Glycobiology vol. 14 no. 10 pp. 859–870, 2004
doi:10.1093/glycob/cwh111
Advance Access publication on June 9, 2004

Purification and characterization of 9-*O*-acetylated sialoglycoproteins from leukemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia

Santanu Pal[1,2,5], Shyamasree Ghosh[1,5], Chhabinath Mandal[6], Guido Kohla[7], Reinhard Brossmer[8], Rainer Isecke[3,8], Anette Merling[9], Roland Schauer[7], Reinhard Schwartz-Albiez[9], Dilip K. Bhattacharya[10], and Chitra Mandal[4,5]

[5]Immunobiology Division, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700032, India; [6]Drug Design Development and Molecular Modelling, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700032, India; [7]Biochemisches Institut, Christian-Albrechts-Universität zu Kiel, Olshausenstr. 40, D-24098 Kiel, Germany; [8]Biochemistry Center, Universität Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany; [9]Schwerpunkt Tumorimmunologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany; and [10]Vivekananda Institute of Medical Sciences, Kolkata 700045, India

Received on January 21, 2004; revised on June 3, 2004; accepted on June 6, 2004

Sialic acids as terminal residues of oligosaccharide chains play crucial roles in several cellular recognition events. Exploiting the selective affinity of Achatinin-H toward *N*-acetyl-9-*O*-acetylneuraminic acid-α2-6-GalNAc, we have demonstrated the presence of 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac$_2$-GPs) on lymphoblasts of 70 children with acute lymphoblastic leukemia (ALL) and on leukemic cell lines by fluorimetric HPLC and flow cytometric analysis. This study aims to assess the structural aspect of the glycotope of Neu5,9Ac$_2$-GPs$_{ALL}$ and to evaluate whether these disease-specific molecules can be used to monitor the clinical outcome of ALL. The Neu5,9Ac$_2$-GPs$_{ALL}$ were affinity-purified, and three distinct leukemia-specific molecular determinants (135, 120, and 90 kDa) were demonstrated by SDS–PAGE, western blotting, and isoelectric focusing. The carbohydrate epitope of Neu5,9Ac$_2$-GPs$_{ALL}$ was confirmed by using synthetic sialic acid analogs. The enhanced presence of anti-Neu5,9Ac$_2$-GP$_{ALL}$ antibody in ALL patients prompted us to develop an antigen-ELISA using purified Neu5,9Ac$_2$-GPs$_{ALL}$ as coating antigens. Purified antigen was able to detect leukemia-specific antibodies at presentation of disease, which gradually decreased with treatment. Longitudinal monitoring of 18 patients revealed that in the early phase of the treatment patients with lower anti-Neu5,9Ac$_2$-GPs showed a better prognosis. Minimal cross-reactivity was observed in other hematological disorders ($n = 50$) like chronic myeloid leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma as well as normal healthy individuals ($n = 21$). This study demonstrated the potential of purified Neu5,9Ac$_2$-GPs$_{ALL}$ as an alternate tool for detection of anti-Neu5,9Ac$_2$-GP antibodies to be helpful for diagnosis and monitoring of childhood ALL patients.

Key words: 9-*O*-acetylated sialoglycoconjugates/acute lymphoblastic leukemia/Achatinin-H/anti-Neu5,9Ac$_2$ antibody/Neu5,9Ac$_2$-binding lectin

Introduction

Childhood acute lymphoblastic leukemia (ALL) is highly responsive to chemotherapy; with current treatment protocols, virtually all patients achieve remission and nearly 80% are eventually cured (Campana *et al*., 2001). However, the 20% relapse implies that leukemic cells, although undetectable, are not completely eradicated (Uzunel *et al*., 2003). Considering that ~2000 new ALL cases per year are diagnosed in the United States alone, an urgent need exists to find easily detectable and stably expressed leukemia-specific markers, whose altered expression could be applied for comprehensive and reliable monitoring of ALL (Chan, 2002). The mandatory technical expertise required for detection of minimal residual disease limits its widespread applicability. Therefore, user-friendly techniques should be developed to identify biochemical markers whose altered expression could be explored for risk stratification.

Sialic acids are important constituents of the lymphocyte cell membrane and influence many biological reactions either by reacting with specific surface receptors or via masking of carbohydrate recognition sites (Angata *et al*., 2002; Kelm and Schauer, 1997; Mandal *et al*., 2000; Schauer, 2004; Sinha *et al*., 2000). Among the diverse derivatives of sialic acid, the most frequently occurring substitutions are *O*-acetylation at positions C-7, C-8, and C-9 to form *N*-acetyl-7-, -8-, and -9-*O*-acetyl sialic acids, respectively, leading to a family of *O*-acetylated sialoglycoconjugates (Klein and Roussel, 1998; Schauer and Kamerling, 1997). However, because *O*-acetyl esters from C-7 and C-8 positions are known to spontaneously migrate to C-9 even under physiological conditions, *O*-acetylation at C-9 is considered the most common biologically occurring modification (Vandamme-Feldhaus and Schauer, 1998).

Lectins or lectin-like molecules have been used to predict changes in sialylation patterns (Angata *et al*., 2002; Mandal and Mandal, 1990; Mandal *et al*., 2000; Sinha *et al*., 1999a).

[1]These authors contributed equally to this work.
[2]Present address: Gurudas College, Department of Botany, Kolkata 700054, India
[3]Present address: Chess GmbH, D-68526 Ladenburg, Germany
[4]To whom correspondence should be addressed; e-mail: cmandal@iicb.res.in

A lectin, *Cancer antennarius* agglutinin, which recognizes sialic acids that are *O*-acetylated at both C-4 and C-9 positions, has been used to identify an *O*-acetylated disialoganglioside, Neu5,9Ac_2-GD3, as a biomarker in human melanoma cells (Ravindranath *et al*., 1988). The enhanced presence of 9-*O*-acetylated GD3 has been reported in several tumors, including melanomas, basaliomas, breast cancer, and tumors of neuroectodermal origin (Fahr and Schauer, 2001; Kohla *et al*., 2002). In the gastrointestinal tract, the concentration of *O*-acetylated sialic acids of colonic mucin decreases in colorectal cancers and Hirschsprung's disease (Aslam *et al*., 1999; Mann *et al*., 1997).

9-*O*-acetylated sialoglycans are detectable at low levels on human B lymphocytes, (Kamerling *et al*., 1982) and altered expression in disease conditions has been found (Bandyopadhyay *et al*., 2004; Chava *et al*., 2002, 2004a; Mandal *et al*., 2000; Sharma *et al*., 1998; Sinha *et al*., 1999a). These sialoglycotopes have also been reported on parasites (Chatterjee *et al*., 2003; Chava *et al*., 2004b,c). However, owing to the lack of detailed biochemical characterization, their biological significance, especially as a potential biomarker, remains obscure in ALL. The preferential specificity of a lectin, Achatinin-H, toward Neu5,9Ac_2-α2,6-GalNAc (Mandal and Basu, 1987; Mandal *et al*., 1989; Sen and Mandal, 1995) allowed us to identify these glycotopes on lymphoblasts of ALL patients. We have reported an increased amount of Neu5,9Ac_2-GPs on erythrocytes (Mandal *et al*., 1997) and peripheral blood mononuclear cells (PBMCs) of ALL patients (Mandal *et al*., 1997; Pal *et al*., 2004a; Sinha *et al*., 1999a,b,c,d). Subsequently, we detected an enhanced level of antibodies against Neu5,9Ac_2-GPs in ALL patients as compared to normal individuals (Pal *et al*., 2000, 2001, 2004b).

The present study reports (1) confirmation of the occurrence of Neu5,9Ac_2 on PBMCs of ALL patients by fluorimetric high-performance liquid chromatography (HPLC) and flow cytometry using Achatinin-H as a probe; (2) affinity purification of Neu5,9Ac_2-GPs_{ALL} from PBMC of ALL patients using Achatinin-H; (3) demonstration of three leukemia-specific Neu5,9Ac_2-GPs_{ALL} by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), western blotting, and isoelectric focusing (IEF); (4) examination of the structural aspects of the binding of Neu5,9Ac_2-GPs_{ALL} with Achatinin-H using synthetic sialic acid analogs by inhibition enzyme-linked immunosorbent assay (ELISA); and (5) the potential application of Neu5,9Ac_2-GPs_{ALL} to monitor leukemia-specific antibodies at different phases of treatment by an antigen ELISA. Given the importance of these disease-specific molecules, we propose that Neu5,9Ac_2-GPs_{ALL} on lymphoblasts may serve as potential biomarkers for diagnosis and monitoring the disease status.

Results

Expression of O-acetylated sialoglycoconjugates on PBMCs of ALL ($PBMC_{ALL}$) patients as analyzed by fluorimetric HPLC

The presence of Neu5,9Ac_2 on lymphoblasts of ALL patients was demonstrated by preparing acid hydrolysates of PBMCs that were analyzed by fluorimetric HPLC. As shown in Figure 1, the chromatogram exhibited a well-resolved peak that coincided with Neu5,9Ac_2. This peak disappeared by saponification, thus confirming the presence of Neu5,9Ac_2 on $PBMC_{ALL}$. The peak eluting at the position of Neu5Ac was confirmed to be this sialic acid because it was degraded by incubation of the sample with sialate-pyruvate lyase prior to derivatization. The Neu5,9Ac_2 peak partially disappeard by this treatment, too. The peak at retention time 18 (Figure 1B, 1C) represents a reagent peak. The HPLC profile of PBMCs from a normal individual showed only a minimal amount of Neu5,9Ac_2 as compared to PBMCs from an ALL patient.

High expression of Neu5,9Ac_2-GPs on lymphoblasts of ALL patients as detected by flow cytometry using FITC-Achatinin-H

ALL patients were selected based on the binding of their PBMCs with anti-CD10 and/or anti-CD19 for B-ALL ($n = 55$) and anti-CD3 and/or anti-CD7 for T-ALL ($n = 15$). The leukemic blast population ranged from 60% to 97%. A high expression of cell surface Neu5,9Ac_2-GPs on $PBMC_{ALL}$ of both B and T lineage was evidenced by the high binding of fluorescein isothiocyanate (FITC)–Achatinin-H being $90.5 \pm 5.0\%$ and $94.2 \pm 4.2\%$, respectively (Figure 2). A similar degree of FITC-Achatinin-H binding was established in B-ALL cell lines, namely, NALM-6 ($68 \pm 5.6\%$) and REH ($90.6 \pm 3.4\%$); T-ALL cell lines, namely, JURKAT ($94.2 \pm 4.6\%$), CEMC-7 ($86.0 \pm 3.6\%$), MOLT-3 ($82.8 \pm 3.8\%$), and MOLT-4 ($93.4 \pm 4.6\%$). In contrast, the binding of FITC-Achatinin-H to cells from acute myelogenous leukemia (AML) ($n = 6$), chronic lymphocytic leukemia (CLL) ($n = 6$), non-Hodgkin's lymphoma (NHL) ($n = 5$), chronic myeloid leukemia (CML) ($n = 5$), and normal donors ($n = 15$) was minimal ($4.2 \pm 2.7\%$, $6.5 \pm 2.3\%$, $7.5 \pm 2.0\%$, $2.7 \pm 1.2\%$, and $5.0 \pm 2.4\%$, respectively). The negligible binding observed with the cell lines U937 ($1.6 \pm 0.8\%$), U266 ($19 \pm 1.1\%$), TF-1 ($3.8 \pm 1.5\%$), and KG-1A ($1.3 \pm 0.8\%$) corresponded to the minimal expression of Neu5,9Ac_2-GPs in allied hematological diseases, thus establishing Neu5,9Ac_2-GPs as a unique biomarker induced in ALL (Figure 2). The nature of the 9-*O*-acetylated determinant on lymphoblasts was further determined by using the CD60b-specific monoclonal antibody UM4D4 (Kniep *et al*., 1992). The low binding (5.87%) of CD60b confirmed that negligible amounts of the ganglioside Neu5,9Ac_2-GD3 were present on leukemic blasts.

To demonstrate the binding specificity of Achatinin-H toward the 9-*O*-acetyl moiety, PBMCs from an ALL patients, at presentation of disease (i.e., before any treatment) were preincubated with a recombinant *O*-acetylesterase (Figure 2, inset). The resultant de-*O*-acetylation caused a drastic reduction in lectin binding from 80.0% (mean fluorescence intensity was 140) to 20.2% (mean fluorescence intensity 8.5). The presence of the 9-*O*-AcSA glycotope on lymphoblasts of ALL patients was thus reconfirmed.

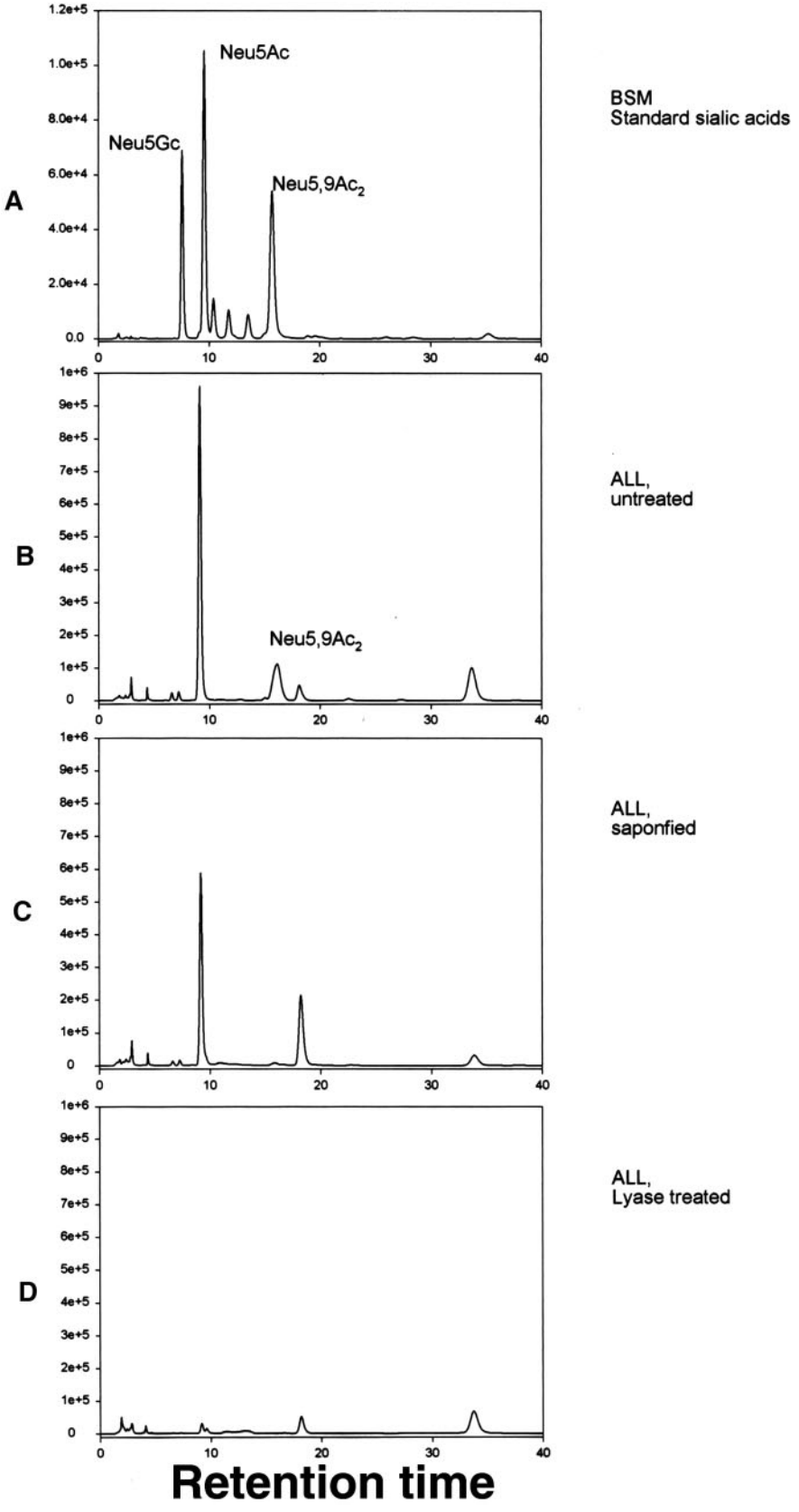

Fig. 1. Determination of Neu5,9Ac$_2$ by fluorimetric HPLC. Representative profile of an HPLC chromatogram of fluorescent derivatives of free sialic acids derived from (**A**) BSM as standard, (**B**) PBMC of ALL before and (**C**) after preincubation with ammonia vapor (0.1 M) and (**D**) acyl sialate-pyruvate lyase. Glycosidically bound sialic acids were subjected to acid hydrolysis, derivatised with 1,2-diamino-4,5-methylenedioxybenzene and analyzed as described in *Materials and methods*. The peak at retention time 18 in b and c represents the reagent peak.

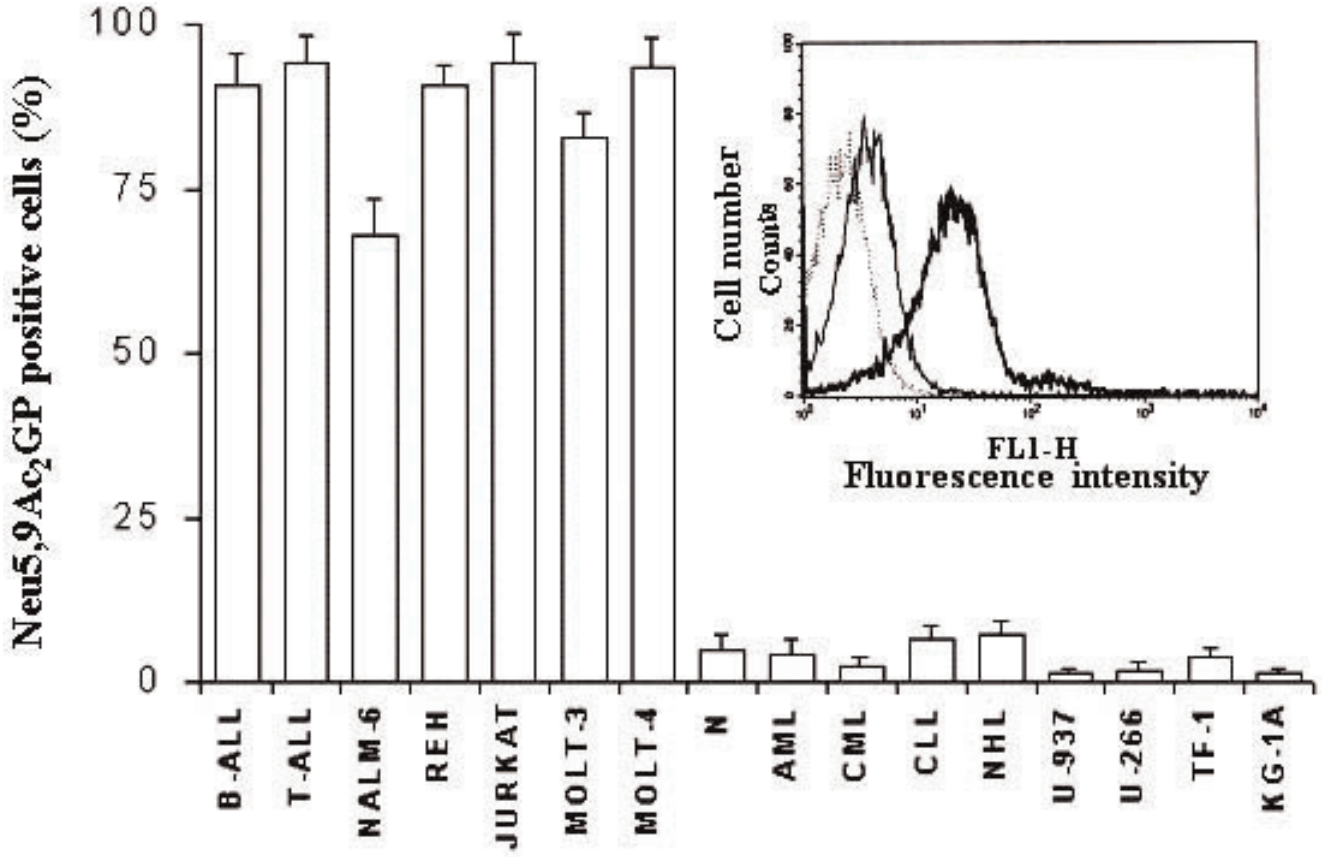

Fig. 2. Comparison of cell surface expression of Neu5,9Ac_2-GPs on ALL patients, patients with other hematological disorders, different cell lines, and on normal donors by single color FACS analysis using FITC-Achatinin-H. Binding of FITC-Achatinin-H (0.10 μg) to PBMCs (1×10^6) of childhood ALL patients of B- ($n = 55$) and T-ALL ($n = 15$), normal individuals (N, $n = 15$) along with established B- (NALM-6, REH) and T- (JURKAT, MOLT-3, MOLT-4) ALL cell lines was determined by single-color flow cytometric analysis as described in *Materials and methods*. PBMCs (1×10^6) from patients with other hematological disorders ($n = 22$) that included CML ($n = 5$), AML ($n = 6$), CLL ($n = 6$), and NHL ($n = 5$) along with JOK-1 (derived from peripheral blood of a patient with hairy cell leukemia), U266 (derived from peripheral blood of a patient with an IgE myeloma), U937 (histiocytic lymphoma), TF-1 (erythroleukemia), and KG-1a (AML) cell lines was also tested with similar dose of FITC-Achatinin-H. Bars depict the mean ± SD of percentage of cells expressing Neu5,9Ac_2-GPs as evidenced by FITC-Achatinin-H binding. *Inset:* Demonstration of Neu5,9Ac_2-GPs on cell surface of PBMCs of an ALL patient, at presentation of disease, by flow cytometric analysis. A representative profile of binding of FITC-Achatinin-H (0.10 μg) to PBMCs (1×10^6) of an ALL patient before (thick line) and after (thin line) treatment with recombinant 9-*O*-acetylesterase derived from the HE1 region of the influenza C esterase gene to remove cell surface 9-*O*-acetylation as described in *Materials and methods*. The dotted line represents unstained cells. The log of fluorescence intensities in arbitrary units is plotted against cell number.

Purification of Neu5,9Ac_2-GPs from PBMCs of ALL patients

Clinically confirmed, immunophenotyped children with B-ALL ($n = 10$) and T-ALL ($n = 5$) with >80% lymphoblasts (as measured using lineage-specific established anti-CD antibodies and Achatinin-H) were selected for purification of Neu5,9Ac_2-GPs_{ALL}. Equal amounts of membrane proteins (0.8 mg) of PBMCs from ALL and normal donors ($n = 5$) were affinity-purified using Achatinin-H-Sepharose 4B as an affinity matrix. The yield of purified Neu5,9Ac_2-GPs was 3.7-fold higher in ALL patients as compared to normal donors—0.37 ± 0.15 mg and 0.10 ± 0.05 mg, respectively. The proportion of membrane proteins from ALL patients that bound to Achatinin-H was 46.54 ± 2.4% as compared with 12.50 ± 1.30% from normal individuals. An ELISA using equal amounts (0.4 μg) of purified Neu5,9Ac_2-GPs from ALL patients (Neu5,9Ac_2-GPs_{ALL}) and normal donors (Neu5,9Ac_2-GPs_N) as the coating antigen showed a ~threefold increase in lectin binding—OD_{405nm} was 1.34 versus 0.45, which indicates the lower amount of Neu5,9Ac_2-GPs in normal individuals.

Molecular analysis of purified Neu5,9Ac_2-GPs

Three distinct leukemia-specific bands corresponding to 135, 120, and 90 kDa were demonstrated in purified Neu5,9Ac_2-GPs_{ALL} on SDS–PAGE (Figure 3A) and western blot using Achatinin-H (data not shown). The presence of *O*-acetylation was strengthened by complete abolition of binding of Achatinin-H following de-*O*-acetylation with alkali treatment. In contrast, two common bands corresponding to 140 and 36 kDa (Figure 3) were visible both in Neu5,9Ac_2-GPs_N and Neu5,9Ac_2-GPs_{ALL}.

The molecular nature of these Neu5,9Ac_2-GPs was further investigated on an IEF gel (Figure 3B). Analysis of Neu5,9Ac_2-GPs_{ALL} showed five distinct bands, indicating five different sialoglycoproteins with similar type of

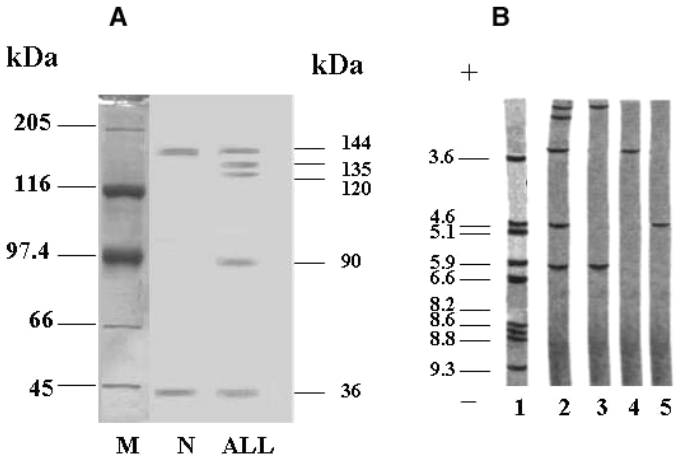

Fig. 3. Molecular characterisation of Neu5,9Ac$_2$-GPs present on PBMCs of an ALL patient. (**A**) SDS–PAGE (7.5%): A representative profile of Neu5,9Ac$_2$-GPs$_{ALL}$ (lane ALL, 10 μg/lane) and Neu5,9Ac$_2$-GPs$_N$ (lane N, 4 μg/lane) were electrophoresed. The gel was fixed and stained for protein with Coomassie brilliant blue R-250. Lane M represents the molecular weight markers. (**B**) IEF: The purified Neu5,9Ac$_2$-GPs (2 μg) were applied to an ampholine polyacrylamide tube gel (4%) in a pH gradient (3.5–10) as described in *Materials and methods*. Representative profiles of Neu5,9Ac$_2$-GPs$_{ALL}$ and Neu5,9Ac$_2$-GPs$_N$ are shown in lanes 2 and 3, respectively. Lanes 4 and 5 are individual 120- and 90-kDa Neu5,9Ac$_2$-GPs$_{ALL}$ that were gel-eluted from SDS–PAGE analysis. Lane 1 represents the pI markers.

glycotopes (lane 2). The single bands implied again their purity and homogeneity. The gel-eluted 120- and 90-kDa bands showed acidic pI values corresponding to 3.4 and 4.6, respectively (lanes 4 and 5). On the contrary, Neu5,9Ac$_2$-GPs$_N$ showed the presence of only two bands (lane 3).

Characterization of the carbohydrate epitope of Neu5,9Ac$_2$-GPs$_{ALL}$ by inhibition-ELISA

To study the binding specificity of Neu5,9Ac$_2$-GPs$_{ALL}$, an inhibition-ELISA was developed with synthetic analogs of sialic acid, using Achatinin-H as coating antigen (Table I). Three regions of sialic acid at C-4, C-2, and C-9 were modified keeping the common core intact. The percent inhibition (*PI*) for a particular inhibitor was calculated as follows:

$$PI = 100 - [100 \times (\text{OD in presence of inhibitor}/\text{OD in absence of inhibitor})]$$

The relative inhibition (*RI*) was calculated using the following normalization equation:

$$RI = 100 \times (PI \text{ of an analog at 68 mM})/(PI \text{ of Me–α–Neu5,9Ac}_2 \text{ at 68 mM})$$

Inhibition was measured at different concentrations of Me-α-Neu5,9Ac$_2$ (compound 1, 10–68 mM). The highest inhibition (*PI* = 89.32%) was achieved using 68 mM of Me-α-Neu5,9Ac$_2$ and the IC$_{50}$ (concentration needed for 50% inhibition) was found to be 30.11 mM. However, due to limited stocks of other analogs, a fixed concentration (68 mM) of all inhibitors was used for determination of *RI*. The relative inhibitions of these analogs with reference to Me-α-Neu5,9Ac$_2$ were calculated using the normalization equation and are presented in Table I.

Table I. Inhibitory potency of sialic acid analogs for the binding of Neu5,9Ac$_2$-GPs$_{ALL}$ with Achatinin-H

	Sialic acids	C-2	C-9	C-4	Relative inhibition (%)
1	Me-α-Neu5,9Ac$_2$	CH_3	O.CO.CH_3	OH	100
2	Me-α-Neu 5Ac	CH_3	OH	OH	28
3	Me-α-Neu5Ac, 9-SAc	CH_3	S.CO.CH_3	OH	20
4	Me-α-Neu5Ac -9-NHAc	CH_3	NH.CO.CH_3	OH	13
5	Me-α-Neu5Ac9 -NHFAc	CH_3	NH.CO.CH_2F	OH	2.8
6	Benzyl-α-Neu4, 5,9Ac$_3$	$C_6H_5CH_2$	O.CO.CH_3	O.CO.CH_3	71
7	Benzyl-α-Neu5Ac-9-*O*-Propionyl	$C_6H_5CH_2$	O.CO.CH_2.CH_3	OH	2
8	Neu5 Ac	H	OH	OH	NI
9	Neu4,5Ac$_2$	H	OH	O.CO.CH_3	NI

NI: not inhibited.

Replacement of the *O*-acetyl group of compound 1 with a hydroxyl group (2) and replacement of oxygen by sulfur (3) or NH (4) at C-9 decreased inhibition to 28%, 20%, and 13%, respectively. Interestingly, introduction of fluorine (F) into the 9-*N*-acetyl group of compound 4 at C-9, that is, compound 5, resulted in a fivefold reduction of inhibition. A striking difference was observed between compounds 6 and 7 being (71 ± 3.8% versus 2.0 ± 0.8%), where the *O*-acetyl group at C-9 of the former had been exchanged with the propionyl group -O.CO.CH_2.CH_3. No inhibition was observed with compounds 8 and 9, possibly due to the lack of *O*-acetylation at the C-9 position. Also, replacement of the CH_3 group of compound 2 with hydrogen at C-2 (compound 8) made the carbohydrate noninhibitory.

Bovine submandibular gland mucin (BSM) with a high Neu5,9Ac$_2$ content was found to be a good inhibitor of Neu5,9Ac$_2$-GPs$_{ALL}$ binding to Achatinin-H. No inhibition was observed with de-*O*-acetylated BSM and asialo-BSM, indicating that the *O*-acetylated glycotope is the vital component for binding of Neu5,9Ac$_2$-GPs$_{ALL}$ with Achatinin-H. Other sialoglycoproteins, such as sheep submaxillary gland mucin (SSM), human chorionic gonadotropin (HCG), fetuin, and α_1-acid glycoprotein, that have no *O*-acetylated sialic acids did not inhibit.

Potential use of purified Neu5,9Ac$_2$-GPs for monitoring the disease status by an antigen-ELISA

Previous studies from our group have demonstrated increased antibody titers against Neu5,9Ac$_2$ in sera of ALL patients (Pal *et al.*, 2000, 2001, 2004b). Therefore, we wished to examine the reactivity of affinity-purified Neu5,9Ac$_2$-GPs$_{ALL}$ as a novel capture antigen to monitor the disease

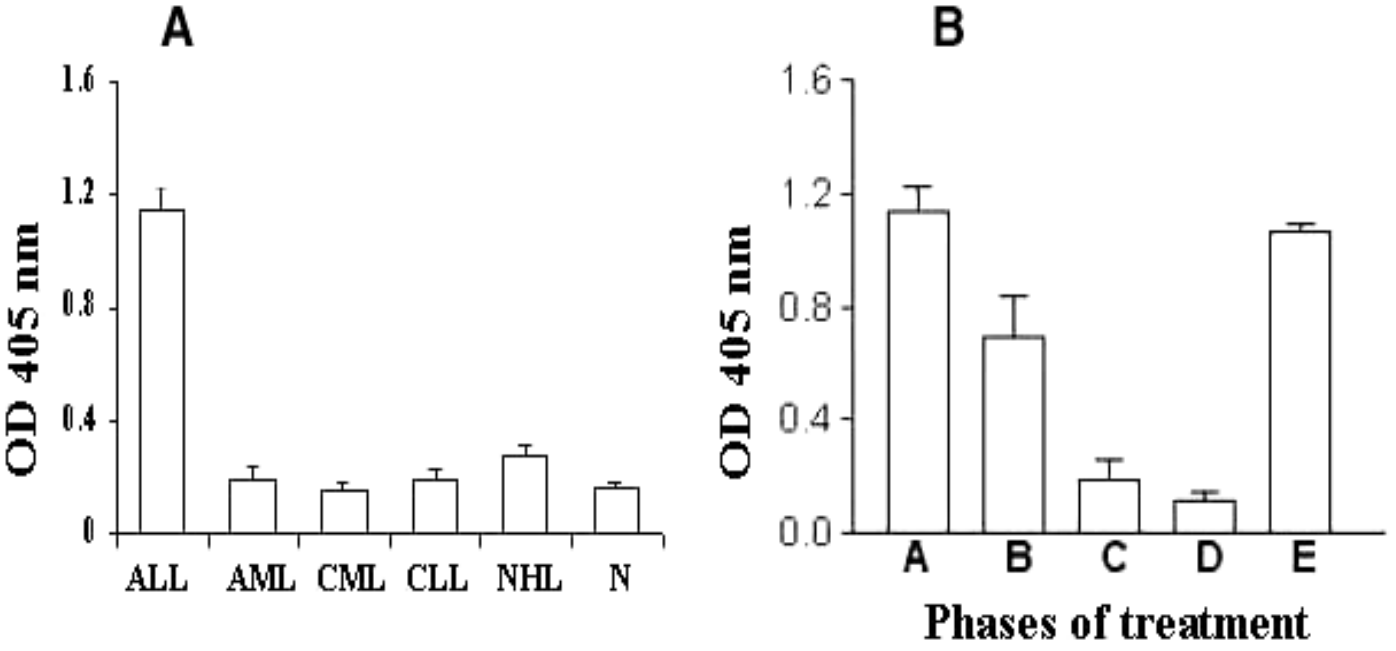

Fig. 4. (**A**) Diagnostic potential of antigen-ELISA using Neu5,9Ac_2-GPs_{ALL} as coating antigen. Purified Neu5,9Ac_2-GPs_{ALL} (0.5 μg) was coated on a microtiter plates, washed, blocked, and incubated with sera (1:10 diluted) from untreated ALL ($n = 70$) patients along with AML ($n = 20$), CML ($n = 15$), CLL ($n = 10$), NHL ($n = 5$), and normal individuals (N, $n = 21$) at 4°C. After washing, the antigen–antibody complex was detected using HRP-protein A as described in *Materials and methods*. Each point is the average of duplicate determinations. (**B**) Prognostic potential of antigen-ELISA. Sera (1:10 diluted) from clinically confirmed ALL patients at different phases of treatment were allowed to bind with Neu5,9Ac_2-GPs_{ALL}-coated wells. Anti-Neu5,9Ac_2-GPs antibodies were monitored by an antigen-ELISA. Data are expressed as mean ± SD of optical density. A, Induction of remission (phase A, $n = 70$); B, consolidation/early intensification (phase B, 4–8 weeks, $n = 58$); C, period of maintenance therapy (phase C, 8 weeks–2.5 years, $n = 40$); D, follow-up case (phase D, 2.5 years onward, $n = 33$) and E, patients who relapsed (phase E, $n = 10$).

status. A strong binding with sera from untreated patients ($n = 70$) was observed in contrast to negligible binding with normal human serum ($n = 21$), mean ± SD of OD_{405nm} being 1.14 ± 0.12 versus 0.16 ± 0.01, respectively (Figure 4A). With treatment, the binding gradually decreased in phases B, C, and D to 0.69 ± 0.01, 0.19 ± 0.01, and 0.12 ± 0.01, respectively (Figure 4B). In patients that relapsed (phase E, $n = 10$), absorbance increased again to 1.06 ± 0.06. In parallel, sera from patients with other hematological disorders (such as AML, CML, CLL, and NHL) were examined and showed no detectable levels of binding (Figure 4A). To minimize the false positivity, the cut-off value was selected as 0.2 based on the mean OD+3 SD obtained from normal controls. A good correlation ($r = 0.92$) was observed between antibody titers from the same set of patients as determined by both antigen-ELISA and BSM-ELISA using Neu5,9Ac_2-GP_{ALL} and BSM as coating antigens, respectively (Figure 5). The data were subjected to a one-way analysis of variance with a post test for linear trend and confirmed a significant decrease of antibody titers with progress in treatment. Sensitivity, specificity, efficiency, positive predictive value, and negative predictive value of both assays are compared (Table II).

Assessment of the anti-Neu5,9Ac_2-GPs antibody levels at early phase of treatment, an indicator for overall disease-free survival (DFS)

Measurement of the anti-Neu5,9Ac_2-GPs antibody levels at an early phase of treatment (phase B) allowed discrimination

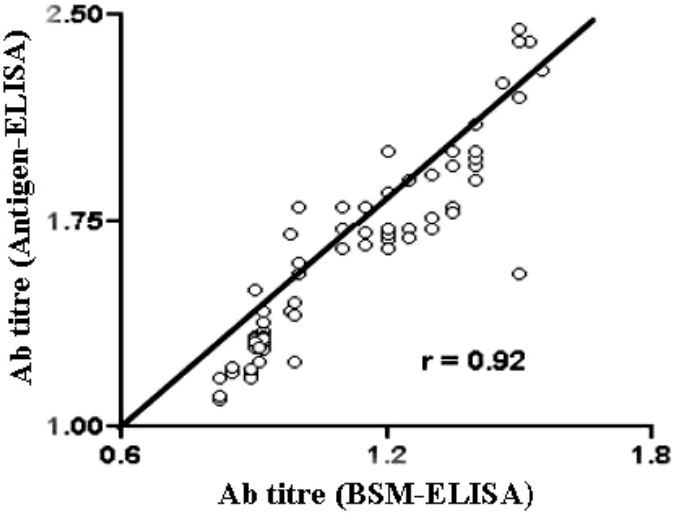

Fig. 5. Correlation between Neu5,9Ac_2-GPs specific antibody levels as measured by antigen-ELISA and BSM-ELISA using Neu5,9Ac_2-GPs_{ALL} and BSM as capture antigens. A correlation graph representing Neu5,9Ac_2-GPs specific antibody (Ab) titers present in ALL patients ($n = 70$), at presentation of disease, as measured by antigen-ELISA (y-axis) and BSM-ELISA (x-axis). Purified Neu5,9Ac_2-GPs_{ALL} (0.5 μg) was coated on microtiter plates, washed, blocked, and incubated with untreated ALL sera at 4°C. After washing, the bound complex was detected using HRP-protein A as described in *Materials and methods.* The antibody titers was similarly quantitated using the same set of ALL samples by BSM-ELISA in which BSM (1 μg) was used as coating antigen (Pal *et al.*, 2000).

Table II. Comparison between antigen-ELISA and BSM-ELISA for detection (by %) of anti-Neu5,9Ac$_2$-GPs$_{ALL}$ antibodies present in the sera of same set of untreated ALL patients

Assay	Antigen-ELISA	BSM-ELISA
Sensitivity	100	98.92
Specificity	98.21	92.10
Efficiency	98.42	96.94
Positive predictive value	100	96.84
Negative predictive value	97.58	97.22

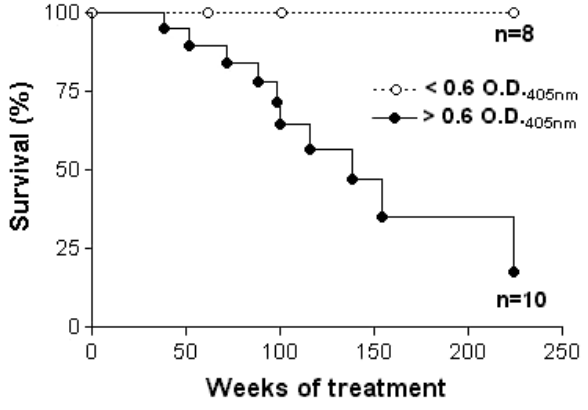

Fig. 6. DFS curves of patients with ALL categorized according to Neu5,9Ac$_2$-GPs-specific antibody titer at phase B as evidenced by antigen-ELISA. Sera from patients ($n = 18$) were longitudinally monitored for 224 weeks at various time points after chemotherapy and anti-Neu5,9Ac$_2$-GPs antibody was detected by antigen-ELISA. A few patients ($n = 10$) persistently showed high antibody titer ($OD_{405nm} \geq 0.6$) at phase B associated with significantly poor DFS. In contrast a few patients ($n = 8$) persistently showed low antibody titer ($OD_{405nm} < 0.6$) at phase B and this was associated with good DFS ($p = 0.002$).

between two groups of patients ($n = 18$) who were followed up for 224 weeks. In one group, children with high antibody levels (≥ 0.6 at OD_{405nm}, $n = 10$) relapsed (10/10), whereas children showing low antibody titer (< 0.6 at OD_{405nm}, $n = 8$) remained in continuous complete remission. This suggests that monitoring of these anti-Neu5,9Ac$_2$-GPs antibody levels may serve as an indicator for DFS (Figure 6).

Discussion

Assessment of the *O*-acetylated sialoglycan profile in ALL is a relatively new domain in leukemia sialobiology. Although the increase of Neu5,9Ac$_2$-GPs has been identified as an important determinant on lymphoblasts (Pal *et al.*, 2004a; Sinha *et al.*, 1999a), little progress has been made in examining the structural aspect of the glycotopes of Neu5,9Ac$_2$-GPs and in evaluating whether these affinity-purified disease-specific molecules can be harnessed to monitor the clinical outcome of ALL. In this study, we have demonstrated an overexpression of Neu5,9Ac$_2$-GPs in a large patient population, several T- and B-ALL cell lines, that collectively substantiate that Neu5,9Ac$_2$-GPs are unique biomarkers of childhood ALL as established by HPLC and FACS analyses (Figures 1, 2). Abolition of lectin binding by esterase-treated cells confirmed the involvement of *O*-acetylated sialic acid (Figure 2 inset). Additionally, the absence of Neu5,9Ac$_2$-GPs in normal healthy individuals and other hematological disorders confirmed their disease specificity (Figure 2). The selective binding affinity of Achatinin-H enabled the purification of three new ALL-specific molecules of molecular weight 135, 120, and 90 kDa having a Neu5,9Ac$_2$α2-6GalNAc moiety as their binding epitope. They were exclusively present on PBMCs of ALL patients, that is, absent on normal PBMCs (Figure 3A–B). In ALL patients, a significant amount of total membrane protein (2 mg from 10^7 cells) was found to be *O*-acetylated sialoglycoprotein (0.925 mg). The detection of leukemia-specific bands both on SDS–PAGE and western blots and their subsequent disappearance after de-*O*-acetylation confirmed the occurrence of *O*-acetylated sialic acid in Neu5,9Ac$_2$-GPs$_{ALL}$.

A major achievement of this investigation is the verification of the nature of the glycotope of Neu5,9Ac$_2$-GPs$_{ALL}$ (Table I) by inhibition-ELISA using synthetic sialic acid analogs that serve as inhibitors preventing binding between Neu5,9Ac$_2$-GPs$_{ALL}$ and Achatinin-H. A comparative analysis of the inhibitory potency of various sialic acid analogs showed that Neu5,9Ac$_2$ is the most critical neuraminic acid derivative for competition, as compound 1 is the strongest inhibitor (Table I). Interestingly, a minor modification of this group caused drastic reduction in inhibition, thus confirming that *O*-acetylation at C-9 position is essential for binding. The introduction of a highly electronegative atom (−F) at C-9 in compound 5 drastically reduced inhibition, suggesting hindrance of binding. The absence of inhibition with compounds lacking *O*-acetylation at C-9, or the 35.5-fold reduction in inhibition due to the introduction of a bulky group at C-9, suggests the existence of a smaller cleft size in the binding pocket. It can be envisaged that the cleft size around the binding site of C-9 is just right for an acetyl group, and even minor substitutions hinder binding. Introduction of a large group like C_6H_5 at position C-2 in place of a CH_3 group reduces the inhibition only from 100% to 71%, indicating that the cleft size around C-2 is much bigger than that needed to accommodate CH_3. At C-4 substitution of H with an acetyl group does not affect the inhibition properties, suggesting that adequate space for accommodation is present in this region. In summary, our data suggest that the region where C-9 is bound is highly specific for an *O*-acetyl group and does not allow any modification of this group, whereas binding criteria in other regions of the binding cleft are less stringent.

To show the involvement of the Neu5,9Ac$_2$ moiety of Neu5,9Ac$_2$-GPs$_{ALL}$ in Achatinin-H binding, BSM was used as an inhibitor, which is known to contain this glycosidic group (Sen and Mandal, 1995). However, BSM is not a proper control, as Neu5,9Ac$_2$α2-6GalNAc is only one of the oligosaccharide structures present along with mono-, di-, or tri-*O*-acetylated sialic acid. Ideally, to elucidate the sugar specificity one should use the disaccharides isolated from BSM. It is not possible to prepare the disaccharides with Neu5,9Ac$_2$, because the alkali treatment of BSM required

for the elimination of the O-glycosidically linked disaccharides would destroy the *O*-acetyl groups. Presently, no enzyme or process is available for releasing this glycoside from the sialoglycoprotein. Synthetic analogs are therefore the only alternative, but presently they are not available.

The detection of residual leukemic cells by FACS analysis mainly suffers from the lack of leukemia-specific CD markers. So far, progress in the identification of new leukemia-specific markers relied on testing the expression of known CD markers (Björklund *et al.*, 2003). This approach, largely based on trial and error, is slow. We believe that purified and well-characterized Neu5,9Ac_2-GPs_{ALL} will now be the new tool to reach the desired goal.

Previous observations suggested that a humoral response was directed specifically toward Neu5,9Ac_2-GPs_{ALL} (Pal *et al.*, 2000, 2001). Accordingly, we developed a BSM-ELISA for the measurement of this antibody using BSM as a coating antigen that contains a mixture of mono-, di-, and tri-*O*-acetylated forms of Neu5,9Ac_2. Therefore, it may be envisaged that not all forms of Neu5,9Ac_2 present in BSM are equally available for binding with anti-Neu5,9Ac_2-GPs antibodies. Although a comparable correlation ($r = 0.92$) between antigen and BSM-ELISA was observed, antigen-ELISA revealed a higher specificity using a similar set of patients, and it may be considered as better capture antigen (Figure 5, Table II). The absence of cross-reactivity with other hematological diseases makes Neu5,9Ac_2-GPs_{ALL} a suitable coating antigen for diagnosis and monitoring of ALL (Figure 4A). To the best of our knowledge, this is the first demonstration of the applicability of these unique Neu5,9Ac_2-GPs_{ALL} molecules to monitor the clinical outcome of ALL (Figure 4B).

In a similar assay, following chemotherapy, anti-Neu5,9Ac_2-GP antibody titers progressively decreased, reflecting clinical remission. However, a few children showed an increase in OD_{405nm}, which correlated with clinical relapse. Therefore, monitoring of antibody titers using Neu5,9Ac_2-GPs_{ALL} as capture antigen may be an alternate tool to assess the clinical status of patients.

Interestingly, we observed with a small population of children ($n = 18$) a good correlation of the antibody titers at early phase of treatment (phase B) with disease status (Figure 6). High antibody titers at phase B that showed close association with relapse ($n = 10$) were observed in 55.5% of patients. The relapse rate was 33% during maintenance therapy and 22% after completion of therapy. The higher incidence of relapse observed in these follow-up studies may be attributed to inclusion of patients irrespective of the risk factors (Riyat, 1995; Viana *et al.*, 1994). These patients may benefit from extensive treatment to avoid relapse. Therefore it may be recommended that patients with high antibody titers should be closely monitored for recurrence of disease. Additionally, a low-dose therapy may be considered for patients showing a persisting low antibody titer. In the population group of our study, we successfully correlated the antibody titers with the state of the disease, thus proving the potency of this assay for monitoring the disease. Of course, the study should be evaluated with a larger population group. Studies are ongoing to build a foundation for the predictive value of these antibodies.

The role of two different Neu5,9Ac_2-GPs_N, corresponding to 144 kDa and 36 kDa were detected on normal PBMCs, remains unclear. Earlier results suggest that they are constitutively present on normal PBMCs. This is corroborated by the presence of low anti-Neu5,9Ac_2-GPs antibody in normal human serum, indicating that they are either less immunogenic or inadequately exposed on the cell surface of normal PBMC (Pal *et al.*, 2000, 2001).

In summary, our results indicate that leukemia-specific Neu5,9Ac_2-GPs_{ALL} recognized by Achatinin-H are novel lymphoblastoid antigens. It may be envisaged that these antigens will allow designing primers for RT-PCR–based detection of minimal residual disease. In the future, development of monoclonal antibodies against these Neu5,9Ac_2-containing glycoproteins will be useful for immunophenotyping and drug targeting. Studies on the production of humanized monoclonal antibodies are under way and will be helpful for future immunotherapy in childhood ALL.

Materials and methods

Patients

The study population included clinically confirmed ALL patients at presentation ($n = 70$). The group was made up of 46 male and 24 female patients, the median age being 6 years (range 0.8–16 years). The median presenting white blood cell count was 12×10^9/L (range 0.4–1000×10^9/L). A further 141 samples were obtained during chemotherapy, and subsequently 10 samples were obtained from children who relapsed. The diagnosis was established by cytological examination of the bone marrow smears according to the French-American-British Group recommendations (Burns *et al.*, 1981), belonging to L1 or L2 and immunophenotyping using antibodies against terminal deoxynucleotidyl transferase, cytoplasmic μ, surface membrane Ig, CD3, CD7, CD10, CD19, and CD34. The group included B-ALL (n = 55) and T-ALL (n = 15).

Children had been entered into UK ALL X (Eden *et al.*, 2000) with addition of further drugs, that is, etoposide and cytosar, for intensification. They were broadly grouped as follows: induction of remission (phase A, $n = 70$), consolidation/early intensification (phase B, 4–8 weeks, $n = 58$), period of maintenance therapy (phase C, 8 weeks–2.5 years, $n = 40$), follow-up case (phase D, 2.5 years onward, $n = 33$), and patients who relapsed (phase E, $n = 10$). A few patients ($n = 18$) were longitudinally monitored for 224 weeks. Normal healthy individuals ($n = 21$) of both sexes and different blood groups and patients with other hematological disorders ($n = 50$) that included CML ($n = 15$), AML ($n = 20$), CLL ($n = 10$), and NHL ($n = 5$) served as controls.

Venous blood (3–4 ml) or bone marrow was collected at Vivekananda Institute of Medical Science (Kolkata, India) and then sent to the Indian Institute of Chemical Biology, where PBMCs were separated by Ficoll-Hypaque density centrifugation; sera or plasma was stored at −20°C. Informed consent was obtained from donors, patients, and parents or guardians. The study was approved by the institutional human ethical committee as per protocol of the Indian Council of Medical Research.

Probes and reagents

Achatinin-H, a lectin, purified from the hemolymph of the African giant land snail *Achatina fulica*, has been shown to preferentially bind to glycoconjugates with terminal Neu5,9Ac$_2$-α2,6-GalNAc residues mainly based on inhibition study with several monosaccharides and sialoglycoproteins, for example, BSM, SSM, HCG, fetuin and α_1-acid glycoprotein. BSM having terminal Neu5,9Ac$_2$ and a subterminal GalNAc in an α2,6-linkage showed highest inhibition (Sen and Mandal, 1995). SSM, HCG, fetuin, and α_1-acid glycoprotein with terminal sialic acid either in α2,6- or α2,3-linkage did not show any inhibition, reconfirming the specificity of Achatinin-H toward Neu5,9Ac$_2$α2,6GalNAc glycotope (Mandal and Basu, 1987; Mandal *et al*., 1989; Sen and Mandal, 1995).

The cell lines used included B-ALL (Nalm-6 and REH), T-ALL (CEM-C7, MOLT 3, MOLT-4, and JURKAT), JOK-1 (derived from peripheral blood of a patient with hairy cell leukemia), U266 (derived from peripheral blood of a patient with an IgE myeloma), U937 (histiocytic lymphoma), TF-1 (erythroleukemia), and KG-1a (AML). All cell lines were maintained in RPMI-1640 supplemented with glutamine (2 mM), gentamycin, and 10% heat-inactivated human AB serum (medium A). Other chemicals and biological reagents were from Sigma (St. Louis, MO) unless otherwise stated.

Fluorimetric HPLC analysis of sialic acids

PBMCs (5×10^8) of ALL patients were extensively washed in phosphate buffered saline, and the cell pellet was resuspended in 1 ml double-distilled water. Cell lysis was completed by sonication (three pulses of 16 s each, keeping samples on ice in between). Glycosidically bound sialic acids were then subjected to acid hydrolysis with an equal volume of 4 M propionic acid. Samples were heated to 80°C for 4 h, cooled on ice for 10 min, separated into three portions, and then lyophilized. Controls included (1) saponification of sialic acids by placing one of the lyophilized samples in an ammonium atmosphere overnight and (2) treatment by sialate-pyruvate lyase by resolving a sample in 250 mM phosphate buffer, pH 7.2, containing 25 mU lyase and incubation for 2 h at 37°C. All samples were then derivatized with 1,2-diamino-4,5-methylenedioxybenzene for fluorimetric reverse-phase HPLC analysis (Chatterjee *et al*., 2003).

Flow cytometric analysis

The expression of Neu5,9Ac$_2$-GPs on lymphoblasts of ALL patients was evaluated by flow cytometry (FACS Calibur flow cytometer) using FITC-Achatinin-H. Briefly, PBMCs (1×10^6 cells/100 ml) from both T- and B-ALL patients and several established cell lines in medium A was blocked with goat serum (10%) and individually labeled with FITC-Achatinin-H or phycoerythrin-conjugated anti-CD10, anti-CD19, anti-CD7 (Pharmingen, San Diego, CA) in ice for 1 h (Pal *et al*., 2000). The cells were washed, fixed in paraformaldehyde (1%), and analyzed. FITC–bovine serum albumin (BSA) or FITC-Achatinin-H in the presence of the inhibitor (BSM) or unconjugated lectin control (preincubation of cells with Achatinin-H followed by incubation with FITC-Achatinin-H) was used as different sets of controls. The % positive cells were recorded based on the threshold or background fluorescence provided by all these sets of controls, which gave a similar level of background fluorescence.

Alternatively, cells were incubated with Achatinin-H followed by rabbit anti-Achatinin-H polyclonal antibody, and the bound complex was detected by FITC-conjugated second antibody. The percentage of lymphoblasts that bind to this template was calculated relative to appropriate isotype matched antibodies, which served as background fluorescence.

The binding was also measured using FITC-labeled anti-CD60b, a 9-*O*-acetyl GD3-specific monoclonal antibody. FITC-IgM served as an isotype control. Analysis and calculations were performed using Cell Quest software.

Esterase treatment of lymphoblasts of ALL patients

The presence of *O*-acetyl sialoglycan groups on PBMC of ALL membrane was demonstrated by taking advantage of the 9-*O*-acetyl hemagglutinin esterase of influenza C virus (Chatterjee *et al*., 2003). It had been originally cloned in an SV40 vector (Vlasak *et al*., 1987) to construct a gene consisting of the influenza C virus HE1 domain fused to the eGFP gene. Briefly, the entire HE1 coding region was isolated as a Sac I/Cla I restriction fragment. The Cla I site was filled in to allow blunt end ligation with the filled-in BamH I site immediately upstream of the eGFP gene derived from plasmid pEGFP-N3 (Clontech Laboratories, Austria). The resulting chimeric gene contains the entire HE1 domain and the first four codons of the HE2 domain linked via a five-codon spacer to the coding region of eGFP. This construct was ligated into the recombination vector pBakPAK8. The resulting plasmid pBacPAK-CHE1-eGFP was cotransfected with baculovirus DNA (Pharmingen) into Sf9 cells. Recombinant baculovirus Bak-CHE1-eGFP was plaque-purified and used to express the recombinant HE1-eGFP fusion protein. The expression of the HE-1 domain was sufficient to obtain a specific 9-*O*-acetyl esterase activity. Accordingly, cells (1×10^6) were incubated with the culture supernatant (100 µl) containing recombinant protein for 1 h at 20–25°C, washed, and processed for flow cytometric analysis as described.

Purification of Neu5,9Ac$_2$-GPs

PBMC membranes from clinically confirmed, immunophenotyped children of B-ALL ($n = 10$) and T-ALL ($n = 5$), at presentation of disease, that is, before any drug treatment, having >80% lymphoblasts (as measured using lineage specific established anti-CD antibodies and Achatinin-H) and normal donors ($n = 5$) were prepared according to Weissman *et al*. (1988). In brief, cells (1×10^7) were washed in ice-cold phosphate buffered saline (1.9 mM disodium hydrogen phosphate, 154 mM sodium chloride, pH 7.2) and suspended in lysis buffer containing Tris–HCl (50 mM, pH 7.6), NaCl (300 mM), Triton X-100 (0.5%), phenylmethyl sulfonyl fluoride (0.01 M), and iodoacetamide (1.8 mg/ml). Following incubation for 45 min on ice, the nuclear pellet was discarded by centrifugation at

15,000 × *g* for 15 min at 4°C. To the supernatant, SDS (10%) and sodium deoxycholate (10%) were added to a final concentration of 0.2% each, centrifuged, and the supernatant containing membrane-enriched fraction was stored at −70°C. The purity of membrane fractions was confirmed by measuring 5′ nucleotidase activity, and the protein concentration was determined using BSA as the standard.

The purified Achatinin-H was covalently linked to Sepharose-4B (1.0 mg of Achatinin-H/ml of gel). Equal amount of membrane fractions (0.8 mg) from each patient or normal donors were separately passed through this affinity column (1 × 2 cm) previously equilibrated with Tris-buffered saline (TBS) containing Tris–HCl (0.05 M), NaCl (0.15 M), and sodium azide (0.02%), pH 7.2, with $CaCl_2$ (0.03 M) at 4°C. After nonspecific washing, bound Neu5,9Ac_2-GPs were eluted with TBS containing sodium citrate (0.04 M, pH 7.2), dialyzed against TBS at 4°C and stored at −70°C. The biological activity of Neu5,9Ac_2-GPs_{ALL} and Neu5,9Ac_2-GPs_N was compared by an ELISA where equal amounts of purified fractions (0.4 mg/100 µl/well) were allowed to bind with Achatinin-H on a 96-cell plates, probed with anti-Neu5,9Ac_2-GP antibodies and processed as described in inhibition-ELISA (see later discussion).

Molecular analysis of purified Neu5,9Ac_2-GPs by SDS–PAGE, western blot, and IEF

Affinity-purified Neu5,9Ac_2-GPs were separated by SDS–PAGE (7.5%) according to the method of Laemmli (1970) and stained with Coomassie brilliant blue R-250. For western blot analysis, Neu5,9Ac_2-GPs were transferred after SDS–PAGE onto nitrocellulose at 100 V for 2 h. After blocking the nonspecific binding sites with BSA (10%) in TBS (0.1 M, pH 7.4) the membranes were probed with Achatinin-H (160 µg/ml) in TBS-BSA-Ca^{2+} (0.03 M). After washing, the blot was incubated with rabbit anti-Achatinin-H (diluted 1:500) at 4°C and washed, and the antigen-antibody complex was detected using horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG (Cappel, St. Louis, MO; 1:10,000). For de-*O*-acetylation, the blots were incubated with NaOH (0.1 N) for 45 min at 4°C, neutralized, and processed similarly.

Following SDS–PAGE, the 90- and 120-kDa Neu5,9Ac_2-GP_{ALL} bands were gel-eluted using an Electro-Eluter (Model 422; BioRad, Hercules, CA) according to the manufacturer's instructions and analyzed by IEF on ampholine polyacrylamide gel (4%) using a Mini-Protean II tube cell apparatus (BioRad) at a constant voltage of 400 V for 6 h. The gels were washed with sulfosalicylic acid (5%), methanol (30%), and acetic acid (10%) solution, fixed in trichloroacetic acid (10%), and stained with silver nitrate. The isoelectric point (pI) of individual proteins was determined as a function of their migration from the cathode using standard pI markers ranging from 3.5 to 10 (Bio Rad).

Synthesis of sialic acid analogs

Me-α-Neu5,9Ac_2 (1) and benzyl-α-Neu4,5,9Ac_3 (6) were obtained by partial acetylation of the respective Neu5Ac glycoside using acetimidazole. Me-α-Neu5Ac (2) was prepared as reported earlier (Meindl and Tuppy, 1965; Kuhn *et al*., 1966). Me-α-Neu5Ac9-SAc (3) and Me-α-Neu5Ac9-NHAc (4) were synthesized as previously described (Brossmer and Gross, 1994; Isecke and Brossmer, 1995). Conversion of Me-α-9-amino-Neu5Ac with nitrophenyl fluoroacetate gave the corresponding *N*-fluoroacetyl derivative (5). Reaction of benzyl-α-Neu5Ac with propionic anhydride afforded after purification the corresponding 9-*O*-propionic ester (7). Catalytic hydrogenation of benzyl-α-Neu4,5Ac_2 produced Neu4,5Ac_2 (9). All analogs were characterized by nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectroscopy.

Competitive binding of purified Neu5,9Ac_2-GPs_{ALL} with synthetic sialic acid analogs by inhibition-ELISA

To substantiate the binding specificity of purified Neu5,9Ac_2-GPs_{ALL}, an inhibition-ELISA was developed. Several sialic acid analogs (Troncoso *et al*., 2000) were used as inhibitors to compare their potency to inhibit binding of Neu5,9Ac_2-GPs_{ALL} to immobilized Achatinin-H. A microtiter plate was coated with Achatinin-H (1 µg/100 µl/well in 0.05 M TBS, pH 7.4) and incubated overnight at 4°C. Following three washes, the wells were blocked with TBS–2% BSA for 2 h at 25°C. A constant amount of purified Neu5,9Ac_2-GPs_{ALL} (1 µg/50 µl TBS-BSA) was preincubated separately with inhibitor (50 µl, 10–68 mM) in the presence of 30 mM Ca^{2+} at 4°C for 30 min. The mixture (100 µl) was added to lectin-coated wells, incubated overnight at 4°C, and washed thrice with TBS containing 0.1% Tween-20 (TBS-T). The binding of Neu5,9Ac_2-GPs_{ALL} to Achatinin-H was detected by incubating anti-Neu5,9Ac_2-GPs_{ALL} antibodies (1 µg), affinity-purified from sera of ALL patients (Pal *et al*., 2000), overnight at 4°C. The plate was washed thrice with TBS-T, and the specific antigen–antibody complex was measured using HRP-conjugated protein A (diluted 1:10,000, Cappel) and azino-bis-thio-sulfonic acid. The absorbance was recorded at 405 nm in an ELISA reader.

Based on the long-standing evidence that BSM contains a high percentage of 9(8)-*O*-acetylated sialic acid derivatives as estimated fluorimetrically (Sharma *et al*., 1998) and by fluorimetric HPLC (Chatterjee *et al*., 2003) it was also used as an inhibitor in the inhibition-ELISA. In parallel, de-*O*-acetylated BSM, desialylated BSM, and other sialoglycoproteins (such as SSM, HCG, fetuin, and α_1-acid glycoprotein) having no *O*-acetylated sialic acids were also used.

Monitoring disease status by measuring the reactivity of Neu5,9Ac_2-GPs_{ALL} with ALL serum in an antigen-ELISA

Purified Neu5,9Ac_2-GPs_{ALL} (0.5 µg/100 µl TBS/well) were coated overnight on 96-well plate at 4°C. Following three washes with TBS-T, the wells were blocked with 2% BSA. Sera (diluted 1:10) from ALL patients at different stages of treatment, and from patients with other hematological disorders and normal donors were added and incubated overnight at 4°C. After washing thrice with TBS-T, the antigen–antibody complex was detected using HRP-protein A as described. In addition, anti-Neu5,9Ac_2-GPs_{ALL} present in ALL serum was quantified by BSM-ELISA using BSM (1 µg/100 µl/well) as coating antigen and binding of

anti-Neu5,9Ac$_2$-GPs$_{ALL}$ was measured as previously described (Pal *et al*., 2000).

Statistical analysis

Statistical analysis was performed using the Graph-Pad Prism statistics software program (Graph-Pad Software, San Diego, CA). Student's unpaired or paired *t*-tests were used. Reported values are two-tailed and *p*-values lower than 0.05 were considered statistically significant. The Spearman correlation test was used for the comparison of independent variables. Life table analysis according to Kaplan and Meier were performed for relapse-free intervals and overall survival.

Acknowledgments

S. Ghosh received her senior research fellowship from University Grants Commission, New Delhi, India. Dr. R. Vlasak (Applied Biotechnology GmbH, Salzburg, Austria) is acknowledged for his generous gift of esterase fusion protein. We are thankful to A. Mallick for excellent technical help and Dr. M. Chatterjee (Dr. B.C. Roy P.G. Institute of Basic Medical Sciences, IPGMER, Kolkata, India) for helpful discussions. This work was supported by the Department of Science and Technology and Indian Council of Medical Research, government of India, Deutscher Akademischer Austauschdienst (Bonn, Germany), the Sialic Acids Society (Kiel, Germany), and the Fonds der Chemischen Industrie (Frankfurt, Germany).

Abbreviations

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BSA, bovine serum albumin; BSM, bovine submandibular gland mucin; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; DFS, disease-free survival; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCG, human chorionic gonadotropin; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IEF, isoelectric focusing; NHL, non-Hodgkin's lymphoma; PBMC, peripheral blood mononuclear cell; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SSM, sheep submaxillary gland mucin; TBS, Tris-buffered saline.

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