Glycoconj J (2009) 26:57–73
DOI 10.1007/s10719-008-9163-3

High level of sialate-*O*-acetyltransferase activity in lymphoblasts of childhood acute lymphoblastic leukaemia (ALL): enzyme characterization and correlation with disease status

Chandan Mandal · G. Vinayaga Srinivasan · Suchandra Chowdhury · Sarmila Chandra · Chhabinath Mandal · Roland Schauer · Chitra Mandal

Received: 8 February 2008 / Revised: 13 June 2008 / Accepted: 16 June 2008 / Published online: 3 August 2008
© Springer Science + Business Media, LLC 2008

Abstract Previous studies had established an over-expression of 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac$_2$-GPs) on lymphoblasts of childhood acute lymphoblastic leukaemia (ALL). Here, we report the discovery and characterization of sialate-*O*-acetyltransferase enzyme in ALL-cell lines and lymphoblasts from bone marrow of children diagnosed with B- and T-ALL. We observed a positive correlation between the enhanced sialate-*O*-acetyltransferase activity and the enhanced expression of Neu5,9Ac$_2$-GPs in these lymphoblasts. Sialate-*O*-acetyltransferase activity in cell lysates or microsomal fractions of lymphoblasts of patients was always higher than that in healthy donors reaching up to 22-fold in microsomes. Additionally, the V_{max} of this enzymatic reaction with AcCoA was over threefold higher in microsomal fractions of lymphoblasts. The enzyme bound to the microsomal fractions showed high activity with CMP-*N*-acetylneuraminic acid, ganglioside GD3 and endogenous sialic acid as substrates. *N*-acetyl-7-*O*-acetylneuraminic acid was the main reaction product, as detected by radio-thin-layer chromatography and fluorimetrically coupled radio-high-performance liquid chromatography. CMP and coenzyme A inhibited the microsomal enzyme. Sialate-*O*-acetyltransferase activity increased at the diagnosis of leukaemia, decreased with clinical remission and sharply increased again in relapsed patients as determined by radiometric-assay. A newly-developed non-radioactive ELISA can quickly detect sialate-*O*-acetyltransferase, and thus, may become a suitable tool for ALL-monitoring in larger scale. This is the first report on sialate-*O*-acetyltransferase in ALL being one of the few descriptions of an enzyme of this type in human.

Keywords Achatinin-H · Acute lymphoblastic leukaemia (ALL) · Lymphoblast · Sialate-*O*-acetyltransferase · 9-*O*-acetylated sialic acids

C. Mandal · S. Chowdhury · C. Mandal (✉)
Infectious Diseases and Immunology Division,
Indian Institute of Chemical Biology,
4 Raja S.C. Mullick Road, Jadavpur,
Kolkata 700 032, India
e-mail: cmandal@iicb.res.in

S. Chandra
Haematology, Kothari Medical Center,
8/3, Alipore Road,
Kolkata 700027, India

C. Mandal
Structural Biology and Bioinformatics,
Indian Institute of Chemical Biology,
4, Raja S. C. Mullick Road,
Kolkata 700032, India

G. V. Srinivasan · R. Schauer
Biochemisches Institut, Christian-Albrechts Universität,
Olshausenstr. 40,
24098 Kiel, Germany

Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
AcCoA	acetyl-coenzyme A
ALL	acute lymphoblastic leukaemia
AML	acute myelogenous leukaemia
BM	bone marrow
BSA	bovine serum albumin
BSM	bovine submandibular gland mucin
CML	chronic myeloid leukaemia

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMP-Neu5Ac	cytidinmonophosphate *N*-acetylneuraminic acid
CoA	coenzyme A
cyt-μ	cytoplasmic μ
Cy5	cyanin 5
de-*O*-AcBSM	de-*O*-acetylated BSM
DAB	3,3′-diamino benzidine
DMB	1,2-diamino-4,5-methylene-dioxybenzene
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GD3	ganglioside GD3
HPLC	high-performance liquid chromatography
HRP	horse radish peroxidase
IgG	immunoglobulin G
Mab	monoclonal antibodies
GalNAc	*N*-acetylgalactosamine
$Neu5,7Ac_2$	*N*-acetyl-7-*O*-acetylneuraminic acid
$Neu5,8Ac_2$	*N*-acetyl-8-*O*-acetylneuraminic acid
$Neu5,9Ac_2$	*N*-acetyl-9-*O*-acetylneuraminic acid
$Neu5,9Ac_2$-GPs	*O*-acetylated sialoglycoproteins
PCA	perchloric acid
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
Sia	sialic acids
SOAT	sialate-*O*-acetyltransferase
SD	standard deviation
SIg	surface membrane Ig
TdT	terminal deoxynucleotidyl transferase
TLC	thin-layer chromatography
TBS	Tris-buffered saline

Introduction

Childhood acute lymphoblastic leukaemia (ALL) is highly responsive to chemotherapy and with current treatment protocols, virtually all patients achieve remission and nearly 80% are eventually cured. However, considering the increasing number of new incidences and relapse amongst the cases, the need exists for easily detectable and stably expressed specific markers, whose altered expression profile could be used for comprehensive and reliable monitoring of ALL [1].

Sialic acids (Sia) constitute a family of *N*- and *O*-substituted 9-carbon monosaccharides, the most ubiquitous member being *N*-acetylneuraminic acid (Neu5Ac). Frequent are *O*-acetylations at positions C-7, C-8 or C-9 of sialic acids that generate a family of *O*-acetylated sialoglycoconjugates [2–5]. These are found in nearly all higher animals studied and in certain bacteria and have been recognized to play a pivotal role in modulating various biological and pathological processes such as cell–cell and virus–cell adhesion, signalling, differentiation, immune reactions including apoptosis and malignancy [2–5]. Recently, differential expression and possible function of 9-*O*- and 7-*O*-acetylated GD3 during apoptosis of human erythrocytes, embryonic kidney cells, epithelial cells and lymphocytes has been reported [6–8]. Since the appearance of *O*-acetylated sialic acids on glycoconjugates, glycoproteins or glycolipids is cell type-specific and developmentally regulated, it is expected that their synthesis and turnover is a finely tuned phenomenon [9]. Therefore, it is necessary to get more information about the enzymes responsible for regulation of the expression of *O*-acetylated sialoglycotopes. Following the translocation of CMP-sialic acid into the Golgi apparatus, sialyltransferases catalyze the transfer of sialic acid onto an appropriate acceptor molecule [10]. Subsequently, the sialoglycoconjugate thus generated may be further modified by sialate-*O*-acetyltransferases (SOATs) [3,4,9, 10]. Lrhorfi *et al,* however, have shown that in bovine submandibular gland *O*-acetylation can occur even at the CMP-sialic acid level [11]. Exogenous factors seem to induce sialic acid *O*-acetylation, as was observed in several cell lines after transfection of an α2,6-sialyltransferase [12], the GD3 synthase, and exogenously added ganglioside GD3 [13].

Sialate-*O*-acetyltransferase activity has been reported in bovine submandibular gland [11,14], livers of rat [15] and guinea pig [16], and human colon [9, 17,18]. Because of the frequent occurrence of *O*-acetylated sialic acids in the deuterostome animal lineage and their important functions, understanding the regulation of this enzyme is required. An increased expression of *O*-acetylated GD3 was reported in human melanoma [19, 20], basalioma, [21] and human breast cancer [22]. However, human skin contains very little *O*-acetylated sialic acids.

The role of *O*-acetylation in these tumours and the activity of SOAT possibly involved in their *O*-acetylation have not yet been investigated. Alternatively, the reduced enzyme activity in human colon carcinoma corroborated with decreased *O*-acetylation in the course of tumour development [18].

Another malignant disease, which is accompanied by an increase of *O*-acetyl sialic acid expression on glycoproteins, is childhood ALL. For these studies we used the preferential specificity of the snail lectin Achatinin-H for glycoproteins with terminal 9-*O*-acetylated sialic acid ($Neu5,9Ac_2$) derivatives α2–6-linked to subterminal *N*-acetylgalactosamine (GalNAc) to identify and quantify these specific sialoglycotopes [23]. In previous studies, we have detected 9-*O*-acetylated sialoglycoproteins

(Neu5,9Ac$_2$-GPs) on peripheral blood mononuclear cells (PBMC) of ALL patients but not in corresponding cells of healthy children or in patients with other haematological disorders [24–27]. The high level of Neu5,9Ac$_2$-GPs and antibodies against Neu5,9Ac$_2$-GPs, characteristics of this disease, may help these lymphoblasts to evade apoptosis [28, 29]. The cell surface Neu5,9Ac$_2$-GPs [30] and anti-Neu5,9Ac$_2$-GPs antibodies [31] have been used for monitoring the disease status [24]. Therefore, it may be envisaged that, in disease condition, modification of the activity of an enzyme like sialate-*O*-acetyltransferase, would be reflected in an altered concentration of *O*-acetylated sialoglycoconjugates. In this context, identification of SOAT activity may provide insight into the pathogenesis of this disease, its progression and may even provide clues for designing new drugs.

Due to the possible association of the expression of Neu5,9Ac$_2$-GPs with SOAT activity, we have investigated this enzyme and report the identification, characterization and status of SOAT in lymphoblasts isolated from bone marrow (BM) of children with B- and T-ALL and in normal PBMC. A significant increase in the relative activities of SOATs, both in cell lysates and microsomal fractions of lymphoblasts from BM of B- and T-ALL, as compared to healthy donors, was demonstrated by a radiometric assay and a non-radiometric ELISA. The transfer of *O*-acetyl groups specifically to sialic acids was demonstrated and the types of these neo-*O*-acetylated sialic acids were identified by means of radio-TLC and radio-HPLC. The SOAT activity increased rapidly with the onset of disease, decreased with clinical remission and sharply increased again with clinical relapse and correlated well with high levels of cell surface Neu5,9Ac$_2$-GPs on lymphoblasts.

Materials and methods

Chemicals and substrates

Chemicals were of analytical grade. [^{3}H]AcCoA (specific activity, 208 mCi/mmol) and [^{14}C]AcCoA (59 mCi/mmol) were from Amersham Bioscience (Braunschweig, Germany). CMP-Neu5Ac was obtained from Calbiochem–Novabiochem (Bad Soden, Germany). Unless otherwise stated, TLC plates (cellulose, 20×20 cm^2), HPTLC silica gel 60 plates (10×10 cm^2), reversed-phase columns (RP18, LiChrospher 100, particle size 5 mm) and HPLC solvents (gradient grade) were obtained from Merck (Darmstadt, Germany). Dowex 2×8 (200–400 mesh) was purchased from Pharmacia Biosystems (Freiburg, Germany). DMB was obtained from Dojindo Laboratories (Tokyo, Japan). Mini complete protease inhibitor, ABTS and Pefabloc SC were from Roche Molecular Biochemicals (Mannheim, Germany). Ganglioside GD3, AcCoA, Neu5Ac, CMP, CoA, FCS, transferrin [32], α_1-acid glycoprotein [33] and fetuin [34] were from Sigma-Aldrich, St. Louis. All PE- and Cy5-conjugated lineage-specific monoclonal antibodies (Mabs) were from BD Pharmingen, San Diego.

Bovine submandibular gland mucin (BSM) [35] and ovine submandibular mucin (OSM) [36] were isolated from the corresponding tissues. BSM contains 38% Neu5,9Ac$_2$ (α2–6 linked to GalNAc) as measured fluorimetrically [37]. It was saponified in the presence of NaOH (0.2 M) for 30 min at 25°C followed by neutralization with HCl (0.2 M) and this de-*O*-acetylated BSM (de-*O*Ac-BSM) was used as an acceptor.

The lectin, Achatinin-H, was purified from the haemolymph of the snail *Achatina fulica* by affinity chromatography using BSM as affinity matrix [23]. The preferential affinity of Achatinin-H towards Neu5,9Ac$_2\alpha$2–6GalNAc was exploited to identify the selective presence of Neu5,9Ac$_2$-GP on lymphoblasts. However, as the binding of this lectin towards 7-*O*- or/and 8-*O*-Ac sialic acids cannot be excluded, therefore such linkages in *O*-acetylated sialoglycoproteins may also be present. Achatinin-H was conjugated with fluorescein isothiocyanate (FITC) for flow cytometry and coupled to sepharose 4B for purification of neo-*O*-acetylated sialoglycoproteins to establish the identity of the reaction products following acetyl transfer to sialoglycoproteins.

Cell lines

Human B- (REH) and T-ALL (CEMC7, MOLT-4) cell lines were sourced from American Type Culture Collection (Maryland, USA), ALLPO, a human B-ALL cell line, was a kind gift from Prof. Bruno Venerando, University of Milan, Italy. Cells were cultured in RPMI-1640 medium supplemented with heat-inactivated FCS [10% (*v*/*v*), L-glutamine (0.002 M), antibiotics and antimycotics.

Selection of lymphoblasts from diseased and healthy donors

The study included clinically confirmed ALL patients, at presentation (20 males and five females, median age: 6 years, range: 0.8–15 years, median white blood cell count: 12×10^9/L, range 0.4–1,000×10^9/L of the Kothari Medical Centre, Kolkata. The diagnosis of ALL was established by cytological examination of BM smears according to the FAB group recommendations, belonging to L1 or L2. Bone marrow (1–2 ml) was collected from diagnosed patients (>65% leukaemia blasts as per morphological estimation) and sent to the Indian Institute of Chemical Biology, Kolkata, where lymphoblast from BM were prepared by density gradient centrifugation (Ficoll-

Hypaque, Amersham Pharmacia, Uppsala, Sweden) and used for immunophenotyping by flow cytometry (FACS-Calibur, Becton Dickinson, San Jose, CA). The data was analyzed with the CellQuestPro software (Becton Dickinson). The PE- and PECy5-conjugated lineage-specific Mabs against terminal deoxynucleotidyl transferase (TdT), cytoplasmic μ (cyt-μ), surface membrane Ig (SIg), CD3, CD7, CD10, CD13, CD19, CD33 and CD34 (Pharmingen, San Diego, CA) were used for immunologic subgroups of these patients and they were defined as follows: common ALL ($CD19^+$, $CD10^+$, cyt-μ^-, SIg^-, n=10), pre B-ALL ($CD19^+$, $CD10^{\pm}$, cyt-μ^+, SIg^-, n=7), T-ALL ($CD3^+$, $CD7^+$, n=5) and mixed lineages ($CD19^+$, $CD10^+$, $CD7^+$, n=3). These lymphoblasts were $CD45^{low}$ as compared to normal human peripheral blood mononuclear cells ($PBMC_{N,}$ $CD45^{high}$) separated from healthy donors. Lymphoblasts (70–95%) from each of these clinically confirmed patients showed ALL-associated antigens ($Neu5,9Ac_2$-GPs) using FITC–Achatinin-H by flow cytometry [25–26]. The presence of $Neu5,9Ac_2$ on these lymphoblasts was reconfirmed with FITC–Achatinin-H after 1 h at 37°C pre-treatment or no pre-treatment with 9-*O*-acetyl esterase of influenza C virus, known to specifically cleave off *O*-acetyl groups from sialic acids. The sera of these patients were accompanied by high levels of anti-$Neu5,9Ac_2$-GPs antibodies against $Neu5,9Ac_2$-GPs, A_{405nm} being 0.95–1.12 as compared to 0.04–0.20 observed in normal sera by an ELISA using BSM as coating antigen developed in our laboratory [27].

Children were entered into MCP841 protocol and samples were assayed at diagnosis, clinical remission and during clinical relapse. A few children (n=20) were longitudinally monitored for 90 weeks. Controls included age-matched healthy individuals of both sexes and different blood groups having low positivity of $Neu5,9Ac_2$-GPs^+ cells (5±2%, n=15). Patients diagnosed for other haematological disorders (n=5) were included. Chronic myeloid leukaemia (CML) and acute myelogenous leukaemia (AML), exhibiting a low percentage of $Neu5,9Ac_2$-GPs^+ cells (8.58±0.69), served as controls. The Institutional Human Ethical Committee had approved the study and samples were taken with the consent of donors, patients, or their parents or guardians.

Subcellular fractionation of lymphoblasts

Lymphoblasts from BM of individual patients ($1–2\times10^8$) and PBMC of healthy donors were homogenized (six pulses of two strokes each giving break of 1 min on ice) with a glass homogenizer in 0.025 M sucrose, 20 mM Tris/HCl, pH 7.2, and centrifuged at 600×*g* for 10 min. The Golgi vesicles were obtained by using differential sucrose density gradient centrifugation [15, 18, 38] and immediately used for enzyme assays. The intactness of these membranes was determined using sialyltransferase [39] as a marker enzyme, with asialofetuin and CMP-[^{14}C]Neu5Ac in the presence and absence of Triton X-100 (0.2%). In a typical preparation, sialyltransferase was enriched about 4-fold in the microsomal fraction compared to cell homogenate, radioactivity being 936±63 cpm vs. 226±29 cpm (n=6) using equal amounts of protein. Sialyltransferase latency towards Triton X-100 indicated that approximately 85% of the microsomal membranes were intact and correctly orientated. Protein was estimated by the method of Lowry [40].

Chemical analysis of sialic acids

Quantification of sialic acids by fluorimetric estimation using acetylacetone method

Cell lysates of lymphoblasts from BM of ALL patients (n=5) were prepared by sonication (three pulses of 8 s each giving break of 1 min) of $1–2\times10^8$ cells in water on ice and used as a source of sialate-*O*-acetyltransferases in the presence of exogenous substrates, *e.g.* de-*O*-AcBSM and unlabelled AcCoA (5 μM) as donor. SOAT reaction was performed in Tris/HCl (10 mM), KCl (150 mM) and $MgCl_2$ (1 mM) designated as TKM buffer (pH 7.0) at 37°C for 60 min. Microsomal fractions containing endogenous substrates were also used as source of SOAT. The reaction was performed under identical condition using unlabelled AcCoA (5 μM) as donor. Quantification of *O*-acetylated sialic acids (%) both in the microsomal fractions (25 μg) and cell lysates (100 μg), before and after SOAT treatment was performed fluorimetrically by oxidizing an aliquot of SOAT-reaction products (25 μg) and processed using the acetylacetone method with and without saponification of the *O*-acetyl group of sialic acids [37]. The relative fluorescence intensity [$\lambda_{max(excitation\ =\ 410\ nm)}/\lambda_{max(emission\ =\ 510\ nm)}$] of each sample was measured against reagent blanks on a Hitachi F-4010 spectrofluorimeter (Tokyo, Japan). The sialic acid content was determined from a standard curve obtained using pure sialic acid. The values obtained for the de-*O*-acetylated samples indicated total sialic acid content, while the percentage of *O*-acetylated sialic acid was determined by subtracting the respective unsubstituted sialic acids from that obtained after de-*O*-acetylation. Cell lysates alone or only the acceptor was used as controls.

Analysis of sialic acids in microsomal fractions

Sialic acids present in the microsomal fraction were detected and quantified either fluorimetrically by using the acetylacetone method described above or with the orcinol/

Fe^{3+}/HCl reagent or by integrating the corresponding peaks from fluorimetric HPLC of sialic acids liberated by propionic acid from the microsomes, using sialic acids from BSM as standards [41,42].

Sialate-O-acetyltransferase assays

1. Radiometric assay using endogenous substrate, glycoproteins and [^{3}H]AcCoA

The reaction was carried out by incubating at 37°C for 60 min the microsomal fractions (0–30 μg protein) as source of enzyme and [^{3}H]AcCoA (10 μl, 0.05 μCi, 2.4 μM) in TKM buffer (pH 7.0). Reaction mixtures (100 μl) were quenched with ice-cold perchloric acid (PCA, 10%), incubated for 1 h on ice and centrifuged at 10,000×*g* for 10 min at 4°C. The precipitated protein was washed sequentially with 1.0 ml ice-cold PCA (8% followed by 4%), dissolved in 0.70 ml NaOH (1 N) and incubated overnight at 25°C. The protein solution was vortexed, neutralized with HCl (1 N, 0.70 ml) and radioactivity was measured in a β-counter (Packard Bioscience Company, USA) using Cocktail-W (4.0 ml). Optimal conditions of incubation regarding temperature, time, pH, concentrations of microsomal fractions and [^{3}H] AcCoA were determined. The enzyme activity was also checked in the presence of Triton X-100, CHAPS and $ZnCl_2$. The specificity of the acetylation reaction was established by pre-incubating microsomal fractions with CMP (0–1.0 mM) and CoA (0–1.0 mM) [9]. For some studies, the reactions were quenched with ice-cold buffer (4 ml) and vesicles were re-isolated by centrifugation, washed, sonicated and PCA-soluble and insoluble radioactivities were separately determined [43].

In parallel, the activity of sialate-*O*-acetyltransferase was determined using cell lysate prepared by sonication (three pulses of 8 s each giving break of 1 min) of $1–2\times10^8$ lymphoblasts in water on ice. SOAT activity was measured using cell lysate (0–150 μg protein) and [^{3}H]AcCoA (10 μl, 0.05 μCi, 2.4 μM) in TKM buffer (10 μl) in a total volume of 100 μl in the presence of equal amounts (5.0 μg Sia) of exogenous acceptor substrates such as de-*O*-AcBSM, OSM, fetuin, α_1-acid glycoprotein and transferrin according to the method described above using perchloric acid for precipitation. Enzyme stability was established by heat-inactivation of the cell lysate (30 min at 57°C) and activity was also checked in the presence of Triton X-100, CHAPS and $ZnCl_2$. In the control experiment buffer instead of exogenous substrates was added.

Incorporation of acetyl groups from AcCoA into amino- and hydroxyl groups were determined by mild saponification of the reaction product using 0.10 N NaOH at 4°C for 30 min after radiometric assay [44]. The supernatant was neutralized and the radioactivity was determined. As *O*-acetylation is labile to mild alkali hydrolysis, the count in this fraction will represent *O*-acetylation. The residual pellet was dissolved in 1.0 N NaOH and processed similarly for measurement of possible *N*-acetylation.

2. Radiometric assay using endogenous substrate, CMP-Neu5Ac, ganglioside GD3 and [^{14}C]AcCoA and analysis of the reaction product by TLC and HPLC

In order to obtain chemical proof of the reaction products from sialate-*O*-acetyltransferase reaction, we employed the methods described by G.V. Srinivasan *et al.* (Glycoconj J. doi:10.1007/s10719-008-9131-y-2008, 9, 18). Shortly, microsomal fractions from cell lines and ALL patients (0.025–0.10 mg protein) without exogenous substrate or in the presence of CMP-Neu5Ac (600 μM) or ganglioside GD3 (100 μM) as exogenous substrates were incubated in phosphate buffer, pH 6.5, with ^{14}C-AcCoA (0.04 μCi) in a total volume of 0.20 ml and incubated at 37°C for 60 min.

For analysis of the radioactive neo-*O*-acetylated sialic acids formed with endogenous substrate, sialic acids were released by 4 h hydrolysis in 2 M propionic acid at 80°C of the microsomal pellet and the soluble products, after centrifugation at 14,000×*g* for 30 min were analyzed by radio-TLC on cellulose (see below).

In experiments with CMP-Neu5Ac, after incubation, the glycosidic bonds of CMP-sialic acids were hydrolyzed by heating the mixture in 2 M propionic acid for 15 min at 80°C. After removal of precipitated proteins by centrifugation at 14,000×*g* for 30 min in the cold, the supernatant was lyophilized. The liberated sialic acids were analyzed by radio-TLC on cellulose or by radio-HPLC (see below). When assaying enzyme activity with GD3, the reaction mixture, after centrifugation, was directly analyzed by radio-TLC. For radio-HPLC, the ganglioside sialic acids were liberated by 4 h hydrolysis in 2 M propionic acid at 80°C.

The nature of radioactive neo-*O*-acetylated sialic acids liberated from endogenous substrates was identified by radio-TLC and saponification. Sialic acids from the particle-free incubation mixtures or hydrolysates were developed on cellulose thin layers (0.1 mm) on glass plates using ethanol/ammonium acetate (7:3) as solvent. Radioactive sialic acids were co-chromatographed with an authentic sialic acid standard mixture from BSM. Gangliosides were identified by TLC on silica gel using chloroform/methanol/water containing 25 mM $CaCl_2$ (5:4:1) as solvent. 9-*O*-Acetylated GD3, kindly donated by Dr. B. Kniep, University of Dresden, Germany, was co-chromatographed. The radiolabelled compounds were detected using an LB 284 automatic TLC linear analyzer (Berthold, Wildbad, Germany), followed by staining with orcinol reagent.

Fluorimetrically coupled radio-HPLC was also applied for the analysis of the sialic acids formed by SOAT

reaction. Sialic acids were prepared from the microsomal fractions reaction product using the procedure as described above and purified on a column of Dowex 2×8 (200–400 mesh). Purified sialic acids were derivatized with DMB reagent, mixed with an appropriate amount of derivatized BSM standard sialic acids and analyzed by fluorimetric HPLC (instrument parts from Hitachi and Merck, Darmstadt, Germany) using the method described by Hara *et al.* [42] coupled to a radio-HPLC equipment (Berthold, Wildbad, Germany). The retention times of the various sialic acids detected by HPLC were compared with the authentic DMB derivatized sialic acid standards co-injected. The amount of individual sialic acids separated by this method was calculated via a standard curve constructed from known amounts of Neu5Ac (5–20 ng) against the corresponding area of the integrated peak. There was a time delay of about 1.5 min of monitoring the radioactive peaks compared to the electrometrically registered sialic acids, which was verified by running a radioactive Neu5Ac standard in parallel.

3. Isolation of neo-*O*-acetylated glycoproteins from microsomes of lymphoblasts

The proportion of incorporation of [^{3}H]acetyl group into the glycerol side chain of sialic acids of sialoglycoproteins by sialate-*O*-acetyltransferase was determined by purifying newly formed *O*-acetylated sialoglycoproteins using Achatinin-H as an affinity matrix. Microsomal fractions from lymphoblasts of BM of ALL patients (n=3) were incubated at 37°C for 60 min at pH 7.0 with [^{3}H]AcCoA as described above. The reaction mixture was incubated with solubilization buffer containing 0.5% CHAPS, β-octyl glucoside, 0.01 mM $MgCl_2$, 0.03 mM $CaCl_2$ and protease inhibitors followed by passing through a P-10 column to deplete low molecular weight radioactivity ([^{3}H]AcCoA) and the void volume containing reaction product was allowed to bind with Achatinin-H covalently linked to sepharose-4B (1.0 mg Achatinin-H per milliliter gel) previously equilibrated with Tris-buffered saline (TBS) containing Tris–HCl (0.05 M, pH 7.2), NaCl (0.150 M) and $CaCl_2$ (0.03 M), TBS/Ca^{+2} at 4°C [27]. After washing of the unbound radioactivity, radioactive neo-*O*-acetylated sialoglycoproteins were eluted with TBS containing sodium citrate (0.04 M, pH 7.2) and radioactivity in unbound and bound fractions was determined. Radioactivity bound to the column corresponds to the neo-*O*-acetylated sialoglycoproteins.

4. Quantitation of neo-*O*-acetylated sialoglycoprotein by a newly developed ELISA

De-*O*-AcBSM (20 ng in 0.02 M TBS, pH 7.5/100 µl/well) was immobilized on a 96 well flat-bottomed polystyrene microtitre plates (Nunc-Immunoplate, USA) for overnight at 4°C. After discarding the unbound protein the wells were washed thrice with TBS and the non-specific binding sites were blocked for 2 h at 4°C with TBS containing bovine serum albumin (BSA, 2%). The reaction mixture (100 µl) containing microsomal fraction (50 ng protein) of ALL patients (n=10), and AcCoA (5 nM) in TKM buffer (pH 7.2) was added to the well and incubated for 60 min at 37°C. Subsequently, the wells were washed thrice with TBS and the reaction product was quantified by incubating the wells with Achatinin-H (0.50 µg) in TBS-BSA (2%) in the presence of 30 mM $CaCl_2$ overnight at 4° C. The extent of specifically bound lectin was detected with rabbit anti-Achatinin-H antibody. The bound complex was estimated using HRP-goat anti-rabbit IgG (1:5000, Sigma) with the addition of the chromogenic substrate, ABTS (100 µl/well) and absorbance measured at 405 nm by an ELISA reader (Win Read V.2.1, Anthos Labtec, UK). Absorbance values greater than mean plus three times the standard deviation (SD) of normal controls were considered as positive. The extent of Achatinin-H binding gave a measure of the degree of *O*-acetylation of de-*O*-AcBSM. The assay was initially carried out by varying the concentration of microsomal fractions (0–100 ng protein).

Kinetics of sialate-*O*-acetyltransferase catalysis

The kinetics (K_M and V_{max}) of sialyl-*O*-acetyltransferase catalysis were studied by varying the concentration of [^{3}H]AcCoA (0–10 µM) using 25 µg of microsomal fractions prepared from lymphoblasts isolated from BM of patients. Additionally, cell lysates (100 µg) from the same patients and de-*O*-AcBSM containing 5 µg of Sia were incubated with varying concentrations of [^{3}H]AcCoA. Similarly, mixtures of cell lysates (100 µg) and [^{3}H]AcCoA (2.4 µM) were used with different concentrations of de-*O*-AcBSM. K_M and V_{max} values were obtained from plots of [V] vs. [S] where [V] is the initial velocity at a substrate concentration [S]. Equal amounts of both microsomal fractions and cell lysates from healthy donors were used for comparison.

Statistical analysis

This was performed using the Graph-Pad Prism statistics software program (Graph-Pad Software Inc., San Diego, CA, USA). Results are expressed as mean ± SD. for individual sets of experiments. Each experiment was performed four to five times and the results are representative of each set of experiment. Reported values are two-tailed, with $p<0.05$ considered statistically significant. The correlation coefficient was determined between individual SOAT activities of ALL patients, as determined by radiometric method, and percentage of Neu5,9Ac_2-GPs^+ cells, as determined by FACS analysis using FITC–Achatinin-H, present in these children along with normal PBMC.

Results

The microsomes from a representative T-ALL cell line (CEMC7) contained 33 µg sialic acid/mg protein after mild acidic liberation and measurement by HPLC (Fig. 1). The main sialic acid was Neu5Ac (96%) together with small amounts of Neu5,9Ac$_2$ (3%) and Neu5Gc (1%).

Various enzymatic assays were developed which show that the microsomes from both normal and ALL lymphoblasts or cell lines express significant and variable sialate-*O*-acetyltransferase activities. The reaction products were shown to represent neo-*O*-acetylated sialic acids both indirectly by the incorporation of alkali-labile radioactive acetyl groups or with the aid of Achatinin-H on the one hand and as direct chemical proof by the isolation of radioactively labelled *O*-acetylated sialic acids formed which were identified by TLC and HPLC on the other.

Optimization of the enzyme assay using [^{3}H]AcCoA

The assay was initially standardized at different temperatures, time and pH using microsomal fractions (25 µg protein) of lymphoblasts from BM of both B- (n=3) and T- (n=2) ALL patients, as source of SOAT and [^{3}H]AcCoA as the donor in the absence of any exogenous acceptor. However, when cell lysate (100 µg) was used as source of SOAT, an exogenous acceptor like de-*O*-AcBSM was used. The incorporation of radioactivity following transfer of [^{3}H]acetyl groups to the endogenous or exogenous acceptors in the pellet-associated acid-insoluble fraction served as a measure of SOAT activity. The maximum transfer of *O*-acetyl groups was at 37°C (Fig. 2a) when incubated for 60 min (Fig. 2b) at pH 7.0 (Fig. 2c). To obtain maximum transfer of acetyl groups to the acceptor, increasing concentrations of microsomal fractions (Fig. 2d) and cell lysates (Fig. 2e) were used for this assay. A linear increase in radioactivity was observed up to 100 µg of cell lysate and 25 µg of microsomal fraction in all five patients.

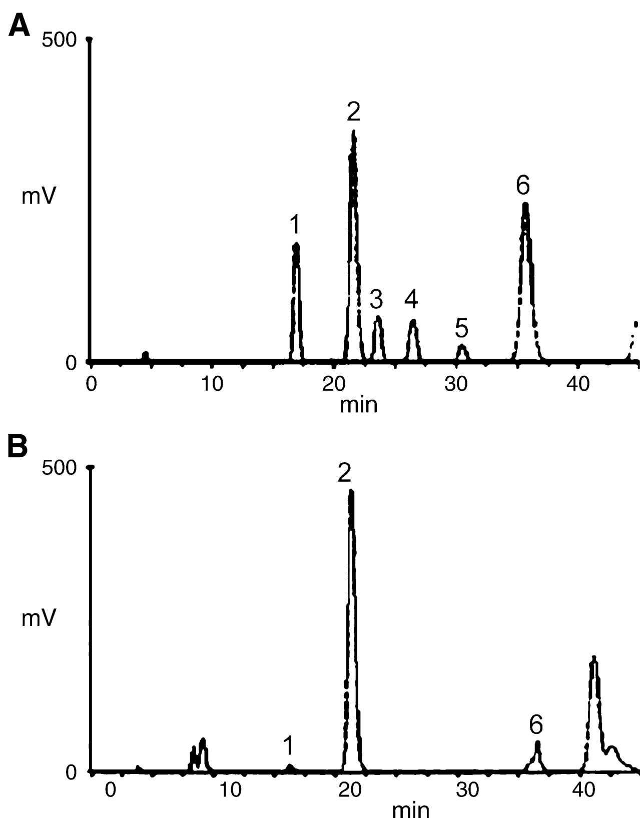

Fig. 1 Fluorimetric HPLC analysis of sialic acids isolated from microsomes of a representative T-ALL cell line (CEMC7). **a** BSM standard sialic acids. *1* Neu5Gc, *2* Neu5Ac, *3* Neu5,7Ac$_2$, *4* Neu5Gc9Ac, *5* Neu5,8Ac$_2$, *6* Neu5,9Ac$_2$. **b** Microsome sialic acids. The *numbering* of the identified peaks corresponds to that in **a**. For further experimental details see "Materials and methods"

Negligible radioactivity in the absence of cell lysate (87.77±12.6 cpm) served as control. Cell lysates from all these five patients showed an approximately threefold lower radioactivity in the absence of an acceptor having all other incubation components, indicated the presence of some endogenous activity in the lysate; average radioactivity in presence and absence of acceptor being 3,392±167 vs. 1,164±159 cpm.

The enzyme was heat-labile as it lost 75% of activity at 57°C for 30 min. Both microsomal fractions and cell lysates showed a sharp decrease in SOAT activity in the presence of increasing concentrations of a non-ionic detergent. Approximately 50% and 94% activity was lost with 0.015% and 0.10% of Triton X-100. In contrast, approximately 29% increase in SOAT activity in microsomal fraction was observed in the presence of CHAPS (12 mM). Incorporation of [^{3}H]AcCoA into microsomal fraction was gradually inhibited with increasing concentrations of CMP and CoA leading to a reduction of 43% and 45% respectively, in the presence of 1.0 mM CMP and CoA. $ZnCl_2$ (1 mM) inhibited approximately 60% of the SOAT activities of ALL. Similar results were observed using cell lysate.

Incorporation of AcCoA as *N*- or *O*-acetylation was checked by mild saponification of the reaction product using cell lysate from lymphoblasts of a B-ALL patient. The radioactivity (2,877±145 cpm, 96.0%) in the supernatant was due to *O*-acetylation. Negligible radioactivity (118±12 cpm) was obtained in the residual pellet probably representing *N*-acetylation.

Utilization of incorporated [^{3}H]AcCoA into intact microsomal fraction

To determine the utilization of incorporated [^{3}H]AcCoA, after SOAT reaction, microsomal fractions from ALL

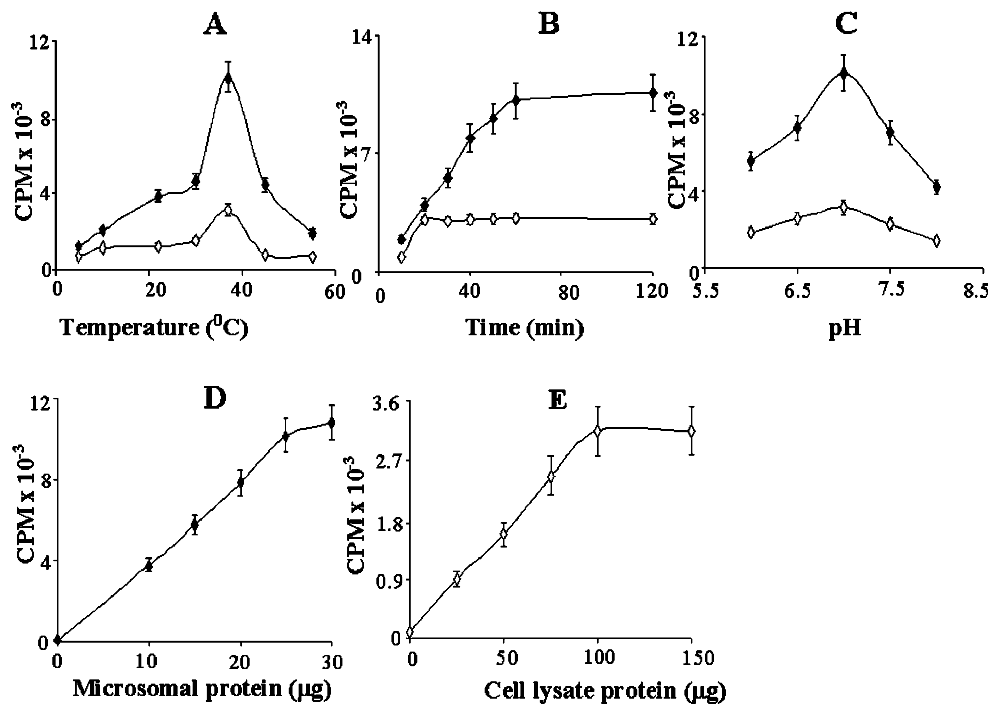

Fig. 2 Optimization of sialate-*O*-acetyltransferase assay in microsomal fractions or cell lysate. The enzyme assay was performed by varying several parameters using endogenous acceptors of microsomal fractions (25 μg protein, *filled diamond*) of BM lymphoblasts from clinically confirmed B- (n=3) and T- (n=2) ALL patients and [^{3}H] AcCoA as donor substrate described in "Materials and methods". In parallel, cell lysates (100 μg protein, *open diamond*) from these patients were processed similarly in which de-*O*-AcBSM containing 5.0 μg of sialic acid served as exogenous acceptor. The radioactivity was counted in a β-counter. **a** The dependence of SOAT activity on temperature. Cell lysates (*open diamond*) and microsomal fractions (*filled diamond*) were incubated at different temperature for 60 min at pH 7.0 separately and the reaction was carried out as above. **b** The dependence of SOAT activity on time of incubation. Cell lysates (*open diamond*) and microsomal fractions (*filled diamond*) were incubated for different time points at 37°C at pH 7.0, separately keeping all the other conditions of the reaction as above. **c** The dependence of SOAT activity on pH of incubation buffer. Cell lysates (*open diamond*) and microsomal fractions (*filled diamond*) were incubated for 60 min at 37°C by varying the pH of the buffer, separately keeping all the other conditions of the reaction as above. **d**, **e** The dependence of SOAT activity on protein concentrations of microsomal fractions and cell lysates. The enzyme assay was similarly performed at 37°C for 60 min, pH 7.0 with increasing concentration of microsomal fractions (**d**, *filled diamond*) and cell lysates (**e**, *open diamond*) of lymphoblasts

patients and ALL-cell lines were centrifuged separately to distinguish incorporated radioactivity between the acid soluble and insoluble fractions (Table 1). The average radioactivity incorporated into the acid-insoluble fractions, *i.e.* protein-associated radioactivity was 6,628±453 cpm, 6,366±360 cpm, 6,262±387 cpm for B- (n=5), T- (n=3) cells and mixed lineage cells (n=2), respectively, as compared to 4,155±153 cpm, 3,285±149 cpm and 3,433±156 cpm in acid-soluble fractions. Thus, the percentage of utilization for sialoglycoprotein *O*-acetylation was 61, 66

Table 1 Uptake and incorporation of radiolabelled AcCoA into microsomes from malignant and normal lymohoblasts

Samples	Radioactivity[a] in intact microsomes (cpm)	
	Utilization[b] (acid insoluble)	Acid soluble
REH and ALLP0 (B-ALL cell lines)	6,504±423	3,215±161
CEMC7 and MOLT-4 (T-ALL cell lines)	6,170±375	3,527±172
B-ALL patient (n=5)	6,628±453	4,155±153
T-ALL patient (n=3)	6,366±360	3,285±149
Mixed lineage-ALL (n=2)	6,262±387	3,433±156
Normal (n=5)	286±25	2,337±123
AML, CML (n=3)	298±22	2,381±158

[a] After SOAT reaction, microsomal fractions were re-isolated and radioactivity in the acid soluble and insoluble fractions was determined as described in "Materials and methods"

[b] Glycoconjugate-associated radioactivity in the acid insoluble fraction reflecting incorporation of acetyl groups into endogenous glycoproteins

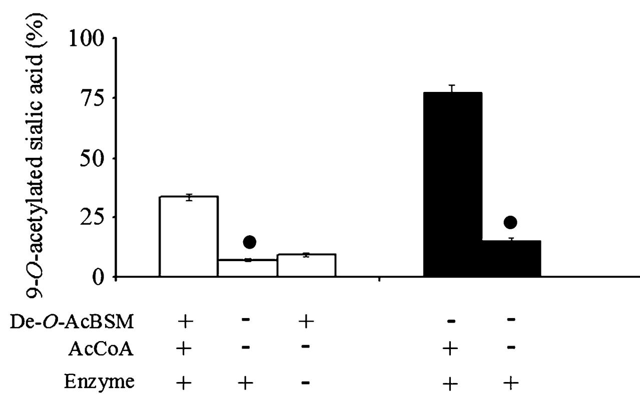

Fig. 3 Quantification of *O*-acetylated sialic acids by fluorimetric estimation using acetylacetone method. Cell lysates (*open bar*) of lymphoblasts from BM of ALL patients ($n=5$) was incubated with exogenous substrates (de-*O*-AcBSM) in the presence of unlabelled AcCoA as donor in TKM buffer (pH 7.0) at 37°C for 60 min. The percent *O*-acetylated sialic acids (percent) present in the reaction product were fluorimetrically quantified as described in "Materials and methods" [37]. In parallel, the incorporation of AcCoA group to from nascent *O*-acetylated sialic acids in microsomal fractions (*filled bar*) containing endogenous substrates of same patients was measured similarly in absence of exogenous acceptor. The non-enzymatic *O*Ac sialic acid level both in lysates and microsomal fractions is marked (·) on top of the column

and 64.5 respectively. ALL-cell lines showed similar results (Table 1). In contrast, microsomal fractions of PBMC from healthy individuals ($n=5$) and cells from AML and CML patients showed very little incorporation of radioactivity in the acid-insoluble fraction as compared to acid-soluble fraction, indicating approximately 10–11% utilization of incorporated radioactivity.

Fluorimetric estimation of sialic acid derivatives after sialate-*O*-acetyltransferase reaction

The transfer of acetyl groups to sialic acids and characterization of the nature of the products formed during incubation of de-*O*-AcBSM with unlabelled AcCoA and cell lysate (100 µg) of lymphoblasts from patients ($n=5$) was confirmed as follows. Native BSM contains 38% *O*-acetylated sialic acids, which on saponification is reduced to 9.2%. After SOAT reaction, the amount of neo-*O*-acetylated sialic acids containing BSM had increased to 33.6% in the reaction product as quantified by the fluorimetric assay using acetylacetone (Fig. 3). In contrast, the cell lysate alone showed only 7.2% *O*-acetylated sialic acids.

The neo-O-acetylated sialic acids formed during incubation of microsomal fractions (25 µg) of lymphoblasts from same patients with unlabelled AcCoA were carried out similarly. Microsomal fractions showed 77.5% *O*-acetylated sialic acids after SOAT reaction, whereas the relative amount of endogenous *O*-acetylated sialic acids present in the same amount of microsomes before the reaction was only 15.4% (Fig. 3). The sialic acid content of the microsomal fraction is 0.24 µg/µg protein.

Affinity purification of *O*-acetylated sialolglycoprotein after sialate-*O*-acetyltransferase reaction

The incorporation and transfer of acetyl groups to endogenous glycoproteins inside the microsomal fraction of lymphoblasts of ALL patients to form *O*-acetylated sialolglycoprotein was further reinforced by affinity purification of the end product. To verify the molecular identity of the reaction product, free [^{3}H]AcCoA was initially removed (Fig. 4a) and the protein peak was affinity-purified (Fig. 4b). The relative amounts of Achatinin-H-bound (11,331±551 cpm, 93.93%) and Achatinin-H-unbound (732±66 cpm, 6.06%) radioactivity showed the proportion of sialoglycoprotein labeled during the sialate-*O*-acetyltransferase reaction (Fig. 4b). The presence of the majority of radioactivity in the affinity-purified reaction product confirmed this transfer of *O*-acetyl groups to endogenous sialolglycoproteins.

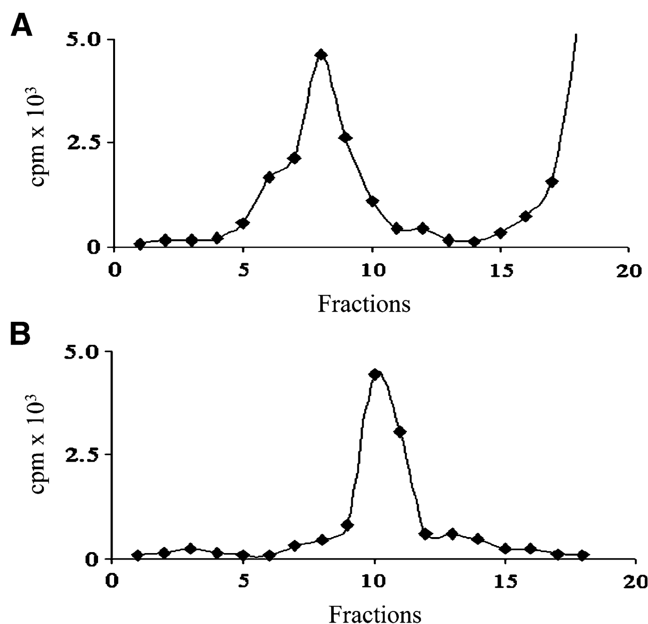

Fig. 4 Analysis of newly formed *O*-acetylated sialoglycoproteins. Microsomal fractions (25 µg protein) from lymphoblasts of BM of ALL patients ($n=3$) were incubated with [^{3}H]AcCoA in TKM buffer (100 µl) for 60 min, pH 7.0 at 37°C. After the SOAT reaction the neo-*O*-acetylated glycoprotein was solubilised and purified by gel filtration (**a**) and affinity chromatography (**b**) on Achatinin-H–Sepharose 4B. For experimental details see "Materials and methods"

Table 2 SOAT activity with various sialoglycoproteins

Acceptors[a]	Type of linkages	Radioactivity[b] (cpm)
De-*O*-AcBSM	Neu5Acα2–6-β-D-GalNAc	2,951±161
OSM	Neu5Acα2–6-β-D-GalNAc	2,922±110
Fetuin	Neu5Acα2–6-β-D-GalNAc, Neu5Ac α2–3-β-D-Gal	2,843±137
α_1-Acid glycoprotein	Neu5Ac α2–6-β-D-Gal, Neu5Ac α2–3-β-D-Gal	2,935±120
Transferrin	Neu5Ac α2–6-β-D-Gal	2,839±145

[a] Equal amounts of acceptors have been used based on their sialic acid content
[b] Reaction was carried out using [3H]AcCoA as donor and cell lysate (100 μg protein) as source of enzyme. Radioactivity is due to transfer of acetyl groups to exogenous sialoglycoproteins

O-Acetylation of exogenous sialylated glycoproteins by ALL sialate-*O*-acetyltransferase using [^{3}H]AcCoA

In order to further establish the specificity of acetyl transfer by SOAT to sialic acids, we used various sialoglycoproteins as acceptors, like de-*O*-AcBSM, *O*SM, fetuin, α_1-acid glycoprotein and transferrin (Table 2). The transfer of radioactive acetyl group to all five sialoglycoproteins was almost similar. In contrast, the transfer of radioactive acetyl group to desialylated BSM and asialofetuin was negligible, being 155±19 cpm and 156±22 cpm (n=5) respectively.

Analysis of neo-*O*-acetylated free sialic acids and ganglioside GD3

Incubation of microsomes from CEMC7 cells with radioactive AcCoA alone or in the presence of CMP-Neu5Ac and ganglioside GD3 as potential exogenous substrates

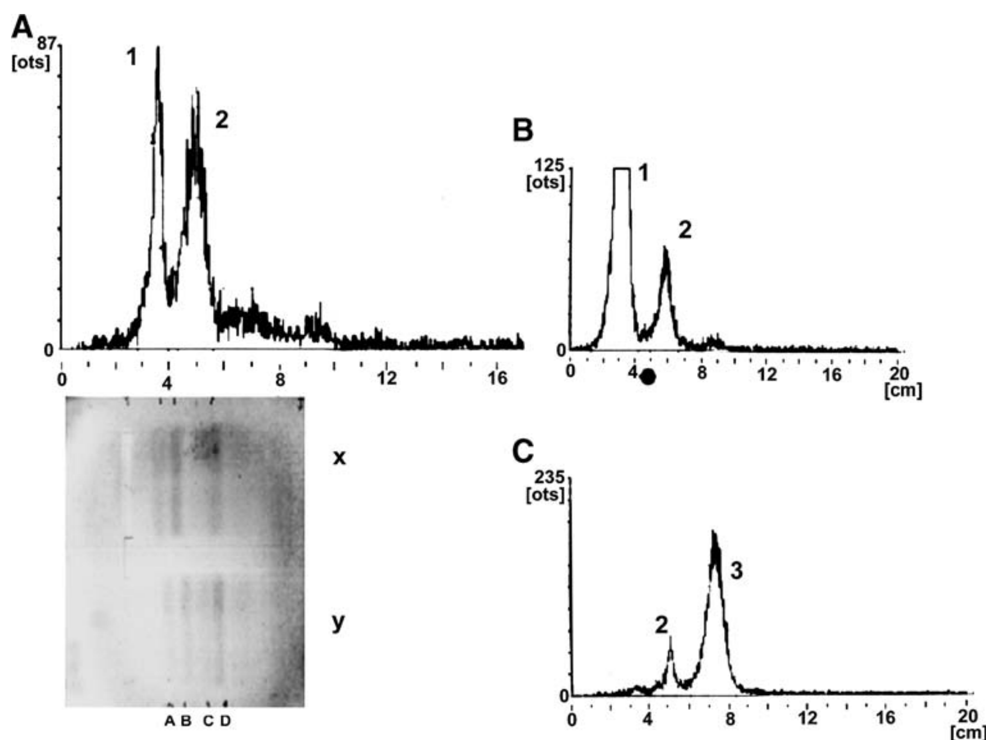

Fig. 5 Analysis of neo-*O*-acetylated sialic acids. Microsomal fractions (25 μg protein) from of a representative T-ALL cell line (CEMC7) were incubated with CMP-Neu5Ac (**a**) or GD3 (**b**, **c**) in the presence of [^{14}C]AcCoA (0.04 μCi) in phosphate buffer (pH 6.5) at 37°C for 60 min. For details see "Materials and methods". Analysis of the reaction product was carried out as follows. Radio-thin layer chromatography of neo-*O*-acetylated sialic acids (**a**) and GD3 (**b**, **c**), respectively. **a** After incubation, CMP-Neu5Ac was shortly hydrolyzed and chromatographed on cellulose together with BSM standard sialic acids (*lane x*). This standard mixture alone was also run in parallel (*lane y*). *1* Residual [^{14}C]AcCoA and *2*, Neu5,7Ac_2. **b** The supernatant from the assay with GD3 was developed on silica gel. The position of GD3 is marked by a *dot*. *1* Residual [^{14}C]AcCoA and *2* 7-*O*-Ac-GD3. **c** The same sample as in **b**, but largely de-*O*-acetylated in ammonia vapour (some residual 7-*O*-Ac-GD3 is still visible). *2* 7-O-Ac-GD3 and *3* free acetate. The plates were stained with the orcinol/Fe^{3+} reagent after radio-scanning. **a** Neu5Ac, **b** Neu5Gc, **c** Neu5,7 Ac_2, **d** Neu5,9 Ac_2

resulted in the formation of neo-*O*-acetylated radioactive sialic acids. The main reaction product was 7-*O*-acetylated Neu5Ac after mild hydrolysis, as shown by radio-TLC (Fig. 5a) and radio-HPLC (Fig. 6) and in Table 3. The latter analysis revealed also the presence of 8-*O*-acetylated sialic acids. Small amounts of radioactive Neu5,9Ac_2 were detected. On incubation with GD3, 7-*O*-acetylated GD3 was the prevailing reaction product (Fig 5b,c). The specific enzyme activity with endogenous substrate, based on the radioactivity of liberated sialic acids from microsomes, was 1.6 nmol/mg protein × hours (Table 3). With exogenous substrates incubated with microsomes, specific activities of 1.5 nmol/mg protein × hours were determined for CMP-Neu5Ac and 1.7 nmol/mg protein × hours for GD3, respectively. The enzyme activity shown by endogenously present GD3 in lymphoblast microsomes was 0.8 nmol/mg protein × hours.

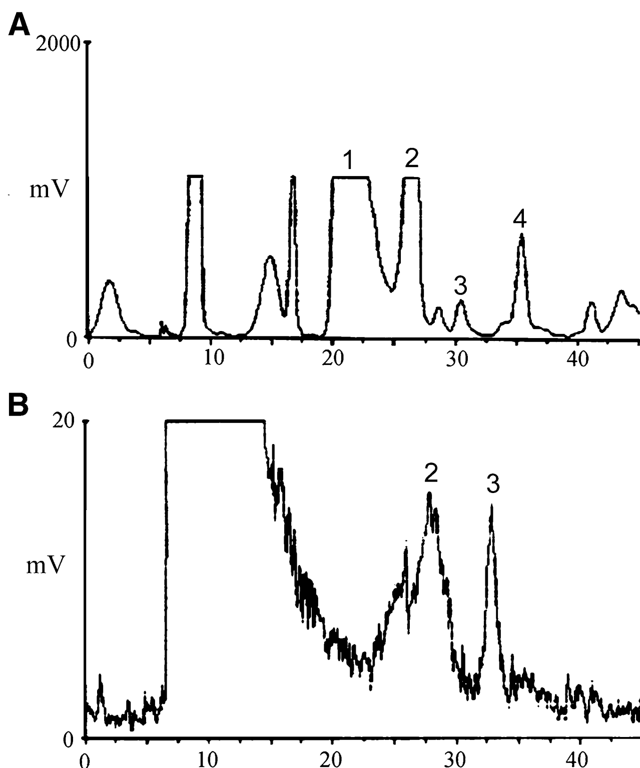

Fig. 6 Radio-HPLC of neo-*O*-acetylated [^{14}C]-labelled sialic acids. Sialic acids from microsomes of a representative T-ALL cell line (CEMC7) incubated with GD3 were liberated by propionic acid hydrolysis, derivatized with DMB and mixed with derivatized BSM standard sialic acids. The HPLC eluate was simultaneously monitored for fluorescence (**a**) and radioactivity (**b**). *1* Neu5Ac, *2* Neu5,7Ac_2, *3* Neu5,8Ac_2 and *4* Neu5,9Ac_2. There is a delay in radioactivity registration of 1.5–2 min, which was checked with radioactive Neu5Ac run in parallel. The high amount of radioactivity eluting first may be AcCoA or free acetate

Table 3 *O*-acetylated sialic acids formed and activities of microsomal SOAT from CEMC7 cells with endogenous and exogenous substrates

Substances added	Reaction product	Specific activity (nmol/mg protein × hours)
0[a]	Neu5,7Ac_2	1.6
CMP-Neu5Ac[b]	Neu5,7Ac_2	1.5
0[c]	7-OAc-GD3	0,8
GD3[c]	7-OAc-GD3	1.7

The specific activity calculation is based on the radioactivity amount (cpm) of the neo-*O*-acetylated enzyme reaction product from radio-TLC. The specific activity of radioactive AcCoA was used for calculation. The values obtained are per hour

[a] Liberation of sialic acids from microsomes by 120 min propionic acid hydrolysis and chromatography on cellulose

[b] Hydrolysis of CMP-sialic acid for 15 min by propionic acid

[c] Direct chromatography of assay supernatant on silica gel

High sialate-*O*-acetyltransferase activity as determined by an ELISA

The status of neo-*O*-acetylated sialoglycoproteins was further evaluated by using a newly developed ELISA. This ELISA allows the determination of SOAT activity even in 25 ng microsomal protein (Fig. 7a). Using this assay, in all ten patients studied, enhanced enzyme activity in microsomal fractions (50 ng protein) was observed, the mean ± SD of A_{405}nm value being 0.883±0.16 as compared to healthy controls (0.128±0.01). Thus, at presentation of the disease, an almost sevenfold increase in SOAT activity was observed in these patients as compared to normal donors (Fig. 7b). Low absorbance (0.174±0.02), similar to ABTS control (0.147±0.017), were observed without acceptor, exogenous addition of AcCoA, cell lysate or microsomal fractions serving as different sets of control.

The V_{max} of sialate-*O*-acetyltransferase reaction is high in ALL

Incorporation of the acetyl group from [^{3}H]AcCoA into sialic acids of endogenous acceptor using microsomal fraction from BM of an ALL patient showed an apparent K_M of 2.92 μM AcCoA with a V_{max} of 21.04 pmol/min × milligram protein (Fig. 8a,b). The experiment, when repeated with three patients having high expression of *O*-acetylated sialoglycoproteins, showed similar V_{max}-values. In contrast, an apparent K_M of 3.16 μM AcCoA with V_{max} being only 6.32 pmol/min × milligram protein was observed with PBMC from healthy persons.

Using cell lysates from the same patient, the kinetics of incorporation of the radioactive acetyl group into sialic acids of de-*O*-AcBSM, showed an apparent K_M for AcCoA

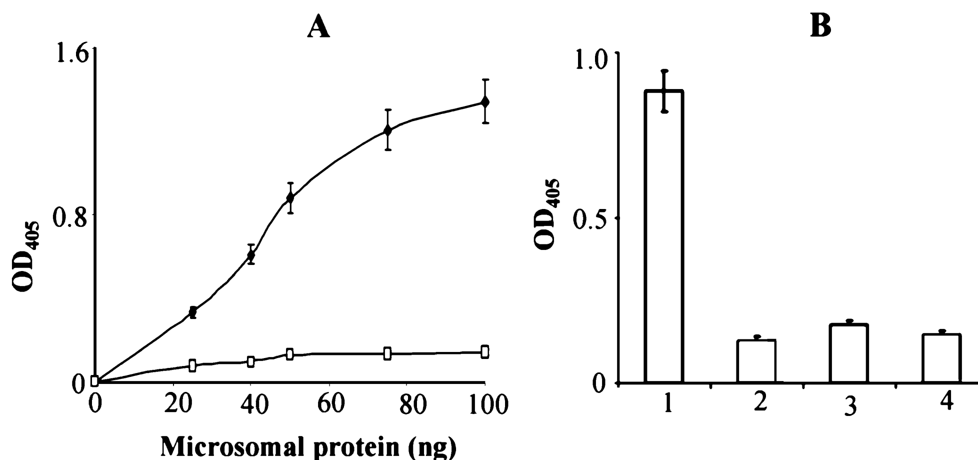

Fig. 7 Development of an ELISA for the detection of SOAT activity. **a** The plot of absorbance A_{405}nm vs. increasing amounts of microsomes. SOAT activity was determined by using immobilized de-*O*-AcBSM containing 20 ng sialic acid was immobilized on the wells of an ELISA plate as described in "Materials and methods". Varying concentrations of microsomal fractions (*filled diamond*) from an ALL patient and AcCoA was mixed and added to the wells. The reaction mixture was incubated for 60 min at 37°C. Microsomal fractions from normal PBMC (*open square*) were used for comparison. **b** The plot of average absorbance A_{405}nm representing SOAT activity of ALL and controls. SOAT activity was detected in microsomal fractions from lymphoblasts of ALL patients (n=10) during presentation of disease (*1*) and normal PBMC (*2*) were used. Absence of exogenous addition of AcCoA (*3*) and only ABTS (*4*) served as controls

of 1.83 μM with a V_{max} of 1.58 pmol/min × milligram protein (Fig. 8c, d). The corresponding values for normal PBMC cell lysate were 2.79 μM (K_M) and 0.209 pmol/min × milligram protein.

Similarly, by varying the concentration of acceptor (de-*O*-AcBSM), SOAT from cell lysate exhibited a K_M=0.94 μM with a V_{max} of 1.57 pmol/min × milligram protein (Fig. 8e, f). The corresponding values for normal PBMC cell lysate were 1.03 μM (K_M) and 0.11 pmol/min × milligram protein.

High sialate-*O*-acetyltransferase activity in cell lysates and microsomes of lymphoblasts of patients with ALL

The SOAT activity was high in cell lysates prepared from both B- (REH, ALLP0) and T- (CEMC7, MOLT4) ALL cell lines as indicated by the high incorporation rate of radioactivity when compared to normal cells (Table 4) using de-*O*-AcBSM as an acceptor. Enhanced expression of SOAT activity was found in cell lysates of all 25 clinically confirmed ALL patients, their mean radioactivity ± SD being 3,178±145 cpm (n=17), 3,125±180 cpm (n=5) and 3,095±166 cpm (n=3) for B-, T- and mixed lineage cells, respectively. The increase of SOAT activity was 24.8, 24.4 and 24.2-fold, respectively, taking average values of B-, T- and mixed lineage-ALL cells at presentation of disease, compared to cell lysate from normal PBMC (n=15) being 128±11 cpm (Table 4). No significant differences were observed with regard to the lineages of these lymphoblasts. Using normal PBMC as the cut-off value, the enzyme source was positive in all patients. Cell lysates from patients with AML and CML showed only low radioactivity incorporation, being 145±13 cpm.

Similar enhanced SOAT activity was observed using microsomal fractions of lymphoblasts isolated from BM of all 25 diagnostically and immunophenotypically proven B-,T- and mixed lineage ALL patients, at presentation of disease, and also of cell lines (Table 4). The mean radioactivity incorporated inside the microsomal fraction was 10,981± 888 cpm, 9,614±502 cpm and 9,724±628 cpm for B-, T- and mixed lineage cells, respectively, as compared to 2,675± 151 cpm for microsomal fraction of normal PBMC.

The SOAT activity in microsomal fractions of each individual patient showed a good correlation with the percentage of Neu5,9$Ac_2$$GPs^+$ lymphoblasts (r=0.9382, Fig. 9a; Table 4), as analyzed with FITC–Achatinin-H. A similar correlation was observed using cell lysates as enzyme source (not shown).

Assessment of sialate-*O*-acetyltransferase for monitoring the disease status

Previous studies from our group have demonstrated differential expression of Neu5,9Ac_2-GPs on lymphoblasts at different phases of treatment [26–27, 30]. Therefore, we wished to examine the SOAT activity in lymphoblasts for monitoring the disease status. We analyzed cell lysates of lymphoblasts from BM of patients (n=20) for this purpose using radioactive AcCoA as donor and de-*O*-AcBSM as substrate.

Two distinct patterns emerged during longitudinal follow-up of these children. Four patients relapsed, whereas 16 remained in clinical remission. There was a progressive decrease of SOAT activity observed in these 16 patients within about 8 weeks and they remained in clinical

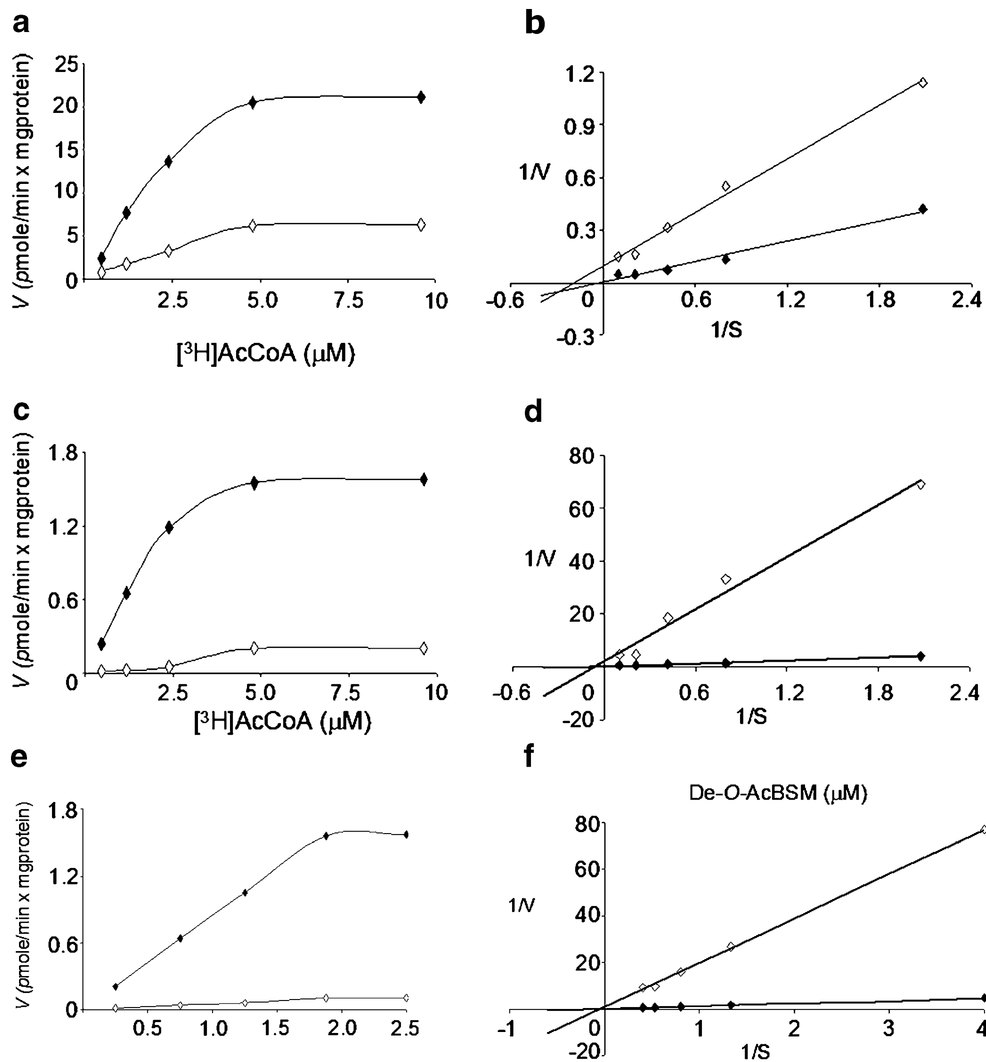

Fig. 8 Kinetics of incorporation of [^{3}H]AcCoA into endogenous acceptors present in microsomes and de-*O*-AcBSM using cell lysates. **a** The plot of reaction velocity (*V*) vs. concentrations of [^{3}H]AcCoA (S). Microsomal fractions (25 μg protein) from lymphoblasts of ALL patient (*filled diamond*) and normal PBMC (*open diamond*) were used as source of sialate-*O*-acetyltransferase. They were incubated separately in the presence of varying concentrations of [^{3}H]AcCoA for 60 min at 37°C in TKM buffer (pH 7.0) as described in "Materials and methods". **b** Double inverse plot of [1/*V*] vs. [1/*S*] of the above. **c** The plot of reaction velocity (*V*) vs. concentrations of [^{3}H]AcCoA (*S*) in cell lysate. Cell lysates from (100 μg protein) from same patient (*filled diamond*) and normal PBMC (*open diamond*) were incubated separately in the presence of varying concentrations of [^{3}H]AcCoA for 60 min at 37°C in TKM buffer (pH 7.0) in which de-*O*-AcBSM containing 5.0 μg of sialic acid served as exogenous acceptor. **d** Double inverse plot of [1/*V*] vs. [1/*S*] of the above. **e** Kinetics of neo-*O*-acetylation of from [^{3}H]AcCoA by cell lysate SOAT. The plot of reaction velocity (*V*) vs. concentrations of de-*O*-AcBSM (*S*). Cell lysates (100 μg protein) from same patient (*filled diamond*) and normal PBMC (*open diamond*) were incubated separately in the presence [^{3}H]AcCoA (2.4 μM) and varying concentrations of de-*O*-AcBSM for 60 min at 37°C in TKM buffer (pH 7.0) as described in "Materials and methods". **f** Double inverse plot of [1/*V*] vs. [1/*S*] of the above

remission throughout the follow-up until week 90 (Fig. 9b). In contrast, in one patient with treatment, enzyme activity remained low only up to week 18. In this patient radioactivity increased in week 30 in the cell lysate of BM (2,168±114 cpm) and SOAT activity continued to increase until week 32. The patient also showed a significant increase in the percentage of Neu5GPAc$_2^+$ lymphoblasts, which was manifested as clinical relapse. All four patients who suffered from relapse at different time points during clinical remission showed a similar pattern.

Discussion

Although the increase of *O*-acetylated sialoglycoproteins has been convincingly demonstrated as an important determinant on lymphoblasts [24–27], little progress has been made in examining the identification and status of sialate-*O*-acetyltransferase activity possibly accounting for the enhanced presence of *O*-acetylated sialic acids in ALL. The major achievements of the current investigation include demonstration of an enhanced activity of SOAT in the

Table 4 SOAT assay using microsomal fractions and cell lysate from malignant and normal lymohoblasts and [^{3}H]AcCoA as donor

Samples	Pellet associated radioactivity in acid insoluble fraction (cpm)		%Neu5,9Ac$_2$-GPs$^+$ cells[a]
	Cell lysate	Microsome fraction, total incorporation	
REH, B-ALL	2,705±155	9,639±600	84±2
ALLP0, B-ALL	2,935±175	10,871±998	95±2
MOLT-4, T-ALL	2,691±171	9,839±565	90±3
CEMC7, T-ALL	3,217±197	10,274±428	85±5
B-ALL patient, n=17	3,178±145	10,981±888	78±8
T-ALL patient, n=5	3,125±180	9,614±502	91±4
Mixed lineage-ALL, n=3	3,095±166	9,724±628	70±4
Normal, n=15	128±11	2,675±151	5±2
AML, CML, n=5	145±13	2,626±138	8±2

The enzyme assay was performed at 37°C at pH 7.0 for 60 min using microsomal fractions of B- and TALL-cell lines and lymphoblasts from BM of clinically confirmed ALL patients. In parallel, cell lysates from each patient were processed similarly in which de-*O*-AcBSM was used as an exogenous substrate as described in "Materials and methods"

[a] Neu5,9Ac$_2$-GP+ cells present in each samples was determined by flow cytometry using FITC–Achatinin-H [25–26]

microsomes of lymphoblasts of children with ALL, irrespective of their lineage. Besides endogenous acceptors, exogenous substrates like five different sialoglycoproteins, CMP-Neu5Ac and GD3 were identified as substrates for the enzyme. Under the experimental condition, mainly Neu5,7Ac$_2$ and Neu5,8Ac$_2$ were isolated both by radio-TLC and fluorimetrically coupled radio-HPLC (Figs. 5, 6), suggesting the primary insertion site of the *O*-acetyl group to be at C-7, followed by C-8 of neuraminic acid. As Neu5,9Ac$_2$ was observed in lymphoblasts (Fig. 1), it may be envisaged that the acetyl group migrates from the seven position to the primary alcohol group of sialic acid at C-9 perhaps via C-8, as was also observed in various other systems [5]. The finding of Neu5,9Ac$_2$ exclusively in isolated microsomes (Fig. 1) fits to this assumption. Therefore, the ALL sialate-*O*-acetyltransferase was denoted sialate-7(9)-*O*-acetyltransferase.

In intact microsomal fractions, incorporation of acetyl groups may depend on the AcCoA-transporter and the utilization of these transported acetyl groups possibly takes place in two ways. Firstly, acetyl groups may be transferred to CMP-Neu5Ac to form CMP-Neu5,7Ac$_2$, which in turn will be transferred to endogenous glycoproteins by sialyltransferases [9]. Alternatively, acetyl groups may be transferred directly to endogenous sialoglycoproteins. The radioactivity (61–66%) in the acid insoluble fraction possibly reflects the direct utilization of incorporated AcCoA by endogenous glycoproteins present in microsomal fractions of ALL (Table 1). Incorporation of radioactivity into gangliosides also suggests higher SOAT activity in lymphoblasts (Figs. 5, 6). Higher radioactivity in ALL patients as compared to healthy individuals indicates higher utilization rate of incorporated AcCoA in lymphoblasts, suggesting higher SOAT activity in patients. Decrease in enzyme activity by the addition of either CMP or CoA suggests a possible blockage of AcCoA transport into the microsomal fraction or a direct inhibition of SOAT. Bovine submandibular gland SOAT can be inhibited well by CoA [9]. The side of activity of SOAT may face the microsomal fraction lumen. The SOAT of cell lysate with specificity for sialic acid attached to various sialoglycoproteins has been demonstrated in ALL as different acceptors used in this study were *O*-acetylated (Table 2).

The *O*-acetylation of exogenously added GD3 by ALL microsomes extends the specificity of this SOAT towards gangliosides. Lymphoblast SOAT is able to *O*-acetylate a number of suitable exogenous substrates; however, it is difficult to find out the selectivity of the *O*-acetyltransferase *in vivo*. It may be envisaged that a number of distinct sialate-*O*-acetyltransferases possibly exist controlling *O*-acetylation of sialic acids attached to glycans via different linkages, thus hinting at another level at which *O*-acetylation may be regulated [9]. This is not so unlikely, as a SOAT activity was reported with high specificity for terminal α2,8-linked sialic acid residues and no detectable activity for α2,3-linked sialic acids [13]. Further studies are needed to unravel the sialic acid of linkage specificity of SOAT in ALL lymphoblasts.

The transfer of acetyl groups to sialic acids has also been demonstrated by spectro-fluorimetric quantitation with acetyl acetone of the reaction product. This method was useful to see the real *O*-acetylation of sialic acids both in the microsomal fractions and cell lysate. High radioactivity (94%) bound to neo-*O*-acetylated sialoglycoprotein shown by affinity purification further demonstrated that a large proportion of sialoglycoprotein got acetylated during sialyl-*O*-acetyltransferase reaction (Fig. 4b). Microsomes showed a fivefold higher amount of *O*-acetylated sialic acids after enzymatic reaction as compared to endogenously present *O*-acetylated sialic acids suggesting the expression of active

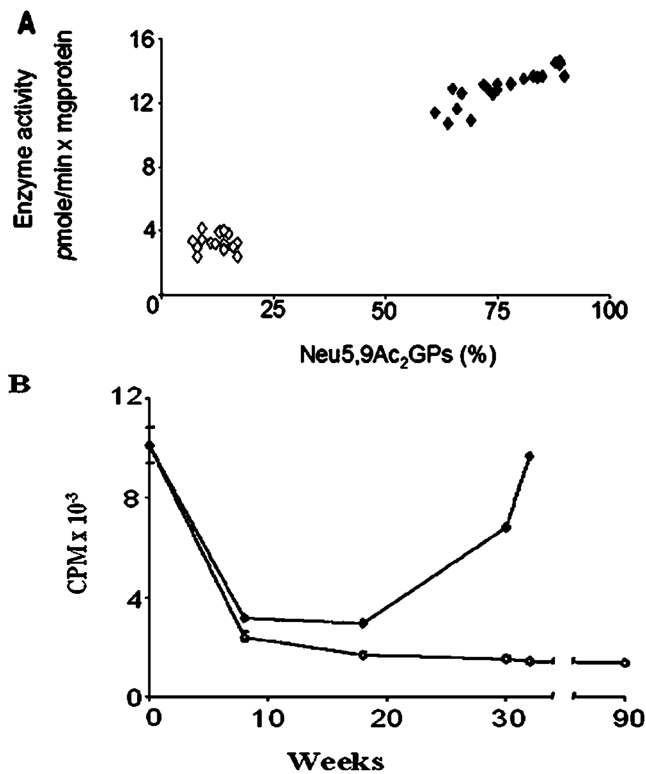

Fig. 9 Dependence of SOAT activity on the expression of *O*-acetylated sialoglycoproteins on the lymphoblasts. **a** Correlation of SOAT activity with the percentage of Neu5,9Ac$_2$-GPs$^+$ cells. The relative SOAT activity values (pmole/min × mg protein) present in microsomal fractions of clinically and immunophenotypically confirmed 25 ALL patients (*filled diamond*) at diagnosis, before drug treatment, were measured by radiometric assay as described in "Materials and methods". The percentage of Neu5,9Ac$_2$-GPs$^+$ cells was determined by FACS analysis using FITC–Achatinin-H [25–26]. Similar correlation plots for normal individuals (*open diamond*, n=15) are also shown. **b** Sialate-*O*-acetyltransferase activity in the course of leukemia treatment and relapse. Children (n=20) were longitudinally monitored using cell lysates (100 µg protein) of lymphoblasts from BM. Incorporation of [^{3}H]AcCoA into de-*O*-AcBSM was monitored. Sixteen patients were in clinical remission (*open diamond*). A representative profile of a child is shown who was in clinical relapse (*filled diamond*). Other three relapsed children showed similar patterns indicating relapse at different time points of treatment (not shown)

SOAT. The increase of $V_{\max}$ of acetyl transfer into the microsomal fraction in ALL further supports the finding of an efficient O-acetyl transfer rate in diseased cells (Fig. 8). Gradual increase of SOAT activity with increasing amount of [^{3}H]AcCoA indicates the sufficient availability of AcCoA for *O*-acetylation (Fig. 8a). However, even at saturating concentration of AcCoA, microsomes from normal cells exhibited very little *O*-acetylation compared to cells from patients showing that SOAT is much more active in microsomes of these lymphoblasts. Furthermore, it may be mentioned that the higher acetylation rate shown in these children may also be partly due to differences in transporters and natural acceptors to increased sialylation.

The efficient acetylation of exogenous substrate, de-*O*-AcBSM, by SOAT present in the cell lysate of ALL is reflected both by fluorimetric quantitation of the reaction product as well as corroborated by increased $V_{\max}$, as determined by a radiometric assay, compared with normal cells. This observation further supports the existence of an efficient SOAT rate in diseased cells (Fig. 8e,f).

At presentation of the disease, increased SOAT activity was demonstrated in both the microsomes and cell lysates of lymphoblasts from BM of B- and T-ALL patients as determined by radiometric and enzymatic assay, suggesting specificity for the disease. Therefore SOAT may be considered as a marker enzyme for childhood ALL (Tables 1, 2 and 3). A sharp decline in SOAT activity in patients who underwent chemotherapy was corroborated by their clinical remission (Fig. 9b). This suggests that measurement of enzyme activity can be effectively used for disease monitoring. The marked increase in SOAT activity with clinical relapse in four patients is also in good agreement with the higher expression of Neu5,9Ac$_2$-GP on lymphoblasts by the radiometric assay, also suggesting that SOAT may serve as a potential marker for ALL.

In summary, it may be concluded that SOAT acetylates the sialic acid of both glycoproteins and glycolipids that are *O*-acetylated on lymphoblasts, which are not found on normal cells in significant quantities. The positive association observed between the level of SOAT activity and the presence of cell surface Neu5,9Ac$_2$-GPs on lymphoblasts suggests that the extent of *O*-acetylation of sialic acids is dependent on the relative activity of this enzyme (Fig. 9a, Table 4). It was described earlier that these typical *O*-acetylated sialoglycoproteins in ALL lymphoblasts exhibit molecular weights of 90, 120, and 135 kDa [27]. Therefore, both the measurement of *O*-acetylated glycoproteins as well as SOAT activity may be useful for diagnosis and monitoring the progress of the disease.

A simple way of measurement of SOAT activity by using a non-radioactive ELISA is a prerequisite to apply this assay for monitoring the disease status. Thus it may be recommended as a practical micro-well assay, which can quickly detect SOAT activity in larger scale and especially in a hospital set-up may become a suitable tool for ALL monitoring. Given the importance of the SOAT activity, we propose that it may serve as an alternative biomarker for continuous monitoring of patients. Accordingly, we have started a long-term longitudinal study for the validation of this non-radioactive assay.

The information reported here adds considerably to our understanding of the *O*-acetylation of sialic acids in ALL and may lead to the identification of a novel drug target. Further studies are necessary to obtain a completely purified enzyme for molecular genetic and structural studies of the sialate-*O*-acetyltransferase, which is still a challenging problem in

animals and man. This information may contribute to new strategies to elucidate the gene(s) of eukaryotic sialate-*O*-acetyltransferases. The studies present advance of our knowledge of the *O*-acetylation of sialic acids in animals and human in general and not only in leukaemia.

Acknowledgement Mr. Chandan Mandal and Ms. Suchandra Chowdhury are recipients of Senior Research Fellowships of the University Grant Commission, Government of India. This work received financial support from the Department of Science and Technology, Department of Biotechnology, Indian Council of Medical Research, Council of Scientific and Industrial Research, New Delhi, India. Our sincere thanks go to Prof. Bruno Venerando (Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Italy) for providing the ALLPO cell line and to Dr. Bernhard Kniep (Institute of Immunology, Technical University of Dresden, Germany) for ganglioside standards. We are thankful to Prof. Tamás Laskay (Institute for Medical Microbiology and Hygiene, University of Lübeck, Germany) for providing laboratory facility to culture the T-ALL cell line. Mr. Arup Sarkar, Dr Anil.K. Chava and Mr. Asish Mallick are also acknowledged for their help. The Sialic Acids Society, Kiel, Germany, also supported this work.

References

1. Pui, C.H., Robison, L.L., Look, A.T.: Acute lymphoblastic leukaemia. Lancet. **371**, 1030–1043 (2008). doi:10.1016/S0140-6736(08)60457-2
2. Schauer, R.: Achievements and challenges of sialic acid research. Glycoconj. J. **17**, 485–499 (2000) doi:10.1023/A:1011062223612 and references therein
3. Klein, A., Roussel, P.: O-Acetylation of sialic acids. Biochimie. 80, 49–57 (1998). doi:10.1016/S0300-9084(98)80056-4
4. Angata, T., Varki, A.: Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective. Chem. Rev. **102**, 439–469 (2002). doi:10.1021/cr000407m
5. Schauer, R.: Sialic acids: fascinating sugars in higher animals and man. Zoology. **107**, 49–64 (2004). doi:10.1016/j.zool.2003.10.002
6. Mukherjee, K., Chowdhury, S., Mondal, S., Mandal, C., Chandra, S., Bhadra, R.K., *et al.*: 9-*O*-acetylated GD3 triggers programmed cell death in mature erythrocytes. Biochem. Biophys. Res. Commun. **362**, 651–657 (2007). doi:10.1016/j.bbrc.2007.08.048
7. Malisan, F., Franchi, L., Tomassini, B., Ventura, N., Condo, I., Rippo, M.R., Testi, R., *et al.*: Acetylation suppresses the proapoptotic activity of GD3 ganglioside. J. Exp. Med. **196**, 1535–1541 (2002). doi:10.1084/jem.20020960
8. Erdmann, M., Wipfler, D., Merling, A., Cao, Y., Claus, C., Kniep, B., *et al.*: Differential surface expression and possible function of 9-*O*- and 7-*O*-acetylated GD3 (CD60 b and c) during activation and apoptosis of human tonsillar B and T lymphocytes. Glycoconj. J. **23**, 627–638 (2006). doi:10.1007/s10719-006-9000-5
9. Shen, Y., Tiralongo, J., Kohla, G., Schauer, R.: Regulation of sialic acid *O*-acetylation in human colon mucosa. Biol. Chem. **385**, 145–152 (2004). doi:10.1515/BC.2004.033
10. Tiralongo, J., Schauer, R.: The enigma of enzymatic sialic acid *O*-acetylation. Trends Glycosci. Glycotechnol. **16**, 1–15 (2004)
11. Lrhorfi, L.A., Srinivasan, G.V., Schauer, R.: Properties and partial purification of sialate-*O*-acetyltransferase from bovine submandibular glands. Biol. Chem. **388**, 297–306 (2007). doi:10.1515/BC.2007.033
12. Shi, W.X., Chammas, R., Varki, A.: Linkage-specific action of endogenous sialic acid O-acetyltransferase in Chinese hamster ovary cells. J. Biol. Chem. **271**, 15130–15138 (1996). doi:10.1074/jbc.271.25.15130
13. Chen, H.Y., Challa, A.K., Varki, A.: 9-*O*-Acetylation of exogenously added ganglioside GD3: the GD3 molecule induces its own *O*-acetylation machinery. J. Biol. Chem. **281**, 7825–7833 (2006). doi:10.1074/jbc.M512379200
14. Vandamme-Feldhaus, V., Schauer, R.: Characterization of the enzymatic 7-*O*-acetylation of sialic acids and evidence for enzymatic *O*-acetyl migration from C-7 to C-9 in bovine submandibular gland. J. Biochem. **124**, 111–121 (1998)
15. Butor, C., Diaz, S., Varki, A.: High-level *O*-acetylation of sialic acids on N-linked oligosaccharides of rat liver membranes. Differential subcellular distribution of 7- and 9-*O*-acetyl groups and of enzymes involved in their regulation. J. Biol. Chem. **268**, 10197–10206 (1993) and references therein
16. Iwersen, M., Vandamme-Feldhaus, V., Schauer, R.: Enzymatic 4-O-acetylation of N-acetylneuraminic acid in guinea-pig liver. Glycoconj. J. 15, 895–904 (1998). doi:10.1023/A:1006911100081
17. Corfield, A.P., Myerscough, N., Warren, B.F., Durdey, P., Paraskeva, C., Schauer, R.: Reduction of sialic acid *O*-acetylation in human colonic mucins in the adenoma–carcinoma sequence. Glycoconj. J. **16**, 307–317 (1999). doi:10.1023/A:1007026314792
18. Shen, Y., Kohla, G., Lrhorfi, A.L., Sipos, B., Kalthoff, H., Gerwing, G.J., *et al.*: *O*-Acetylation and de-*O*-acetylation of sialic acids in human colorectal carcinoma. Eur. J. Biochem. **271**, 281–290 (2004). doi:10.1046/j.1432-1033.2003.03927.x
19. Ritter, G., Boosfeld, E., Markstein, E., Yu, R.K., Ren, S.L., Stallcup, W.B., *et al.*: Biochemical and serological characteristics of natural 9-*O*-acetyl GD3 from human melanoma and bovine buttermilk and chemically *O*-acetylated GD3. Cancer Res. **50**, 1403–1410 (1990)
20. Ravindranaths, M.H., Paulson, J.C., Irie, R.F.: Human melanoma antigen *O*-acetylated ganglioside GD3 is recognized by Cancer antennarius lectin. J. Biol. Chem. **263**, 2079–2086 (1988)
21. Fahr, C., Schauer, R.: Detection of sialic acids and gangliosides with special reference to 9-*O*-acetylated species in basaliomas and normal human skin. J. Invest. Dermatol. **116**, 254–260 (2001). doi:10.1046/j.1523-1747.2001.01237.x
22. Marquina, G., Waki, H., Fernandez, L.E., Kon, K., Carr, A., Valiente, O., *et al.*: Gangliosides expressed in human breast cancer. Cancer Res. **56**, 5165–5171 (1996)
23. Sen, G., Mandal, C.: A unique specificity of the binding site of Achatinin-H, a sialic acid binding lectin from *Achatina fulica*. Carbohydr. Res. **268**, 115–125 (1995). doi:10.1016/0008-6215(94)00311-3
24. Mandal, C., Chatterjee, M., Sinha, D.: Investigation of 9-O-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukaemia. Br. J. Haematol. 110, 801–812 (2000). doi:10.1046/j.1365-2141.2000.02105.x
25. Sinha, D., Mandal, C., Bhattacharya, D.K.: Identification of 9-*O*-acetylated sialoglycoconjugates (9-*O*AcSGs) as biomarkers in childhood acute lymphoblastic leukemia using a lectin, Achatinin$_H$, as a probe. Leukemia. **13**, 119–125 (1999). doi:10.1038/sj.leu.2401312
26. Pal, S., Ghosh, S., Bandhyopadhyay, S., Mandal, C., Bandhyopadhyay, S., Bhattacharya, D.K., *et al.*: Differential expression of 9-*O*-acetylated sialoglycoconjugates on leukemic blasts: a potential tool for long-term monitoring of children with acute lymphoblastic leukaemia. Int. J. Cancer. **111**, 270–277 (2004). doi:10.1002/ijc.20246
27. Pal, S., Ghosh, S., Mandal, C., Kohla, G., Brossmer, R., Isecke, R., *et al.*: Purification and characterization of 9-*O*-acetylated sialoglycoproteins from leukemic cells and their potential as

immunological tool for monitoring childhood acute lymphoblastic leukaemia. Glycobiology. **14**, 859–870 (2004). doi:10.1093/glycob/cwh111
28. Ghosh, S., Bandhyopadhyay, S., Pal, S., Das, B., Bhattacharya, D. K., Mandal, C.: Increased interferon gamma production by peripheral blood mononuclear cells in response to stimulation of overexpressed disease-specific 9-*O*-acetylated sialoglycoconjugates in children suffering from acute lymphoblastic leukaemia. Br. J. Haematol. **128**, 35–41 (2005). doi:10.1111/j.1365-2141.2004.05256.x
29. Ghosh, S., Bandyopadhyay, S., Mukherjee, K., Mallick, A., Pal, S., Mandal, C., Bhattacharya, D.K., Mandal, C.: *O*-Acetylation of sialic acids is required for the survival of lymphoblasts in childhood acute lymphoblastic leukemia (ALL). Glycoconj. J. **24**, 17–24 (2007). doi:10.1007/s10719-006-9007-y
30. Sinha, D., Mandal, C., Bhattacharya, D.K.: A novel method for prognostic evaluation of childhood acute lymphoblastic leukaemia. Leukaemia. **13**, 309–312 (1999). doi:10.1038/sj.leu.2401312
31. Bandhyopadhyay, S., Bhattacharyya, A., Mallick, A., Sen, A.K., Tripathi, G., Das, T., Mandal, C., *et al.*: Over-expressed IgG2 antibodies against *O*-acetylated sialoglycoconjugates incapable of proper effector functioning in childhood acute lymphoblastic leukaemia. Int. Immunol. **17**, 177–191 (2005). doi:10.1093/intimm/dxh198
32. Debray, H., Decout, D., Strecker, G., Spik, G., Montreuil, J.: Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. Eur. J. Biochem. **117**, 41–55 (1981)
33. Schultze, H.E., Schmidtberger, R., Haupt, H.: Studies on bound carbohydrates in isolated plasma proteins. Biochem. Z. **329**, 90–507 (1958)
34. Baenziger, J.U., Fiete, D.: Structure of the complex oligosaccharides of fetuin. J. Biol. Chem. **254**, 789–795 (1979)
35. Murphy, W.H., Gottschalk, A.: Studies on mucoproteins, VII. The linkage of the prosthetic group to aspartic and glutamic acid residues in bovine submaxillary gland mucoprotein. Biochim. Biophys. Acta **52**, 349–360 (1961). doi:10.1016/0006-3002(61)90684-9
36. Bertolini, M., Pigman, W.: The existence of oligosaccharides in bovine and ovine submaxillary mucins. Carbohydr. Res. **14**, 53–63 (1970). doi:10.1016/S0008-6215(00)80699-0
37. Shukla, A.K., Schauer, R.: Fluorimetric determination of unsubstituted and 9(8)-*O*-acetylated sialic acids in erythrocyte membranes. Hoppe-Seylers Z. Physiol. Chem. **363**, 255–262 (1982)
38. Leelavathi, D.E., Easter, L.W., Feingold, D.C., Lombardi, B.: : Isolation of a Golgi-rich fraction from rat liver. Biochim. Biophys. Acta **211**, 124–138 (1970). doi:10.1016/0005-2736(70)90087-8
39. Carey, D.J., Hirschberg, C.B.: Topography of sialoglycoprotein and sialyltransferases in mouse and rat liver Golgi. J. Biol. Chem. **256**, 989–993 (1981)
40. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)
41. Reuter, G., Schauer, R.: Determination of sialic acids. Methods Enzymol. **230**, 168–199 (1984). doi:10.1016/0076-6879(94)30012-7
42. Hara, S., Yamaguchi, M., Takemori, Y., Furuhata, K., Ogura, H., Nakamura, M.: Determination of mono-*O*-acetylated *N*-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. Anal. Biochem. **179**, 162–166 (1989). doi:10.1016/0003-2697(89)90218-2
43. Varki, A., Diaz, S.: The transport and utilization of acetyl coenzyme A by rat liver Golgi vesicles. *O*-Acetylated sialic acids are a major product. J. Biol. Chem. **260**, 6600–6608 (1985)
44. Rosenberg, A., Schengrund, C.L. (eds.): Biological roles of sialic acid. Plenum, New York, 1976, pp. 22.