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Interferon Gamma Promotes Survival of Lymphoblasts Overexpressing 9-*O*-Acetylated Sialoglycoconjugates in Childhood Acute Lymphoblastic Leukaemia (ALL)

Shyamasree Ghosh,[1] Suman Bandyopadhyay,[1] Asish Mallick,[1] Santanu Pal,[1] Reinhard Vlasak,[2] Dilip K. Bhattacharya,[3] and Chitra Mandal[1]*

[1]Immunobiology Division, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Kolkata-700 032, India
[2]Applied BioTechnology, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria
[3]Vivekananda Institute of Medical Sciences, Kolkata 700045, India

Abstract An enhanced linkage-specific 9-*O*-acetylated sialic acid (9-*O*-AcSA) on peripheral blood mononuclear cells (PBMC) of children with acute lymphoblastic leukaemia, ALL ($PBMC_{ALL}$, 9-*O*-$AcSA^+$ cells) was demonstrated by using a lectin, Achatinin-H, whose lectinogenic epitope was 9-*O*-AcSAα2-6GalNAc. Our aim was to evaluate the in vitro contributory role of this glycotope (9-*O*-AcSAα2-6GalNAc) towards the survival of these 9-*O*-$AcSA^+$ cells in ALL patients. For direct comparison, 9-*O*-$AcSA^-$ cells were generated by removing *O*-acetyl group of 9-*O*-AcSA present on $PBMC_{ALL}$ using *O*-acetyl esterase. An elevated level of serum interferon gamma (IFN-γ) in affected children led us to think that $PBMC_{ALL}$ are continuously exposed specifically to this cytokine. Accordingly, 9-*O*-$AcSA^+$ and 9-*O*-$AcSA^-$ cells were exposed in vitro to IFN-γ. A twofold increased NO release along with inducible NO synthase (iNOS) mRNA expression by the 9-*O*-$AcSA^+$ cells was observed as compared to the 9-*O*-$AcSA^-$ cells. The decreased viability of IFN-γ exposed 9-*O*-$AcSA^-$ cells as compared to 9-*O*-$AcSA^+$ cells were reflected from a 5.0-fold increased caspase-3-like activity and a 10.0-fold increased apoptosis in the 9-*O*-$AcSA^-$ cells when production of NO was lowered by adding competitive inhibitor of iNOS in reaction mixture. Therefore, it may be envisaged that a link exists between induction of this glycotope and their role in regulating viability of $PBMC_{ALL}$. Taken together, it is reasonable to hypothesise that *O*-acetylation of sialic acids on $PBMC_{ALL}$ may be an additional mechanism that promotes the survival of lymphoblasts by avoiding apoptosis via IFN-γ-induced NO production. J. Cell. Biochem. 95: 206–216, 2005.

Key words: acute lymphoblastic leukaemia (ALL); achatinin-H: an *O*-acetylated sialic acid binding lectin; 9-*O*-acetylated sialoglycoconjugates; IFN-γ production; nitric oxide (NO); caspase-3-like activity

Nitric oxide, NO, is a potent biological mediator, generated by nitric oxide synthase (NOS). NO plays a key role in physiological as well as pathological processes that includes activities for intracellular signaling, activity as a transcellular messenger and a cytotoxic agent. Most of the tumour masses have been shown to generate NO in vitro and cytokines are capable of influencing the production of this NO [Tachibana et al., 2000]. The role of NO seems to have a complex action including both inhibitory and tumour promoting activities [Alexandrova et al., 2001].

Acute lymphoblastic leukaemia, ALL, is a malignant transformation of lymphoblasts and represents the single most common type of cancer in the paediatric population. With the advent of modern chemotherapy for the treatment of childhood ALL, virtually all patients achieve remission and approximately 80% are cured. The risk of relapse remains at 20% as patients in remission may harbour residual leukaemic blasts referred to as minimal resi-

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*Correspondence to: Dr. Chitra Mandal, Immunobiology Division, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India.
E-mail: cmandal@iicb.res.in or chitra_mandal@yahoo.com

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dual disease (MRD) and prediction of this, still remains a major challenge in leukaemia research [Hoelzer et al., 2002].

Sialic acids, also known as N-acetyl-neuraminic acid (Neu5Ac) together with their substituted derivatives, are reported to regulate biological reactions owing to their strategic terminal locations [Shi et al., 1996; Kelm and Schauer, 1997; Corfield et al., 1999; Mandal et al., 2000; Schauer, 2000, 2004]. The overexpression of 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) on peripheral blood mononuclear cells (PBMC) of ALL patients, $PBMC_{ALL}$ designated as (9-*O*-$AcSA^+$) cells with 9-*O*-AcSAα2-6GalNAc as terminal specific glycotope [Sinha et al., 1999a,b; Mandal et al., 2000; Pal et al., 2004a,b,c] have been reported. The potential of the glycotope in monitoring clinical outcome of these patients have also been established [Sinha et al., 1999c,d; Pal et al., 2001, 2004a,b]. However, the assessment of 9-*O*-AcSGs regarding their structure and function is a relatively new domain in leukaemia sialobiology. A question, currently unanswered is that whether the enhanced presence of disease-specific cell surface 9-*O*-AcSGs plays a contributory role towards their survival. Accordingly, 9-*O*-$AcSA^-$ cells were generated by removing *O*-acetyl group of 9-*O*-AcSGs from patients' PBMC using *O*-acetyl esterase. This study aims to understand the differential reactivity of both 9-*O*-$AcSA^+$ and de-*O*-acetylated (9-*O*-$AcSA^-$) cells in response to in vitro exposure of interferon gamma (IFN-γ). Therefore, $PBMC_{ALL}$ were exposed to IFN-γ and monitored for the (a) NO_2^- release, (b) expression of inducible NO synthase (iNOS) mRNA and (c) effect on cell viability. Cell viability was monitored by detecting: (i) caspase-3-like activity, (ii) appearance of active caspase-3 by Western blot analysis and (iii) apoptosis by Annexin V binding. Given the importance of the disease-specific *O*-acetylation, we propose a possible role of this glycotope in the survival of leukaemic cells, inferring at an alternative mechanism of their escape from immune surveillance.

MATERIALS AND METHODS

Reagents

Monoclonal antibodies used for flow cytometric studies, were purchased from B.D. Pharmingen (San Diego, CA). The primers (Table I) were obtained from Gibco-BRL. ApoAlert NO-Annexin V dual sensor kit (K2013-1) and the kit for intracellular Caspase-3 were from Clontech (Palo Alto CA). Enhanced chemiluminescence (ECL) was obtained from Amersham Bsiosciences (Uppsala Sweden). All other reagents and chemicals were obtained from Life Technologies (Grand Island, NY).

Probes

Achatinin-H. The lectin, Achatinin-H was purified from haemolymph of the African giant land snail *Achatina fulica* using bovine submaxillary mucin (BSM) as the affinity matrix. BSM, purified from bovine submaxillary glands [Chatterjee et al., 1998], was selected based on the established evidence that it contains a high percentage (22.5%) of 7(8)9-*O*-acetylated sialic acid derivatives as determined by fluorimetric analysis [Sharma et al., 1998] and HPLC [Chatterjee et al., 2003]. Purified Achatinin-H was filter sterilised, stored at 4°C and was stable for 2 years [Chava et al., 2002]. The specificity of Achatinin-H towards 9-*O*-AcSA was confirmed through several approaches and its lectinogenic epitope was established as 9-*O*-AcSAα2-6GalNAc [Mandal and Basu, 1987; Sen and Mandal, 1995]. Achatinin-H, either unconjugated or conjugated with fluroescein isothiocyanate (FITC), was used as an analytical probe.

***O*-acetyl haemagglutinin esterase of influenza C virus.** *O*-acetyl group of 9-*O*-acetylated sialoglycoconjugates from 9-*O*-$AcSA^+$ cells was removed by using a recombinant 9-*O*-acetyl haemagglutinin esterase. This enzyme was derived from the cloned HE gene of influenza C virus. The chimeric gene contained the entire HE1 domain and the first four codons of the HE2 domain, which were fused to the coding region of eGFP. The recombinant baculovirus,

TABLE I. Primers for iNOS and GAPDH

Gene	Primer (forward)	Primer (reverse)	Size (bp)
iNOS	5′CTGCATGGATAAGTACAGGCTGAGC3′	5′AGCTTCTGATCAATGTCATGAGCAA3′	225
GAPDH	5′ATGGGGAAGGTGAAGGTCGG3′	5′GGGTGCTAAGCAGTTGGT3′	540

Bak-CHE1-eGFP, secretes HE1-eGFP fusion protein with the desired esterase activity [Vlasak et al., 1987; Chatterjee et al., 2003].

Study Population

The study subjects included clinically and immunophenotypically confirmed childhood ALL patients at presentation of the disease, that is, before drug treatment. Patients were clinically confirmed following French–American–British Group recommendations and immunophenotyped using commercially available anti-CD monoclonal antibodies. In brief, PBMC were gated for lymphocytes by staining with anti-CD45 monoclonal antibody. Within the cases diagnosed, immunologic subgroups were defined as follows: common B- ($n = 16$, $CD19^+$, $CD20^+$, $CD10^\pm$, $CD34^+$, cyt-μ^+), and T- ($n = 6$, $CD2^\pm$, $CD3^+$, $CD34^+$ and $CD7^+$) ALL. The diagnosis was also confirmed by detecting 9-*O*-AcSG antibodies in the sera of ALL patients [Pal et al., 2000, 2001, 2004c]. The study sample comprised males ($n = 14$) and females ($n = 8$), with a M:F ratio of 3:2, median age of 2.25 years (range 0.8–14 years) and a median white blood cell count of 7.75×10^9/L (range 3.5–11×10^9/L). Patients with other cross-reactive haematological diseases, for example, acute myelogeneous leukaemia (AML), chronic myelogeneous leukaemia (CML), chronic lymphoblastic leukaemia (CLL), non-Hodgkin's leukaemia (NHL) and aplastic anaemia were also monitored for 9-*O*-AcSG expression and found to be negative [Sinha et al., 1999a; Pal et al., 2000, 2004a,b] using two 9-*O*-AcSA binding probes, for example, Achatinin-H and anti-9-*O*-AcSG antibody. Normal healthy human donors served as controls.

Venous blood or bone marrow (3–4 ml) was collected at Vivekananda Institute of Medical Sciences, (Kolkata, India) and then sent to the proximal premises of the Indian Institute of Chemical Biology. Informed consent was obtained from donors, patients and parents or guardians. The Institutional Human Ethical Committee as per the protocol of Indian Council of Medical Research approved the study.

Detection of 9-*O*-AcSGs on $PBMC_{ALL}$

By triple colour flow cytometry. Triple colour flow cytometric studies were performed to detect the expression of 9-*O*-AcSGs on $PBMC_{ALL}$ (B and T lineages) and normal individuals ($PBMC_N$). In brief, PBMC were separated by Ficoll gradient, washed and incubated with biotin-conjugated anti-CD-7 for T- and anti-CD-19 for B-ALL patients followed by incubation with PerCP-Strepavidin and FITC-CD-45. To detect the co-expression of 9-*O*-AcSGs on these cells, they were incubated with Achatinin-H (1 μg) followed by anti-Achatinin-H antibody (1:100), developed in albino swiss rabbits, followed by PE-anti-rabbit IgG for 1 h on ice in the dark. The cells were washed and fixed with paraformaldehyde (1%) and analysed using cell quest software. In parallel, $PBMC_N$ were also stained and analysed as above. Appropriate isotype controls were used to determine the background fluorescence.

By leukoagglutination. The binding of Achatinin-H with cell surface 9-*O*-AcSGs on $PBMC_{ALL}$ was determined by a leukoagglutination experiment, in which, cells (1.0×10^3/50 μl RPMI-1640) were allowed to bind with Achatinin-H in the presence of Ca^{++} (0.03 M) for 1 h at 37°C in a carbon dioxide incubator with 5% CO_2 and 95% air. Cells without Achatinin-H and/or calcium served as the controls. The percentage leukoagglutination was scored under a phase contrast microscope and calculated as follows:

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

Removal of Cell Surface *O*-Acetylations by Esterase Treatment

In order to generate 9-*O*-$AcSA^-$ cells, *O*-acetyl groups from 9-*O*-$AcSA^+$ cells of $PBMC_{ALL}$ were removed by recombinant 9-*O*-acetyl haemagglutinin esterase of influenza C virus [Vlasak et al., 1987]. The optimum condition for complete removal of *O*-acetylation was standardised by different variations of cell number, time and dose of esterase and temperature of treatment. Accordingly, $PBMC_{ALL}$ (1×10^6) were incubated with recombinant 9-*O*-acetyl esterase (100 μl, 12.0 mU) for 1 h at 37°C, washed with RPMI-1640 twice and processed for subsequent assays. The viability of esterase treated (9-*O*-$AcSA^-$) cells was checked by trypan blue exclusion. Similarly, $PBMC_N$ was treated with esterase, which served as control. The degree of de-*O*-acetylation was monitored at different times by leukoagglutination assays and FACS analysis using Achatinin-H and FITC-Achatinin-H respectively.

Monitoring the Reappearance of *O*-Acetylation on PBMC by Leukoagglutination

The reappearance of *O*-acetylations on 9-*O*-AcSA$^-$ cells from $PBMC_{ALL}$ patients and $PBMC_N$ was monitored by the leukoagglutination assay. Briefly, washed 9-*O*-AcSA$^-$ cells were kept in RPMI-1640 without phenol red medium supplemented with 5% FCS (Medium A), and an aliquot was tested every hour for gradual reappearance of *O*-acetylations by leukoagglutination. The agglutination titer (%) was used as an index for reappearance of *O*-acetylated moieties on the cell surface of 9-*O*-AcSA$^-$ cells.

Culture Condition Used for all the Following Assays

9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells obtained from $PBMC_{ALL}$ (1.0×10^6 cells/ml) were washed with PRMI-1640 and cultured separately for 6 h in Medium A at 37°C in a carbon dioxide incubator at 5% CO_2 and 95% air. In order to prevent the minimal reappearance of cell surface 9-*O*-acetylations on 9-*O*-AcSA$^-$ cells, the culture media were replenished with *O*-acetyl esterase after every 2 h. Appropriate controls were used for enzyme activity. Cells treated with inactivated esterase, obtained by heating medium containing enzyme at 95°C for 20 min, were also used as medium control.

Nitric Oxide Release Assay

Both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells from $PBMC_{ALL}$ were exposed to IFN-γ (0–10 ng) in Medium A for 6 h. The release of NO was measured by the assessment of NO_2^-, a stable metabolite, by Griess Reaction. Cell-free culture supernatant (100 μl) was transferred into 96-well plates to which the Greiss reagent was added. The reagent was N-1-naphthylethylene diamine HCl in distilled H_2O (50 μl, 0.1% v/v) and sulfanilamide (50 μl, 1% v/v) diluted with phosphoric acid (5% v/v). Absorbance at 540 nm was measured and standard curves were generated with dilutions of $NaNO_2$ (0–100 μM). In parallel, cells cultured in presence of LPS (2.5 ng) served as the positive controls. Both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells in absence of IFN-γ served as the negative controls.

Detection of Intracellular NO of 9-*O*-AcSA$^+$ and 9-O-AcSA$^-$ Cells Upon Exposure With IFN-γ

The NO_2^- producing cells were detected using NO_2^- sensor dye from the ApoAlert NO/Annexin V dual sensor kit. Briefly, NO_2^- sensor dye (1 μl) was added to the 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ (1×10^6/ml) cells, which was incubated for 30 min at 37°C and exposed to IFN-γ as above. Cells were centrifuged at 1,000 rpm for 3 min at 4°C, washed in PBS and fluorescence was recorded. In this instance, cells kept in absence of IFN-γ served as the control.

RT-PCR Studies

Cellular RNA from 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells obtained from $PBMC_{ALL}$, after exposure with IFN-γ (0.1 ng), were extracted using Trizol. RNA (1 μg) was reverse transcribed (RT) to cDNA using a poly (dT) oligonucleotide and 200 U Superscript II reverse transcriptase. Each PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 30 s using a specific primer for inducible nitric oxide synthase (iNOS, Table I) in a Perkin-Elmer DNA thermal cycler. The PCR products (10 μl) were analysed in agarose gel (1%) stained with ethidium bromide and visualised under a UV lamp. A commercially prepared 100 base pair ladder was used as a molecular weight marker. RNA isolated from cells in absence of IFN-γ served as the control. The intensity of the bands for cytokines and iNOS was determined by densitometry using the quantity one software.

Measurement of Caspase-3-Like Activity

Cells (9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) from ALL and normal individuals were exposed to IFN-γ (0.1 ng) for 6 h in Medium A. In another set, cells were exposed to IFN-γ (0.1 ng) in presence of N-omega-nitro-L-arginine-methyl ester (LNAME, 10 mM). The activation of caspase-3 was detected by increased absorbance at 405 nm using the kit and following the manufacturer's protocol. In the control set, an inhibitor specific for caspase-3 (DEVD fmk, 1 μL) was used to detect the specificity of the reaction.

Western Blot Analysis for Detection of Caspase-3

The presence of caspase-3 was also detected by Western blot analysis. Cytosolic fractions (50 μg) of 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells were resolved on SDS–PAGE (15%, Laemmli, 1970), transferred to nitrocellulose, probed with anti-caspase-3 antibody and detected by enhanced chemiluminisance.

Annexin V Staining

Cells (9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) were exposed to IFN-γ (0.1 ng) in presence of LNAME (10 mM) for 6 h in Medium A. They were washed in cold PBS buffer, transferred in FACS binding buffer, stained with PE-Annexin V (5 μl) for 15 min at 25°C in the dark followed by washing as described by the manufacturer's protocol and analysed by flow cytometry. Under similar condition, cells kept in Medium A in absence of IFN-γ and stained with PE-Annexin V served as control.

RESULTS

9-*O*-AcSGs Are Overexpressed on PBMC$_{ALL}$ Irrespective of Lineages

The presence of 9-*O*-AcSGs on PBMC$_{ALL}$ was confirmed by triple colour flow cytometric studies. A high expression of 9-*O*-AcSGs on CD45$^+$CD19$^+$ and CD45$^+$CD7$^+$ cells of patients were observed as reflected by the high binding of Achatinin-H (70–80% positive binding) and (80–90% positive binding) to PBMC from children with B and T ALL respectively (Fig. 1). In contrast, under similar conditions, the binding of Achatinin-H to both CD45$^+$ CD19$^+$ and CD45$^+$ CD7$^+$ cells from normal donors was minimal (2–5% positive binding). The expression of 9-*O*-AcSGs was absent on PBMC from patients with other cross-reactive diseases [Sinha et al., 1999a; Pal et al., 2000, 2004a,b].

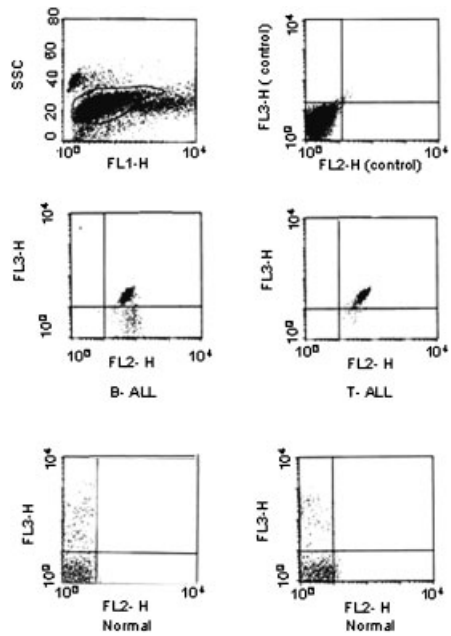

Fig. 1. 9-*O*-AcSGs expressed on PBMC$_{ALL}$ irrespective of lineages. A representative profile of the expression of 9-*O*-AcSGs on PBMC$_{ALL}$ (n = 22) patients detected by triple colour flow cytometric studies. In brief, PBMC were incubated with biotinylated anti-CD-7 for a T- and anti-CD-19 for a B-ALL patient followed by incubation with PerCP-Strepavidin (FL3-H) and FITC-CD-45 (FL1-H). Cells were initially gated for Side Scatter (SSC) versus FL1-H. To demonstrate the co-expression of 9-*O*-AcSGs, these cells were incubated further with Achatinin-H followed by rabbit anti-Achatinin-H and PE-anti-rabbit IgG (FL2-H) as described in Materials and Methods. PBMC from normal individuals were analysed similarly.

De-*O*-Acetylated (9-*O*-AcSA$^-$) Cells Generated by Esterase Treatment of PBMC$_{ALL}$

Since enhanced expressions of 9-*O*-acetylations on PBMC$_{ALL}$ were repeatedly observed, we wanted to investigate the biological role of these glycotopes in their survival. With this aim, de-*O*-acetylated (9-*O*-AcSA$^-$) cells were generated by esterase treatment. Optimum conditions for de-*O*-acetylation were achieved by adjusting concentration of esterase, temperature and duration of treatment. Cells (1×10^6) in presence of esterase (100 μl, 12.0 mU) at 37°C for 1 h showed complete removal of *O*-acetylated groups as assessed by the shift in the mean florescence intensity of Achatinin-H positive cells (Fig. 2A) and absence of leukoagglutination with Achatinin-H. The viability of both 9-*O*-AcSA$^-$ and 9-*O*-AcSA$^+$ cells, when kept in esterase inactivated medium, were found to be unaltered as compared to that of cells kept in Medium A. Thus, demonstrating that other components present in the medium have no direct effect on these cells.

O-Acetylations Reappeared on 9-*O*-AcSA$^-$ Cells

With a view to establish a time frame within which newly generated 9-*O*-AcSA$^-$ cells remained devoid of its cell surface *O*-acetylation, they were kept in Medium A in a carbon dioxide incubator. The percentage of leukoagglutination, typically clumps of 10–16 cells (as an indicator of the reappearance of 9-*O*-AcSA), was monitored at different time intervals (Fig. 2B). As Achatinin-H is known to agglutinate only cells containing 9-*O*-AcSAα2-6GalNAc residues on their cell surface, the percentage of leukoagglutination was considered as a direct measure of the degree of reappearance of 9-*O*-acetylation. No leukoagglutination was observed in the first 2 h of post-esterase treatment.

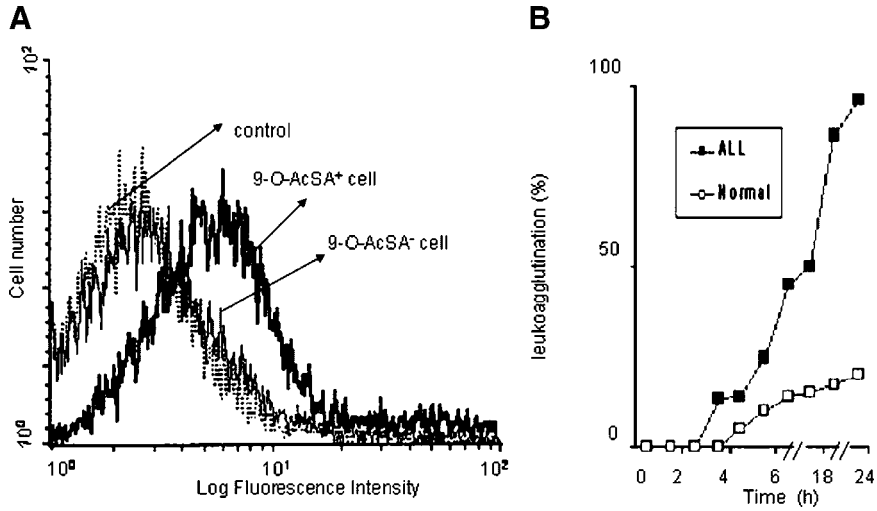

Fig. 2. **A**: Removal of 9-*O*-acetylation from cell surface. PBMC (1×10^6) from ALL patients were treated with esterase for 1 h at 37°C as described in Materials and Methods. Esterase treated (9-*O*-AcSA$^-$) and untreated (9-*O*-AcSA$^+$) cells were stained with FITC-Achatinin-H and analysed by FACS. **B**: Kinetics of appearance of cell surface 9-*O*-acetylation on 9-*O*-AcSA$^-$ cells. After complete removal of 9-*O*-acetylation from cell surface of PBMC from ALL patients and normal individuals by esterase, cells were washed and kept at 37°C in Medium A in a CO_2 incubator for 24 h. Reappearance of *O*-acetylation on 9-*O*-AcSA$^-$ cells were monitored by leukoagglutination, and this titer was plotted against different time point.

Only $13 \pm 1\%$ 9-*O*-acetylations reappeared within 3 h of treatment, while complete reappearance occurred within a 21-h interval as indicated by $98 \pm 2\%$ agglutination. Hence, for all subsequent experiments, the de-*O*-acetylated status of cells was maintained by replenishing the culture medium with *O*-acetyl esterase at every 2-h interval for a typical 6-h culture.

In contrast, $PBMC_N$, reported to have a basal level of *O*-acetylated sialic acid [Sinha et al., 1999a; Pal et al., 2004a,b] showed a low level (15–20%) of agglutination or clumps of only two or three cells. The agglutination was not observed after esterase treatment. The agglutination gradually reappeared along with incubation time and a similar pattern of agglutination (15–20%) was observed at the end of 24 h (Fig. 2B).

Differential Regulation of IFN-γ-Induced NO_2^- Release by 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ Cells

In a separate experiment, we have observed a three- to four-fold increased IFN-γ in serum of ALL patients as compared to that in normal individuals (652–946 pg/ml vs. 171–235 pg/ml). Therefore, it may be envisaged that these cells are continuously exposed specifically to this cytokine. Accordingly, 9-*O*-AcSA$^+$ cells were exposed to IFN-γ (0.1 ng) for 0–48 h and a significant amount (15 ± 2 μM) of NO_2^- release was observed even after 6 h of exposure. Based on this information, for direct comparison, in all subsequent experiments, both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells were exposed to IFN-γ for 6 h. The release of NO_2^- were low in 9-*O*-AcSA$^-$ cells as compared to 9-*O*-AcSA$^+$ cells, the release being 8 ± 2 μM versus 15 ± 2 μM respectively (Fig. 3A). Exposure of both these cells for 6 h with increasing concentration of IFN-γ (0.01–10 ng) led to enhance release of NO_2. However, 9-*O*-AcSA$^+$ cells always produced twofold more NO_2^- release as compared to 9-*O*-AcSA$^-$ cells (Fig. 3B). In a control set, post-esterase treated cells were washed with RPMI-1640 twice and kept in Medium A in absence of IFN-γ for 2 h. Alternatively, the culture of these washed post-esterase treated cells was replenished with fresh esterase and continued to be kept in culture in absence of IFN-γ. In both these sets, the basal level (2 ± 1 μM) of NO was detected indicating that the esterase alone play no significant role. Additionally, cells in presence of esterase-inactivated medium did not show any additional effects on NO production. Either Medium A or esterase medium alone, in absence of cells, revealed only 0.5 ± 0.2 μM NO production and considered as reagent blanks. Similarly, $PBMC_N$ in absence of IFN-γ revealed a low release of NO (1.0 ± 0.05 μM). LPS, serving as a positive control, produced 35 ± 5 μM NO_2^- (Fig. 3A).

To further strengthen this finding, the NO_2^- producing cells in the population of $PBMC_{ALL}$

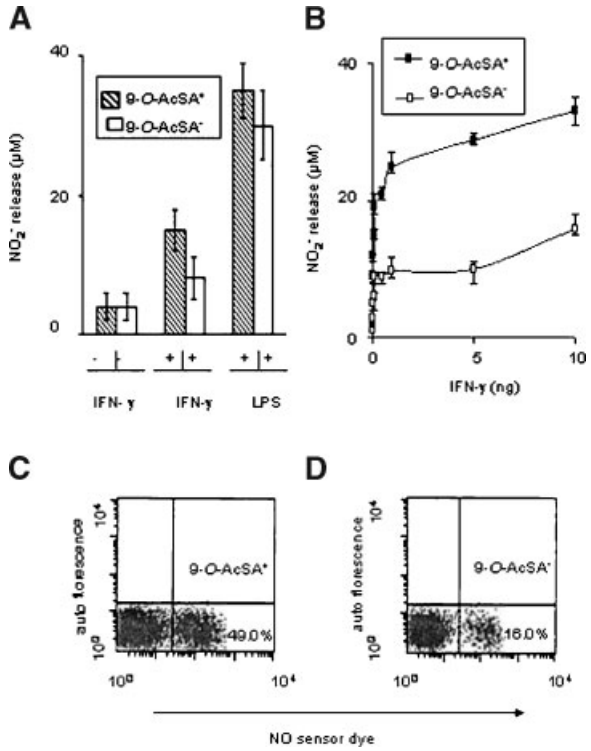

Fig. 3. **A**: Release of enhanced NO_2^- by 9-*O*-AcSA$^+$ cells as compared to 9-*O*-AcSA$^-$ cells. The release of NO_2^- by 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells in response to 6 h exposure of IFN-γ (0.1 ng) was monitored. Cells kept in absence of IFN-γ and in presence of LPS (2.5 ng) served as the negative and positive control respectively. The data shown is a representation of three experiments. **B**: Enhanced release of NO_2^- by 9-*O*-AcSA$^+$ cells with increasing concentration of IFN-γ. The cells were exposed with IFN-γ (0.01–10 ng) for 6 h, and supernatants were assayed spectrophotometrically by Griess reaction as described in the Materials and Methods. The data are the representation of five independent experiments. Cells in absence of IFN-γ served as the controls. **C**: Increased intracellular production of NO by 9-*O*-AcSA$^+$ cells as compared to 9-*O*-AcSA$^-$ cells. The 9-*O*-AcSA$^+$ cells were incubated with NO sensor dye for 30 min at 37°C and subsequently exposed to IFN-γ (0.1 ng) for 6 h at 37°C. The intracellular production of NO was detected by FACS analysis. **D**: 9-*O*-AcSA$^-$ cells were analysed similarly.

were determined by FACS analysis. Cells were initially probed with NO sensor dye and exposed to IFN-γ (0.1 ng) for 6 h. The 9-*O*-AcSA$^+$ cells (Fig. 3C) revealed a threefold ($49 \pm 1\%$ vs. $16 \pm 2\%$) increased NO_2^- production as compared to the 9-*O*-AcSA$^-$ cells (Fig. 3D).

Upregulation of iNOS Expression in 9-*O*-AcSA$^+$Cells

Enhanced release of NO_2^- by the 9-*O*-AcSA$^+$ cells correlated well with their status of iNOS mRNA expression. The increased expression of iNOS transcripts was observed in 9-*O*-AcSA$^+$ cells after 6 h exposure of IFN-γ (0.1 ng) as compared to the 9-*O*-AcSA$^+$ cells. Cells kept in absence of IFN-γ revealed basal level synthesis (Fig. 4A,B).

9-*O*-Acetylated Glycotope Involved in Modulating Survival of $PBMC_{ALL}$

To detect whether viability of $PBMC_{ALL}$ is affected by the presence of 9-*O*-acetylations both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells were exposed to IFN-γ (0.1 ng) for 6 h and assessed for (i) caspase-3-like activity and (ii) Annexin V positivity.

Enhanced caspase-3-like activity in 9-*O*-AcSA$^-$ cells. Caspase-3-like activity was detected spectophotometrically by the absorbance at 405 nm. The activity was found to be twofold higher in 9-*O*-AcSA$^-$ than the 9-*O*-AcSA$^+$ cells, OD 405_{nm} being 0.30 ± 0.10 vs. 0.15 ± 0.08 (Fig. 5A). Caspase-3-like activity in the $PBMC_N$ were found to be 0.14 ± 0.05.

The 9-*O*-AcSA$^-$ cells showed another fivefold increase in caspase-3-like activity, when NO_2^- production was lowered using LNAME (10 mM) in IFN-γ containing culture medium as compared to 9-*O*-AcSA$^+$ cells, $OD_{405\ nm}$ being 1.50 ± 0.10 versus 0.29 ± 0.09 (Fig. 5A).

The specificity of the reaction was confirmed by reduction of caspase-3-like-activity in presence of DEVD fmk ($OD_{405\ nm}$ being 0.08 ± 0.02). To reconfirm this observation, the caspase-3 profile in the cytosol of both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells before and after exposure of IFN-γ was investigated by Western blot analysis.

Western blot detects active caspase-3 in the cytosol of 9-*O*-AcSA$^-$ cells. Western blot analysis of the cytosol of IFN-γ exposed (9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) cells with anti-caspase 3 revealed a 20-kDa band indicative of active caspase only in the 9-*O*-AcSA$^-$ cells (Fig. 5B, lane 4). However, 32-kDa pro-caspase was present in the cytosol of 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells in presence and absence of IFN-γ.

Removal of *O*-acetylation enhanced annexin V positivity. A ten-fold more Annexin V positive 9-*O*-AcSA$^-$ cells (Fig. 6A) was observed after exposure with LNAME (10 mM) in presence of IFN-γ as compared to 9-*O*-AcSA$^+$ cells (Fig. 6B) suggesting a protective role of the glycotope.

In contrast, 9-*O*-AcSA$^-$ (Fig. 6C) and 9-*O*-AcSA$^+$ (Fig. 6D) cells alone in Medium A (in absence of IFN-γ) showed negligible binding

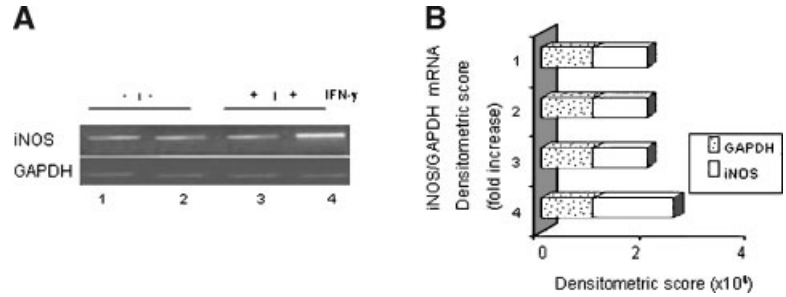

Fig. 4. **A**: 9-*O*-AcSA$^+$ cells expressed more iNOS as compared to 9-*O*-AcSA$^-$ cells. Total RNA was isolated from IFN-γ exposed and unexposed 9-*O*-AcSA$^-$ and 9-*O*-AcSA$^+$ cells, and iNOS expression was detected by RT-PCR. A housekeeping gene, GAPDH, was also assayed by RT-PCR. The products were subsequently analysed by agarose gel and shown as below. **Lane 1**: 9-*O*-AcSA$^-$ cells kept in Medium A in absence of IFN-γ. **Lane 2**: 9-*O*-AcSA$^+$ cells kept in Medium A in absence of IFN-γ. **Lane 3**: IFN-γ exposed 9-*O*-AcSA$^-$ cells. **Lane 4**: IFN-γ exposed 9-*O*-AcSA$^+$ cells. **B**: Densitometric analysis of the agarose gel is presented as a histogram, and the band intensities represented in the form of ratio of densitomentric scores.

with Annexin V. Cells kept under similar conditions in presence of esterase-inactivated medium displayed no binding with Annexin V, confirming that components present in Medium A or esterase medium had no effect in cell death. The decreased Annexin V binding with 9-*O*-AcSA$^+$ cells appears to be due to increased production of NO_2^- in these cells.

DISCUSSION

Earlier studies from this laboratory conclusively established the induction of linkage specific 9-*O*-AcSGs (90 and 120 kDa) containing 9-*O*-AcSAα2-6GalNAc glycotope on PBMC of ALL patients [Sinha et al., 1999a,d; Mandal et al., 2000; Pal et al., 2004a,b]. Absence of 9-*O*-AcSGs both on PBMC [Sinha et al., 1999a,b; Pal et al., 2000, 2001, 2004a,b] and erythrocytes [Ghosh et al., 2004a] of patients with other cross-reactive haematological disorders confirmed its disease specificity. However, the biological role of this disease-specific 9-*O*-AcSGs remained to be understood. Tumour cells are known to live in a cytokine environment that regulates their growth. A three- to four fold increase in concentration of IFN-γ in the sera of ALL patients as compared to normal individuals led us to think that these cells are possibly exposed to a microenvironment rich in IFN-γ hinting at its probable contributory role to the disease biology. The elevated level of IFN-γ may be due to specific stimulation of 9-*O*-AcSGs on $PBMC_{ALL}$ (Ghosh et al., 2004b), however

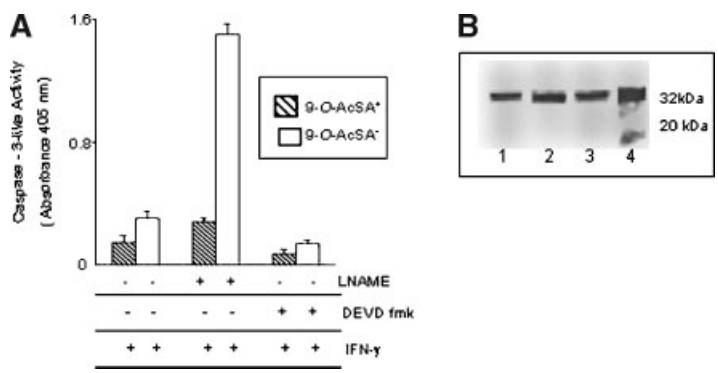

Fig. 5. **A**: Enhanced caspase-3-like activity in 9-*O*-AcSA$^-$ cells. Caspase-3-like activity was determined both in 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells (2×10^6) after exposure with IFN-γ (0.1 ng) for 6 h. The effect of NO on caspase-3-like activity was also detected on IFN-γ exposed cells in the presence of NO inhibitor LNAME (10 mM). The activity was measured in the cytosolic extracts by a colorimetric assay as described in Materials and Methods. Specificity of caspase-3-like activity was determined by treating extracts with caspase-3 inhibitor (DEVD-fmk). The absorbance in absence of IFN-γ was subtracted from each set of the experiments. The results presented are representative of three independent experiments. **B**: Western blot profile of caspase-3 in 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells. Cytosolic extracts of 9-*O*-AcSA$^+$ (**lanes 1**, **3**) and 9-*O*-AcSA$^-$ (**lanes 2**, **4**) cells (2×10^6) were resolved by SDS–PAGE (15%), transferred to nitrocellulose membranes and immunoblotted with anti-caspase-3 antibody without (lanes 1, 2) and with exposure (lanes 3, 4) of IFN-γ respectively.

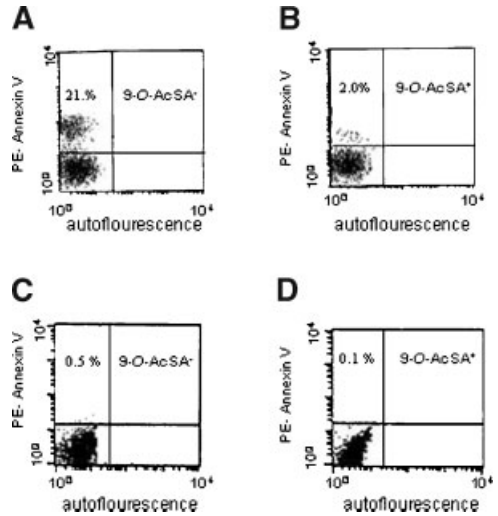

Fig. 6. Enhanced Anexin V positivity in 9-*O*-AcSA$^-$ cells. The 9-*O*-AcSA$^-$ (**A**) and 9-*O*-AcSA$^+$ (**B**) cells were kept in Medium A in the presence of both IFN-γ (0.1 ng) and LNAME (10 mM) for 6 h and stained with PE-Annexin V as described in Materials and Methods. The percent of Annexin V positive cells were detected by flow cytometry. 9-*O*-AcSA$^-$ (**C**) and 9-*O*-AcSA$^+$ (**D**) cells kept only in Medium A served as the controls.

the contributory role of other factors cannot be ruled out.

The major significance of our finding has been the functioning of 9-*O*-acetylated moieties in regulating survival of $PBMC_{ALL}$. Accordingly, 9-*O*-AcSA$^-$ cells were generated (Fig. 2A) and their de-*O*-acetylated status was maintained (Figs. 3–6). Using these 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells, we have demonstrated for the first time that increased caspase-3 activation (Fig. 5A,B) and apoptotic parameters (Figs. 5 and 6) in the 9-*O*-AcSA$^-$ cells through decreased production of NO_2^- (Fig. 3) and iNOS (Fig. 4) as compared to that of 9-*O*-AcSA$^+$ cells suggest a prominent role of this glycotope in the enhanced survival of $PBMC_{ALL}$.

IFN-γ is reported to influence the production of NO_2^- along with iNOS in certain tumours [Weinberg, 1998; Tachibana et al., 2000; Alexandrova et al., 2001] and is a bioregulator of apoptosis [Chung et al., 2001] and caspase 3 [Rossig et al., 1999; Kim et al., 2002]. IFN-γ-induced increased expression of NO_2^- (Fig. 3A–D) and iNOS (Fig. 4A,B) in 9-*O*-AcSA$^+$ cells as compared to 9-*O*-AcSA$^-$ cells, thus demonstrating its differential regulatory effect on these two cell types. A five-fold increase in activation of caspase-3 in 9-*O*-AcSA$^-$ cells was observed when availability of NO in the environment was further reduced (Fig. 5A). This increase in caspase-3-like activity by 9-*O*-AcSA$^-$ cells correlated well with the appearance of a 20 kDa active caspase-3 band in Western blot (Fig. 5B) contributing to a increased apoptosis (Fig. 6A). Taken together, our results appear to confirm the role of NO on modulation of caspase-3 activation in ALL.

Therefore, it may be hypothesised that the increased level of IFN-γ in the sera of these affected children is contributory to the survivility of $PBMC_{ALL}$ having overexpressed 9-*O*-AcSA moiety as the major glycotopes. Thus, a link possibly exists between the inductions of cytokine mediated NO_2^- production by 9-*O*-AcSA$^+$ cells and protection of $PBMC_{ALL}$ from programmed cell death. Our study is in concurrence with other observations of B-cells of chronic lymphocytic leukaemia (B-CLL) where cytokines, including IFN-γ, lead to the promotion of their survival possibly by regulating iNOS [Buschle et al., 1993; Levesque et al., 2003].

Although, sialic acid linked to glycoconjugates of Fas has been reported to regulate caspase-9-dependent apoptosis in Jurkat T-cell lymphoma [Suzuki et al., 2003], no information is available regarding the role of 9-*O*-AcSAα2-6GalNAc glycotope in the survival of lymphoblasts in ALL. The significance of this investigation is the demonstration, for the first time, that *O*-acetylation of sialic acid is helpful for survival of $PBMC_{ALL}$. However, the ideal situation to monitor the role of this glycotope in cell survival would be by generating an *O*-acetylation knockout cell. Such cells are currently unavailable. The exact molecular nature of the ligand for 9-*O*-acetylated sialoglycoconjugates on the $PBMC_{ALL}$ could allow a better understanding of the exact downstream molecular functioning of these molecules. In concordance with this school of thought, such studies are currently underway.

Thus, it appears that 9-*O*-acetylations of sialic acids are unique manifestations of $PBMC_{ALL}$, which possibly mediate the survival of lymphoblasts and may be considered to be one of the pathways for evading programmed cell death. The distribution of immunophenotypes in childhood ALL differs from adult ALL [Plasschaert et al., 2004] and it is therefore not surprising that elevated 9-*O*-AcSGs are only seen in juvenile ALL patients, and not found in adult ALL population indicative of their highly disease specific restricted expression. It has

been suggested that the two diseases are different in their origin, while in childhood ALL, the first genetic event happens in a more mature lymphoid committed progenitor cell, in adult ALL, the first hit occurs in multipotent stem cells [Plasschaert et al., 2004]. Whether the expression of 9-*O*-AcSGs is a developmentally regulated process in childhood ALL needs to be investigated in greater details, which, in turn, may contribute to the better understanding of the etiology of childhood ALL. As little is known about the factors mediating the evasion of the leukaemic cells from immune surveillance and its aggressive nature, studies on factors controlling their aberrant behaviour may contribute to an improved prognosis for ALL patients.

It is our aspiration that these results will have a far-reaching clinical relevance. Ideally, either the functioning of these 9-*O*-AcSGs or the group of enzymes responsible for regulating their expression could be exploited for pharmacological manipulation of the apoptotic pathways leading to clinical application.

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