Interest in the use of biomarkers to evaluate future disease risk has increased greatly in recent years since they are observable end-points in a continuum of events leading from exposure to toxic agents to diseases that ultimately result from exposure. Employing the observation that cytokines in general induce tyrosine phosphorylation in lymphoid progenitor cells and constitutive tyrosine phosphorylation is also observed in B-lineage ALL, attempts have been made to block the growth of leukemic blasts by using blockers against protein tyrosine kinases.[43] Alteration in the pattern of DNA methylation have

been recently reported as a biomarker in various neoplasms, including ALL.[44] Changes in the sialylation pattern of cell surface structures during malignancies are also reported.[1,10–14,17,18] The present study identifies two biomarkers, 9-OAcSGs, appearing exclusively on leukemic blasts of ALL patients employing ATN_H as an analytical probe.

ATN_H selectively binds to leukemic blasts (Figure 2a) inducing their 11-fold increased agglutination as compared to PBMC of normal donors (Table 2). Since ATN_H is a 9-OAcSA binding lectin (Table 1), leukoagglutination and flow cytometry studies indirectly reflect either (1) an increase in % of leukemic blasts, expressing 9-OAcSGs; and/or (2) an increased 9-O-acetylation of surface sialoglycoconjugates on leukemic blasts. These results are further supported by fluorimetric quantitation of surface 9-OAcSA which shows a 4.6-fold increase in ALL patients as compared to normal donors (Figure 1). This observation is consistent with the previous reports which state that although 9-OAcSA are present at a very low concentration in normal human serum and B lymphocytes[19] their level is markedly elevated in melanomas[18] and ALL.[20,21] The preferential binding of ATN_H to leukemic blasts through 9-OAcSGs is further envisaged by an 87% and 91% agglutination of MOLT-4 and MDCK cell lines (Table 2), both showing a five- and eight-fold increase in the % of surface 9-OAcSA compared to normal donors (Figure 1).

Flow cytometry studies of a representative ALL sample state that the % of FITC-ATN_H positive cells (93.81%, Figure 2a) are comparable to the cells expressing CALLA antigen (92%, Figure 2a) as also are the % of leukemic blasts (90%) as per morphological estimates. Comparison of these three observations reiterates the selective binding of ATN_H to leukemic blasts of ALL patients having an increased surface 9-OAcSGs (Figure 1). Furthermore, double-color flow cytometry studies, employing different fluorochromes for ATN_H and anti-CALLA mAb (Figure 3) reflect that they recognize two different receptors, ie 9-OAcSGs and CALLA antigen, on the surface of leukemic blasts. It is of interest that ATN_H binds to leukemic blasts of patient with B cell ALL (Figure 2a, panel 2B) and also MOLT-4 (Figure 2a, panel 3B), a T lymphoblastoid cell line. Therefore, possibly the 9-OAcSGs, are common markers expressed on leukemic blasts of ALL patients irrespective of their B/T lineage. Furthermore, the study extended to patients of other hematological disorders demonstrates no cross-reactivity (Table 2) reiterating that 9-OAcSGs recognized by ATN_H are present exclusively on leukemic blasts of ALL patients.

Western blotting of membrane proteins with ^{125}I-ATN_H confirms the presence of two unique surface 9-OAcSGs (MW 120 kDa and 90 kDa) exclusively present in ALL patients (Figure 4c, Table 3). After de-O-acetylation, these bands disappear indicating that these are O-acetylated sialoglycoproteins. However, in addition many other bands appear on immunoblotting with CHE-FcD (Figure 4b, Table 3). This can be explained by the previous report which states that unlike ATN_H which binds to sialoglycoconjugates with terminal 9-OAcSA in $\alpha 2 \rightarrow 6$ linkage to a subterminal sugar, GalNAc,[25–30] the CHE-FcD binds to any sialoglycoconjugate having terminal 9-OAcSA irrespective of underlying structure.[31–33] Possibly, other 9-OAcSGs reacting with CHE-FcD but not ^{125}I-ATN_H represent either (1) sialoglycoproteins with terminal 9-OAcSA linked to the underlying structure by a linkage different from $\alpha 2 \rightarrow 6$, or (2) their binding epitopes are not in proper conformation required for recognition by ATN_H.

Therefore, both 120 kDa and 90 kDa 9-OAcSGs, in ALL patients, binding to ^{125}I-ATN_H and CHE-FcD are O-acetylated glycoproteins containing a terminal 9-OAcSA. Considering the binding specificity of ATN_H (Table 1), we infer that the lectinogenic epitope of these 9-OAcSGs is presumably 9-OAcSA $\alpha 2 \rightarrow 6$ GalNAc.

The relationship between 9-O-acetylation of sialic acids during neoplastic transformation and abnormalities seen in the immune system remains poorly defined. However, 9-O-acetylation has been reported to abrogate the normal functions of sialic acids in preventing the activation of alternative complement pathway due to involvement of the exocyclic side chain of sialic acid.[45] We have reported earlier that the exocyclic side chains of sialic acid molecules are also important determinants for ATN_H binding.[25,27–29] Certain viruses, ie influenza and corona viruses have been reported to specifically bind to 9-OAcSAs to mediate their effects.[5,31–33] In contrast, removal of 9-OAcSAs has been reported to enhance the binding and invasion of malarial parasites in murine erythrocytes.[46]

Sjoberg *et al*[47] have reported that 9-O-acetylation of sialic acids in melanomas can mask the binding site of adhesion molecules like CD22β which recognizes $\alpha\ 2 \rightarrow 6$ linkages similar to lectinogenic epitope of ATN_H.[25,28,29] This observation is further corroborated as increased binding of CD22β occurs following removal of 9-O-acetylated moiety by a specific enzyme, 9-O-acetyl esterase. We envisage that increased expression of 9-OAcSGs could mask binding sites of adhesion molecule(s) like CD22β, thereby influencing cell adhesion-dependent cellular responses namely cell proliferation and differentiation, immune responses and metastases. Based on these observations, it is reasonable to hypothesize that the increased 9-O-acetylation in ALL could modify host defense functions which may serve as an effective strategy of leukemic blasts to circumvent immune surveillance.

To the best of our knowledge, this is the first report which identifies and partially characterizes two surface 9-OAcSGs as biomarkers in ALL patients. The newly identified biomarkers may help in the development of new therapeutic strategies in the field of ALL research. Studies on the variable expression of these 9-OAcSGs in ALL patients under different phases of treatment have also been recently communicated.[48]

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