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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

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Summary

Disease-specific over-expression of 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) on peripheral blood mononuclear cells (PBMC) of children with acute lymphoblastic leukaemia (ALL, $PBMC_{ALL}$) has been demonstrated using a lectin, Achatinin-H, with specificity towards 9-*O*-AcSAα2-6GalNAc. This study investigated the contributory role of 9-*O*-AcSGs induced on $PBMC_{ALL}$. Stimulation of $PBMC_{ALL}$ with Achatinin-H through 9-*O*-AcSGs led to a lymphoproliferative response with a significantly increased interferon-γ (IFN-γ) production when compared with unstimulated cells as demonstrated by enzyme-linked immunosorbent assay and mRNA expression. Under identical conditions, $PBMC_{ALL}$ ablated of *O*-acetylations did not respond to such stimulation. In summary, it may be concluded that stimulation of over-expressed 9-*O*-AcSGs regulate signalling for proliferation, leading to the release of IFN-γ. Controlled expression of these molecules may be exploited as potential targets for therapy, promising beneficial effects to children with ALL.

Keywords: childhood acute lymphoblastic leukaemia, Achatinin-H, *O*-acetylated sialic acid binding lectin, 9-*O*-acetylated sialoglycoconjugates, interferon gamma.

Sialic acids, commonly referred to as *N*-acetyl neuraminic acid (Neu5Ac) are a family of 9-carbon carboxylated monosaccharides. Amongst 50 known derivatives of sialic acids, 7, 8 and 9-*O*-acetylated derivatives (*O*-AcSA) are important constituents of the cell membrane and known to influence many physiological and pathological processes (Schauer, 2000, 2004) including cell–cell adhesion, signalling, differentiation, and metastasis (Shi *et al*, 1996; Kelm & Schauer, 1997; Corfield *et al*, 1999; Kohla *et al*, 2002).

Childhood acute lymphoblastic leukaemia (ALL) may be defined as a clonal lymphoproliferative disorder characterized by marked overproduction of lymphoblasts and represents the commonest type of paediatric cancer (Hoelzer *et al*, 2002). Although current treatment cures almost 80% of cases, the risk of relapse remains in 20% of patients in remission that harbour residual leukaemic blasts. Therefore, an urgent need exists to study the disease-biology in ALL. In this regard, we have previously reported an over-expression of disease-specific 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) on peripheral blood mononuclear cells (PBMC) of childhood ALL patients ($PBMC_{ALL}$; Sinha *et al*, 1999a; Mandal *et al*, 2000; Pal *et al*, 2004a,b), exploiting the selective binding affinity of a lectin, Achatinin-H, towards 9-*O*-AcSAα2-6GalNAc (Mandal & Basu, 1987; Sen & Mandal, 1995). The importance of these 9-*O*-AcSGs was further shown by demonstrating the gradual decline of 9-*O*-AcSGs with treatment, and their reappearance with frank relapse (Sinha *et al*, 1999b–d; Pal *et al*, 2004a,b). Additionally, lymphoproliferation of $PBMC_{ALL}$, at very low dose of Achatinin-H, has been documented, which was further exploited to develop a non-invasive assay for evaluating the clinical status of these children (Sinha *et al*, 1999b–d). These observations led us to explore the role of disease-specific 9-*O*-AcSGs in regulating signalling for lymphoproliferation of $PBMC_{ALL}$ and therefore, this study aims to understand their biological functioning.

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Accordingly, the present investigation focused on the (i) expression of 9-*O*-AcSGs having terminal 9-*O*-AcSAα2-6GalNAc glycotopes on different subsets of $PBMC_{ALL}$, (ii) stimulation of these 9-*O*-AcSGs and (iii) released cytokine profiles in response to this stimulation.

Materials and methods

Reagents

Monoclonal antibodies both for cytokine assay and flow cytometric studies were obtained from B.D. Pharmingen (San Diego, CA, USA). The primers were from Gibco-BRL. All other reagents and chemicals were obtained from Life Technologies (Grand Island, NY, USA).

Probes: Achatinin-H

The lectin, Achatinin-H was purified from haemolymph of the African giant land snail *Achatina fulica* by affinity chromatography using bovine submaxillary mucin (BSM) as the affinity matrix, because of its high content (22.5%) of 7(8)9-*O*-AcSA (Chatterjee *et al*, 1998; Sharma *et al*, 1998). Purified Achatinin-H was filter sterilized, stored at 4°C and found to be stable for 2 years. The preferential affinity of Achatinin-H towards 9-*O*-AcSAα2-6GalNAc was established (Mandal & Basu, 1987; Sen & Mandal, 1995). The lectin, either un-conjugated or conjugated with flourescein isothiocyanate (FITC), was used as an analytical probe for all experiments.

Study population

The study subjects ($n = 30$) included clinically and immunophenotypically confirmed childhood ALL patients at presentation comprising of 20 males and 10 females, M:F in the ratio of 2:1, with a median age of 2·25 years (range 0·8–14 years) and a median white blood cell (WBC) count of $7{\cdot}75 \times 10^9$/l (range $3{\cdot}5–11 \times 10^9$/l). Cytological examination of both peripheral and bone marrow samples were routinely diagnosed according to the French–American–British Group recommendations (Burns *et al*, 1981) and were immunophenotyped using commercially available anti-CD monoclonal antibodies. Briefly, PBMC were gated for lymphocytes by staining with anti-CD45 monoclonal antibody. Within the cases diagnosed, immunologic subgroups were defined as follows: common B- ($n = 19$, $CD19^+$, $CD20^+$, $CD10^{\pm}$, cyt-μ^+), and T- ($n = 11$, $CD2^{\pm}$, $CD3^+$, and $CD7^+$) ALL. The diagnosis was further validated by fluorescence-activated cell sorting (FACS) analysis using two in-house probes, Achatinin-H and anti-9-*O*-AcSG antibody, specific towards 9-*O*-AcSAα2-6GalNAc glycotope. All the diagnosed patients had 80–90% lymphoblasts detected both by morphological and immunophenotyping studies, irrespective of the lineages. Simultaneously, diagnosis was confirmed by detecting anti-9-*O*-AcSG antibody in the sera of ALL patients (Pal *et al*, 2000, 2001). Age-matched, normal healthy individuals ($n = 20$) of either sex with different blood groups and patients with other cross-reactive haematological diseases e.g. acute myeloid leukaemia (AML, $n = 10$) and chronic lymphocytic leukaemia (CLL, $n = 9$) were included in the study.

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

Detection of 9-O-AcSG expression on $PBMC_{ALL}$ subsets by double colour FACS analysis

Double colour flow cytometric studies were performed to detect the expression of 9-*O*-AcSGs on $PBMC_{ALL}$ (B and T lineages) and normal PBMC ($PBMC_N$). In brief, PBMC were separated by Ficoll gradient, washed and incubated with phycoerythrin (PE)-conjugated anti-CD-7 (T-ALL), anti-CD-19 (B-ALL) and FITC-Achatinin-H (0·1 μg) for 1 h on ice, in the dark, then washed and fixed with paraformaldehyde (1%) and analysed using cell quest software. Similarly, PBMC from clinically-confirmed AML and CLL patients and normal individuals were analysed. Viability of cells was found to be >98%.

The expressions of 9-*O*-AcSGs on $PBMC_{ALL}$ subsets were detected by the whole blood lysis method. Briefly, whole blood (50 μl) was incubated with FITC-Achatinin-H and PE-conjugated anti-CD-19, CD-10, CD-7, CD-13 and CD-14 antibodies separately. The erythrocytes were lysed using FACS lysing solution (BD, 2 ml in 1:10 dilution), centrifuged, washed, fixed and analysed. Isotype-matched antibody and FITC-bovine serum albumin (BSA) or FITC-Achatinin-H, in the presence of unconjugated lectin control (preincubation of cells with Achatinin-H followed by incubation with FITC-Achatinin-H), was used as different sets of controls. Alternatively, cells were incubated with Achatinin-H followed by anti-Achatinin-H antibody and the bound complex was detected by FITC-conjugated second antibody. The percentage of positive cells was recorded, based on the threshold or background fluorescence provided by all these sets of controls, which gave similar level of background fluorescence.

The specificity of Achatinin-H interaction with 9-O-acetylations detected by esterase treatment of cells

The *O*-acetylation from $PBMC_{ALL}$, were removed by 9-*O*-acetyl haemagglutinin esterase of recombinant influenza C virus fusion protein, which specifically hydrolyses the *O*-acetyl groups of sialic acid (Vlasak *et al*, 1987; Chatterjee *et al*, 2003). Accordingly, PBMC (1×10^6) were incubated with recombinant fusion protein (100 μl, 12·0 mU) for 1 h at 37°C and complete removal of *O*-acetylation was confirmed by flow cytometry using FITC Achatinin-H as probe.

In vitro lymphoproliferation assay

$PBMC_{ALL}$ were cultured (1×10^5 cells/ml) separately in the presence of Achatinin-H (0–12 μg), maintained in RPMI-1640 medium supplemented with 5% heat inactivated fetal calf serum (FCS), 0·2 mg/ml penicillin/streptomycin, 0·1% (w/v) L-glutamine (Medium A) at 37°C in an atmosphere of 5% CO_2, in a 96-well microtitre plate for 0–48 h in triplicates. In parallel, PBMC isolated from AML and CLL patients and normal individuals were cultured similarly. Cell free culture supernatants were stored at −70°C for the detection of cytokines.

The specificity of the stimulatory effect of Achatinin-H through 9-*O*-AcSGs on $PBMC_{ALL}$ was established by preincubating Achatinin-H (0·1 μg) with the optimal inhibitory concentration of BSM (0·05 μmol/l) for 1 h at 37°C and cultured for 48 h. Alternatively, esterase-treated cells cultured in presence of Achatinin-H, cells incubated with only BSM or esterase-treated cells alone served as the different controls.

Quantification of cell proliferation

In order to quantify the lymphoproliferation as a consequence of Achatinin-H stimulation, viable cells were assessed both by conventional trypan blue dye exclusion and methylthiazoltetrazolium (MTT) assay. MTT was prepared in phosphate-buffered saline (PBS), filtered through 0·22 μm mesh immediately before use, and diluted in RPMI-1640 medium to 2 mg/ml (Sinha *et al*, 1999b). Cells were incubated with MTT solution (100 μg) for 3 h at 37°C; the formazan crystals formed were dissolved in dimethyl sulphoxide (DMSO). The absorbance at 570_{nm} was a quantitative measure of cell viability.

Quantitative analysis of secreted cytokines by enzyme-linked immunosorbent assay (ELISA)

The concentration of cytokines released into the culture supernatants from Achatinin-H stimulated $PBMC_{ALL}$ was assayed by sandwich ELISA. Briefly, microtitre plates were coated separately with anti-human interleukin (IL)-10, IL-2, IL-12, and interferon-γ (IFN-γ), in 0·03 mol/l carbonate buffer, pH 9·6 for overnight at 4°C, washed and blocked with 1% BSA for 2 h at 37°C and incubated with culture supernatants overnight. Preincubated biotinylated secondary antibodies and horseradish peroxidase (HRP)-streptavidin (1:50) were added, processed as per the manufacturers protocol, using azino-bisthiosulphuric acid (ABTS) as substrate and the OD_{405nm} read in an ELISA Reader (Lab Systems, Helsinki, Finland). The concentrations of cytokines were calculated against the standard curve using standard recombinant cytokines. The lower detection limit for this assay was 1·5 pg/ml for IL-10 and 7 pg/ml for IFN-γ, IL-2, and IL-12. Culture supernatants from un-stimulated cells served as the controls. Cytokines in the sera of ALL patients and normal individuals were quantitated similarly.

Reverse transcription polymerase chain reaction (RT-PCR) Studies

Cellular RNA was extracted using Trizol from Achatinin-H (0·10 μg)-stimulated $PBMC_{ALL}$. RNA (1 μg) was reverse transcribed to cDNA using a poly (dT) oligonucleotide and 200 U Superscript II reverse transcriptase. Each PCR cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min using specific primers for cytokines (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 visualized under UV lamp. A commercially prepared 100 base pair ladder was used as a molecular weight marker. RNA isolated from unstimulated cells served as the control. The intensity of the bands for cytokine was determined by densitometry using the quantity one software.

Results

Over-expression of 9-O-AcSGs on subsets of $PBMC_{ALL}$

In accordance with our earlier studies, the WBC count correlated well with 9-*O*-AcSG expression (Pal *et al*, 2004a) and the disease-specific expression of 9-*O*-AcSGs on $PBMC_{ALL}$ from B- and T-ALL patients were irrespective of their lineages (Sinha *et al*, 1999a; Mandal *et al*, 2000; Pal *et al*, 2004a,b). The distribution of 9-*O*-AcSGs on different haematopoetic cells present in the population of $PBMC_{ALL}$ showed that, although 89–95% cells were CD-19^+-9-*O*-$AcSG^+$, typically in a B-ALL patient, low numbers of other cells (T, myeloid and monocyte cells) were also present. Interestingly, even these low number of cells (CD-7^+; 2–3%, CD-13^+; 3–6% and CD-14^+; 8–10%) co-expressed 9-*O*-AcSGs (Fig 1A). Similarly, in T-ALL patients expressing (CD-7^+-9-*O*-$AcSG^+$; 93–97%) a small number of other cells present also co-expressed 9-*O*-AcSGs. PBMC subsets from normal individuals however reflected low level (3–9%) 9-*O*-AcSG expression (Fig 1B).

The shift in the mean fluorescence intensity (83 to 5%) of untreated and esterase-treated cells confirmed the *O*-acetylated status of these disease-specific molecules (data not shown). Lack of expression of 9-*O*-AcSGs on PBMC from AML (0·05–0·8%) and CLL (0·2–0·7%) patients (data not shown) further confirmed the restricted over-expression of 9-*O*-AcSGs only on $PBMC_{ALL}$.

Achatinin-H induces proliferation of $PBMC_{ALL}$

To observe the stimulatory effect of Achatinin-H on $PBMC_{ALL}$, cells were cultured in the absence or presence of Achatinin-H at different doses for 48 h. While high concentration of Achatinin-H (10–12 μg) induced cell death after 48 h, the lower doses (0·01, 0·05 μg) could not induce any significant lymphoproliferative response (Fig 2A). Maximal lymphoproliferative response by Achatinin-H (0·1 μg) was observed at

Table I. Primers for cytokines.

Gene	Forward	Reverse	Size (bp)
IFN-γ	5′TCTGCATCGTTTTGGGTTCT 3′	5′CAGCTTTTCGAAGTCATCTC 3′	300
IL-10	5′AGATCTCCGAGATGCCTTTCA3′	5′TTTCGTATCTTCATTGTCATGTA3′	408
IL-2	5′ATGTACAGGATGCAACTCCTGTCTT 3′	5′GTCAGTGTTGAGATGATGCTTGAC 3′	458
IL-12(P40)	5′CCAAGAACTTGCAGCAGCTGAAG3′	5′ TGGGTCTATTCCGTTGTGTC 3′	360
GAPDH	5′ATGGGGAAGGTGAAGGTCGG 3′	5′ GGGTGCTAAGCAGTTGGT3′	540

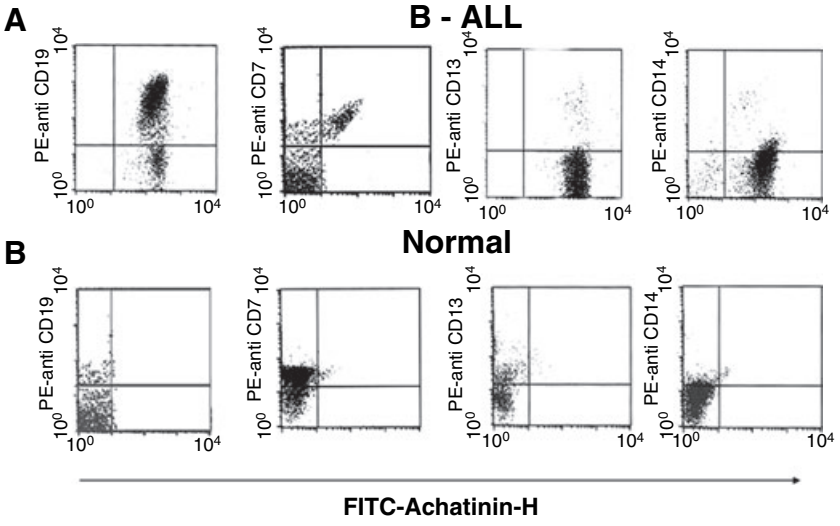

Fig 1. Overexpression of 9-*O*-AcSGs on subsets of $PBMC_{ALL}$. (A) A representative profile of disease-specific expression of 9-*O*-AcSGs on different PBMC of a B-ALL patient, demonstrated by staining cells with FITC-Achatinin-H and PE-conjugated anti-CD-19, anti-CD-7, anti-CD-13 and anti-CD-14 monoclonal antibodies individually. Appropriate isotype matched controls were used as described in *Materials and methods*. (B) A representative profile of whole blood from a normal individual, processed similarly.

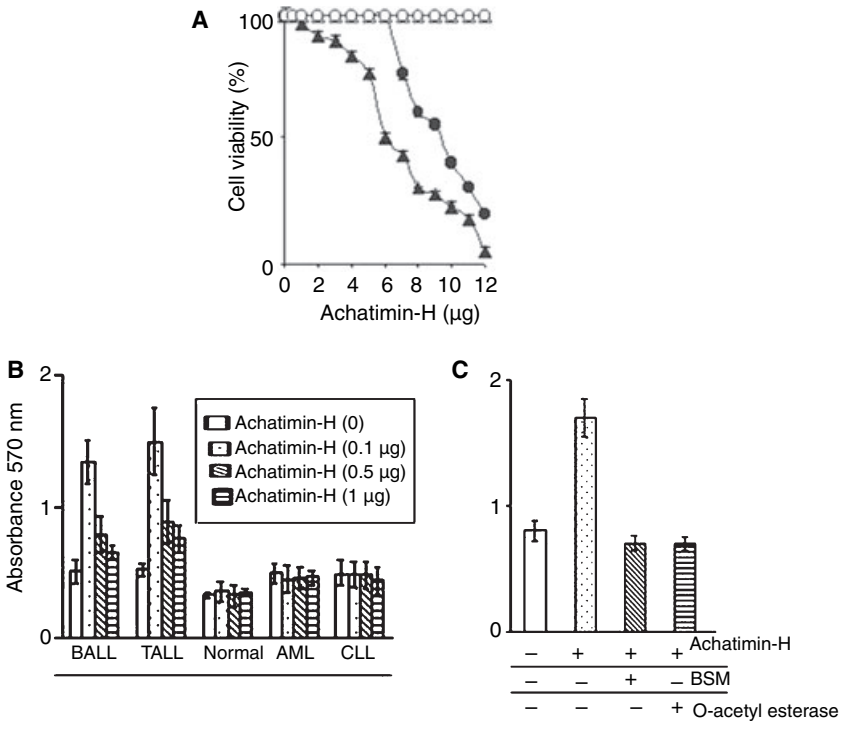

Fig 2. Achatinin-H specifically interacts with 9-*O*-AcSGs on $PBMC_{ALL}$. (A) Dose response of Achatinin-H on viability. A representative profile of $PBMC_{ALL}$ from a B- and T-ALL patient were cultured in presence (B-▲ and T-●) or absence (B-△ and T-○); of Achatinin-H (0–12 μg) for 48 h and the viability of cells were detected by MTT assay. The data is a representative profile of $n = 10$ patients. (B) Proliferation of PBMC from B, T-ALL, AML, CLL patients and normal donors. Cells (1×10^5/ml) were cultured in the absence and presence of Achatinin-H for 48 h and proliferation was quantitated by the MTT assay. The data is a representative profile of 10 independent experiments. The points represent mean ± SEM of triplicate determinants of one of the two replicate experiments. Cells in absence of Achatinin-H served as control. (C) Lymphoproliferation needs interaction between Achatinin-H and 9-*O*-AcSGs. Cells were cultured in absence (□) or presence of Achatinin-H before (▨) and after pre-incubation with BSM (▩) and $PBMC_{ALL}$ ablated of *O*-acetylations (▤) as described in *Materials and methods*.

48 h. At this dose and time, a two- to threefold lymphoproliferative response was observed in $PBMC_{ALL}$ from both B and T lineages as compared with that of unstimulated cells as detected by the MTT assay (Fig 2B), $O.D._{570nm}$ being 1·34 ± 0·25, 1·5 ± 0·28 for B and T ALL, respectively, as compared with 0·522 ± 0·09 (Fig 2B).

No lymphoproliferation was observed when $PBMC_{ALL}$ were cultured in presence of Achatinin-H preincubated with the optimum inhibitory concentration of BSM (Fig 2C). $PBMC_{ALL}$ ablated of *O*-acetylations, obtained by treating cells with *O*-acetyl esterase, cultured in presence of Achatinin-H, did not show any proliferation (Fig 2C). BSM alone, in the absence of Achatinin-H, under similar condition, showed no proliferative response. However, the addition of BSM did not induce cell death.

PBMC from AML and CLL patients (Fig 2B) did not show any proliferation with this dose of Achatinin-H (0·1 μg) under identical conditions. PBMC from normal individuals, although expressing a minimum level of 9-*O*-AcSGs showed low affinity towards Achatinin-H (Pal *et al*, 2004a) and did not show any proliferation with 0·1 μg of Achatinin-H.

Achatinin-H stimulation predominantly induces IFN-γ

To understand the effect of stimulation of $PBMC_{ALL}$ by Achatinin-H (0·1 μg), we investigated the (i) the cytokine release, (ii) cytokine in serum (iii) expression of these cytokines at the genetic level.

(i) Enhanced IFN-γ secretion by stimulated $PBMC_{ALL}$. An enhanced release of IFN-γ by cells stimulated with Achatinin-H (0·1 μg) was observed even after 6 h (105 ± 5 pg/ml) of exposure, increasing gradually at 24 h (456 ± 10 pg/ml) and at 48 h (673 ± 11 pg/ml; Fig 3A). The secretion of IL-10 followed a similar pattern, but with a significantly low amount of release, 40 ± 15 and 70 ± 20 after 24 and 48 h of stimulation respectively. The release of IL-2 and IL-12 were 25 ± 10 and 17 ± 6 pg/ml, respectively, under similar conditions (Fig 3A).

(ii) Increased IFN-γ level in serum of ALL patients at presentation. In order to understand the *in vivo* situation, we have monitored the level of IFN-γ both in ALL patients, at presentation, i.e. prior to therapy and normal sera. Elevated level of IFN-γ (650–950 pg/ml) was observed in the sera of ALL patients, which was almost three- to fourfold higher than normal serum (Fig 3B). However, the level of other cytokines in these patients was low.

(iii) Increased mRNA expression of IFN-γ. Cytokine transcripts of IFN-γ were consistently detected in $PBMC_{ALL}$, at the genetic level, in response to Achatinin-H (0·1 μg) stimulation (Fig 4). IFN-γ transcripts were over-expressed after 48 h of stimulation while the expression of IL-12 and IL-2 remained constant, reflected by densitometry scanning (Fig 4). The release of IL-10 after stimulation with Achatinin-H was much less than that of IFN-γ. Un-stimulated cells revealed synthesis of basal level of cytokines.

Discussion

Assessment of the *O*-acetylated sialoglycoconjugates in ALL is a relatively new domain in leukaemia sialobiology. Over-expression of 9-*O*-AcSGs (90 and 120 kDa) on $PBMC_{ALL}$ (Sinha *et al*, 1999a; Pal *et al*, 2004a,b) and the concomitant elevated level of anti-9-*O*-AcSG antibodies in sera (Pal *et al*, 2000, 2001, 2004c) of ALL patients prior to therapy have been documented. The gradual reduction in the expression of the disease-specific 9-*O*-AcSGs (90 and 120 kDa) with therapy established their

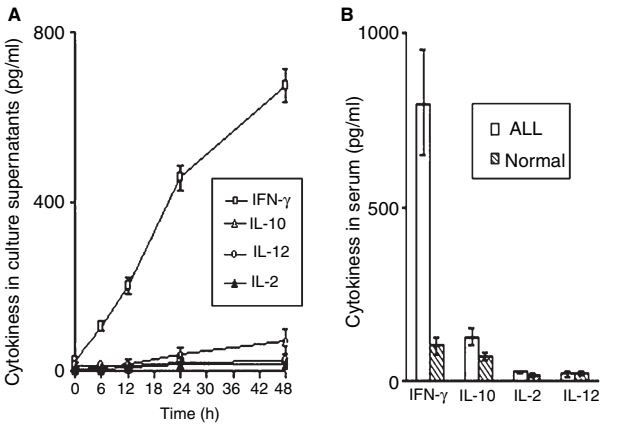

Fig 3. Release of cytokines by $PBMC_{ALL}$ due to stimulation with Achatinin-H. (A) $PMBC_{ALL}$ were cultured in the presence of Achtatinin-H (0·1 μg) for 48 h. The concentration of the released cytokines in the culture supernatant was detected by ELISA as described in *Materials and methods*. Release of cytokines by the unstimulated cells served as the control. The data is a representative profile of ALL patients ($n = 30$). The points represent mean ± SEM of triplicate determinants of one of the two replicate experiments. (B) Increased level of IFN-γ in sera of ALL patients. Levels of cytokines in the sera of B- and T-ALL patients, prior to therapy ($n = 10$), and normal individuals ($n = 10$) were quantitated by ELISA.

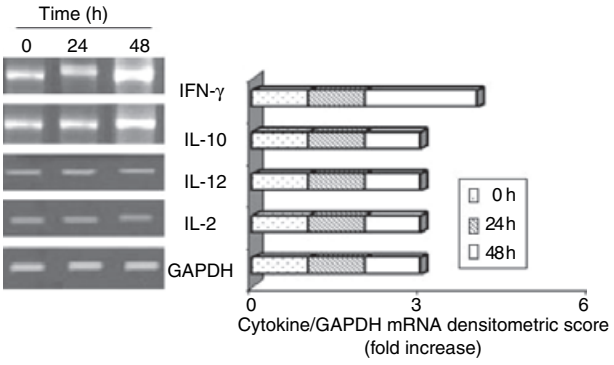

Fig 4. Cytokine mRNA expression in Achatinin-H stimulated $PBMC_{ALL}$. Total RNA from Achatinin-H-stimulated (0·1 μg) $PBMC_{ALL}$ was isolated and the expression of cytokines at different time points (0–48 h) was analysed by RT-PCR as described in *Materials and methods*. GAPDH served as the housekeeping gene. The mRNA expression of unstimulated cell served as the control. The values were compared by densitometric score and this is represented in the fold of increase in band intensity as compared with the expression of GAPDH at the respective time points. The data is a representative profile of 10 ALL patients.

diagnostic and prognostic potential (Pal *et al*, 2004a). Previous studies have conclusively established the expression of high affinity 120 and 90 kDa 9-*O*-AcSGs on leukaemic lymphoblasts (Pal *et al*, 2004a). These high affinity 9-*O*-AcSGs were found to be highly active as they can be stimulated even with a very low dose of Achatinin-H (Sinha *et al*, 1999b–d). The observation suggests that 9-*O*-AcSGs possibly play a key role in regulating signalling for proliferation.

The major achievement of this finding has been the (i) expression of 9-*O*-AcSGs on different haematopoetic cells in ALL, irrespective of lineage (ii) stimulation of such disease-specific 9-*O*-AcSGs, leading to enhanced production of IFN-γ demonstrating the biological consequence to lymphoproliferation.

Our results are suggestive of a major defect in the glycosylation pattern on the haematopoetic cells of these children, as demonstrated by the overexpression of 9-*O*-AcSGs not only on T- and B-lymphocytes, but also on monocytes, and myeloid cells of these patients (Fig 1A). The key role in generating such aberrant glycosylations may be attributed to a group of enzymes, namely *O*-acetyltransferase, sialyltransferases, sialidase and *O*-acetylesterases regulating the metabolism of sialoglycoconjugates (Shen *et al*, 2004). Currently, no molecular information is available about these enzymes and studies are underway to detect their altered expression in ALL.

The elevated levels of 9-*O*-AcSG on all cells in the peripheral blood suggest that this may well be a general response to the proliferation of acute leukaemic cells. This has clearly been demonstrated by activating the newly-induced 9-*O*-AcSAα2-6GalNAc glycotopes on $PBMC_{ALL}$ with Achatinin-H (Fig 2A-C). This specific interaction led to a significant lymphoproliferative response, as reflected by the variations in stimulation index with different dose of Achatinin-H (Fig 2B), suggesting the functionally active nature of the disease-specific 9-*O*-AcSGs. Absence of lymphoproliferation of $PBMC_{ALL}$ by Achatinin-H was observed when 9-*O*-AcSA present on $PBMC_{ALL}$ was blocked by preincubation with BSM, or *O*-acetylation ablated $PBMC_{ALL}$ were used for stimulation, reconfirming the involvement of *O*-acetyl group in this stimulation (Fig 2C). In contrast, the lack of proliferation of PBMC of AML and CLL patients under similar conditions suggested disease-specific stimulation via interaction with 9-*O*-AcSGs (Fig 2B). $PBMC_N$ could not be stimulated with this dose of lectin, corroborating our earlier observation (Sinha *et al*, 1999b–d) and further confirming the disease-specific interaction (Fig 2B).

In vitro interaction of 9-*O*-AcSGs on $PBMC_{ALL}$ with Achatinin-H led to the release of a high amount of IFN-γ (Fig 3A) in the culture supernatant together with an increased mRNA level (Fig 4) indicating the potential of 9-*O*-AcSGs in mediating the signalling responses. Normal B cells have been reported to be incapable of IFN-γ production (Young, 1997). Since the study population included patients with around 80–90% lymphoblasts in the peripheral blood it may be envisaged that the release of IFN-γ was solely mediated by leukaemic lymphoblasts. However, as a small population of other cells also expressed 9-*O*-AcSGs, their collective interplay cannot be ruled out. The exact pathway of the downstream events leading to IFN-γ production in ALL is yet to be investigated.

Cytokines are released in response to a diverse range of cellular stresses that profoundly affect several stages of cancer formation, growth of tumours *in vivo*, progression and playing a significant role in immunosurveillance against malignant cells (Colombo & Trinchieri, 2002). Abundant interactions have been reported to transform the complex cytokine network regulation of normal haematopoiesis into an even more interlaced patchwork controlling leukaemic haematopoiesis (Dranoff, 2004). IFN-γ is reported to influence many biological processes along with the production of NO_2^- and iNOS in certain tumours (Tachibana *et al*, 2000; Alexandrova *et al*, 2001) and a link between IFN-γ-induced iNOS expression and inhibition of spontaneous apoptosis in B-CLL has been reported (Levesque *et al*, 2003). The contributory role of other molecules in aiding lymphoblast survival remains to be investigated in greater details.

In addition, the observed three- to fourfold increase in the serum concentration of IFN-γ as compared with normal individuals (Fig 3B) is probably indicative of the presence of ligands for 9-*O*-AcSAα2-6GalNAc glycotope in the patients' serum, regulating the functional level of IFN-γ in ALL. The presence of anti-9-*O*-AcSGs in the sera of these children (Pal *et al*, 2000, 2001, 2004c) may acts as ligand for newly-induced 9-*O*-AcSGs on $PBMC_{ALL}$. Thus, it may be hypothesized that the presence of anti-9-*O*-AcSG antibody in the milieu of other molecules in patients' serum can mediate the observed effects. However, the exact contributory molecules to the pathway are yet to be elucidated and studies are currently ongoing to address this hypothesis.

In summary, we have established that the disease-specific 9-*O*-AcSGs are abundantly overexpressed on different haematopoietic cells in ALL. These *O*-acetylated sialoglycoproteins are functionally active molecules, which, when stimulated with a ligand specific for its glycotope, is capable of mediating downstream effects revealed by lymphoproliferative responses with elevated levels of IFN-γ.

Thus, this observation is suggestive of a possible role of over-expressed disease-specific 9-*O*-AcSGs in regulating signalling for proliferation. Controlled expression of these molecules may be exploited for therapeutic applications, promising beneficial effects to children with ALL.

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References

Alexandrova, R., Mileva, M. & Zvetkova, E. (2001) Nitric oxide and cancer. *Experimental Pathology and Parasitology*, **4,** 13–18.

Burns, C.P., Armitage, J.O., Frey, A.L., Dick, F.R., Jordan, J.E. & Woolson, R.F. (1981) Analysis of the presenting features of adult acute leukemia: the French–American–British classification. *Cancer*, **47,** 2460–2469.

Chatterjee, M., Sharma, V., Mandal, C., Sundar, S. & Sen, S. (1998) Identification of antibodies directed against O-acetylated sialic acids in visceral leishmaniasis: its diagnostic and prognostic role. *Glycoconjugate Journal*, **15,** 1141–1147.

Chatterjee, M., Chava, A.K., Kohla, G., Pal, S., Merling, A., Hinderlich, S., Unger, U., Strasser, P., Gerwig, G.J., Kamerling, J.P., Vlasak, R., Crocker, P.R., Schauer, R., Reinhard, S.A. & Mandal, C. (2003) Identification and characterization of adsorbed serum sialoglycans on Leishmania donovani promastigotes. *Glycobiology*, **13,** 351–361.

Colombo, M.P. & Trinchieri, G. (2002) Cytokines and cancer. *Cytokines and Growth Factor Reviews*, **13,** 93–94.

Corfield, A.P., Myerscoug, N., Warren, B.F., Durdey, P., Paraskeva, C. & Schauer, R. (1999) Reduction of sialic acid *O*-acetylation in human colonic mucins in the adenoma-carcinoma sequence. *Glycoconjugate Journal*, **16,** 307–317.

Dranoff, G. (2004) Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer*, **4,** 11–20.

Hoelzer, D., Gokbuget, N., Ottmann, O., Pui, C.H., Relling, M.V., Appelbaum, F.R., van Dongen J.J. & Szczepanski, T. (2002) Acute lymphoblastic leukemia. *Hematology*, **2002,** 162–192.

Kelm, S. & Schauer, R. (1997) Sialic acids in molecular and cellular interactions. *International Review of Cytology*, **175,** 137–140.

Kohla, G., Stockfleth, E. & Schauer, R. (2002) Gangliosides with *O*-acetylated sialic acids in tumors of neuroectodermal origin. *Neurochemical Research*, **7,** 583–592.

Levesque, M.C., Misukonis, M.A., O' Loughlin, C.W., Chen, Y., Beasley, B.E., Wilson, D.L., Adams, D.J., Silber, R. & Weinberg, J.B. (2003) IL-4 and interferon gamma regulate expression of inducible nitric oxide synthase in chronic lymphocytic leukemia cells. *Leukemia*, **17,** 442–450.

Mandal, C. & Basu, S. (1987) A unique specificity of a sialic acid binding lectin AchatininH from the hemolymph of *Achatina fulica* snails. *Biochemistry Biophysics Research Communication*, **148,** 795–801.

Mandal, C., Chatterjee, M., & Sinha, D. (2000) Investigation of 9-*O*-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukemia. *British Journal of Haematology*, **110,** 801–812.

Pal, S., Chatterjee, M., Bhattacharya, D.K., Bandyopadhyay, S. & Mandal, C. (2000), Identification and purification of cytolyic antibodies directed against *O*-acetylated sialic acid in childhood acute lymphoblastic leukemia. *Glycobiology*, **10,** 539–549.

Pal, S., Chatterjee, M., Bhattacharya, D.K., Bandhyopadhyay, S., Mandal, C. & Mandal, C. (2001) *O*-acetyl sialic acid specific IgM in childhood acute lymphoblastic leukaemia. *Glycoconjugate Journal*, **18,** 529–537.

Pal, S., Ghosh, S., Bandyopadhyay, S., Mandal, C.N., Bandhyopadhyay, S., Bhattacharya, D.K. & Mandal C. (2004a) Differential expression of 9-*O*-acetylated sialoglycoconjugates on leukemic blasts: a potential tool for long-term monitoring of children with acute lymphoblastic leukemia. *International Journal of Cancer*, **111,** 270–277.

Pal, S., Ghosh, S., Mandal, C., Kohla, G., Brossmer, R., Isecke, R., Merling, A., Schauer, R., Schwartz-Albiez, R., Bhattacharya, D.K. & Mandal, C. (2004b) Purification and characterisation of 9-O-acetylated sialoglycoproteins from leukaemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukaemia. *Glycobiology*, **14,** 859–870.

Pal, S., Bandyopadhyay, S., Chatterjee, M., Bhattacharya, D.K., Minto, L., Hall, A.G. & Mandal, C. (2004c) Antibodies against 9-O-acetylated sialoglycans: a potent marker to monitor clinical status in childhood acute lymphoblastic leukemia. *Clinical Biochemistry*, **37,** 395–403.

Schauer, R. (2000) Achievements and challenges of sialic acid research. *Glycoconjugate Journal*, **17,** 485–499.

Schauer, R. (2004) Sialic acids: fascinating sugars in higher animals and man. *Zoology*, **107,** 49–64.

Sen, G. & Mandal, C. (1995) The specificity of the binding site of AchatininH, a sialic acid-binding lectin from *Achatina fulica*. *Carbohydrate Research*, **268,** 115–125.

Sharma, V., Chatterjee, M., Mandal, C., Sen, S. & Basu, D. (1998) Rapid diagnosis of Indian visceral leishmaniasis using Achatinin H, a 9-*O*-acetylated sialic acid binding lectin. *American Journal of Tropical Medicine and Hygiene*, **58,** 551–554.

Shen, Y., Kohla, G., Lrhorfi, A.L., Sipos, B., Kalthoff, H., Gerwig, G.J., Kamerling, J.P., Schauer, R. & Tiralongo, J. (2004) *O*-acetylation and de-*O*-acetylation of sialic acids in human colorectal carcinoma. *Europen Journal of Biochemistry*, **271,** 281–290.

Shi, W.X., Chammas, R. & Varki, A. (1996). Regulation of sialic acid 9-*O*-acetylation during the growth and differentiation of murine erythroleukemia cells. *Journal of Biological Chemistry*, **271,** 31517–31525.

Sinha, D., Mandal, C. & Bhattacharya, D.K. (1999a) Identification of 9-*O*-acetyl sialoglycoconjugates (9-OAcSGs) as biomarkers in childhood acute lymphoblastic leukemia using a lectin, Achatinin H, as a probe. *Leukemia*, **13,** 119–125.

Sinha, D., Bhattacharya, D.K. & Mandal, C. (1999b) A colorimetric assay to evaluate the chemotherapeutic response of children with acute lymphoblastic leukemia (ALL) employing Achatinin H: a 9-*O*-acetyl sialic acid binding lectin. *Leukemia Research*, **9,** 803–809.

Sinha, D., Mandal, C. & Bhattacharya D.K. (1999c) Development of a simple, blood based lymphoproliferation assay to assess the clinical status of patients with acute lymphoblastic leukemia. *Leukemia Research*, **23,** 433–439.

Sinha, D., Mandal, C. & Bhattacharya, D.K. (1999d) A novel method for evaluation of childhood acute lymphoblastic leukemia. *Leukemia*, **13,** 309–312.

Tachibana, H., Kakuta, S., Yagami, K. & Nagumo, M. (2000) Effects of cytokines on the production of nitric oxide in a chondrogenic cell line established from human osteogenic sarcoma. *Oral Disease*, **6,** 303–309.

Vlasak, R., Krystal, M., Nacht, M. & Palese, P. (1987) The influenza C virus glycoprotein (HE) exhibits receptor binding (hemagglutinin) and receptor destroying (esterase) activities. *Virology*, **160,** 419–425.

Young, H.A. (1997) Interferon expression by B cells. *Methods*, **11,** 112–115.