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Comparative analysis of differential expression of sialic acids and adhesion molecules on mononuclear cells of bone marrow and peripheral blood in childhood acute lymphoblastic leukaemia at diagnosis and clinical remission

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Childhood acute lymphoblastic leukaemia (ALL) is characterized by the neoplasm of immature haematopoietic precursor cells (HPCs). We report significant differences between the expression of sialoglycoproteins and adhesion molecules on mononuclear cells (MNCs) of bone marrow (BM) and peripheral blood (PB) from individual children at diagnosis of the disease. Lymphoblasts in PB predominantly expressed 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac_2-GPs), sialic acid, α2-3 linked sialic acid, L- and P-selectins and vascular cell adhesion molecule -1 (VCAM-1) on their surface compared to BM, as determined with selective lectins and monoclonal antibodies (mAbs) by flow cytometric analysis. $CD34^+CD38^+$ cells present either in diagnostic PB or BM always showed enhanced expression of both α2-3 and α2-6 linked sialic acids, Neu5,9Ac_2-GPs, L- and P-selectins and VCAM-1, compared to $CD34^+CD38^-$ population, as confirmed by higher mean fluorescence intensity (MFI). Expression of ICAM-1 was reverse. However, MFI of Neu5,9Ac_2-GPs was always higher both in $CD34^+CD38^+$ and $CD34^+CD38^-$ population in PB compared to BM. Diverse trend of these cell surface macromolecules was observed during clinical remission. This is the first comparative study between PB and BM, where significant differential distribution of sialylated macromolecules and adhesion molecules was observed. Hence, supervising these cell surface macromolecules at various stages of treatment might help in minimal residual disease detection, identifying mobilization factor(s) and in isolation of normal HPCs for autologous BM transplantation.

Keywords: 9-*O*-Acetylated sialoglycoproteins, Childhood acute lymphoblastic leukaemia, Flow cytometry, Haematopoietic precursor cells, Immunoglobulin (Ig) superfamily cell adhesion molecules, Sialic acids, Selectins.

Childhood acute lymphoblastic leukaemia (ALL) is defined as a clonal lymphoproliferative disorder characterized by malignant transformation and marked overproduction of lymphoblasts, representing the commonest type of paediatric cancer[1]. The phenomenon of normal haematopoiesis, which is regulated by the communication between haematopoietic cells and bone marrow compartment brought about by the ligand-receptor interaction of several adhesion molecules is disrupted in ALL. Neoplastic transformation is often associated with a variety of structural changes on the cell surface, most notably increased sialic acid densities[2]. Sialic acids as terminal residues of oligosaccharide chains play crucial roles in several cellular detection proceedings[3].

N-acetylneuraminic acids (Neu5Ac) commonly known as sialic acids, are a family of 9-carbon carboxylated monosaccharides that can have a variety of modifications[4]. They are important constituents of the cell membrane and influence many biological reactions either by reacting with specific surface receptors or via masking of carbohydrate recognition sites[5-9]. 9-*O*-Acetyl-GD3 gangliosides, considered an

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Abbreviations: ALL, acute lymphoblastic leukaemia; BM, bone marrow; BM_{CR}, BM at clinical remission; BSM, bovine submaxillary mucin; CAMs, cell adhesion molecules; CR, clinical remission; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; HPCs, haematopoietic precursor cells; ICAMs, intercellular adhesion molecules; LECAMs, leucocyte endothelial cell adhesion molecules; LPA, *Limulus polyphemus* agglutinin; MAA, *Maackia amurensis* agglutinin; mAbs, monoclonal antibodies; MFIs, mean fluorescence intensities; MNCs, mononuclear cells; Neu5,9Ac_2-GPs, 9-*O*-acetylated sialoglycoproteins; Neu5Ac, N-acetylneuraminic acid; PB, peripheral blood; PB_{CR}, PB at clinical remission; SNA, *Sambucus nigra* agglutinin; SSC, side scatter; VCAM-1, vascular-cell adhesion molecule-1.

oncofetal marker has been identified using a lectin *Cancer antennarius,* which recognizes sialic acids (Neu5Ac) that are *O*-acetylated at both C-4 and C-9 and biomarkers of human melanoma[9]. The 9-*O*-acetylated disialosyl carbohydrate sequence of CDw60 has been reported as a marker on activated human B lymphocytes[10]. The 9-*O*-acetylated sialoglycans are detectable at low levels on human B lymphocytes[11], and pathologic variations have been found[12-15]. However, their detailed biological significance, especially as potential biomarkers remains ambiguous.

Cell surface adhesion molecules play vital roles in numerous cellular processes like cell growth, differentiation, embryogenesis, immune cell transmigration and response and cancer metastasis. They are capable of transmitting information from the extracellular matrix to the cell. Four major families of cell adhesion molecules (CAMs) include the immunoglobulin (Ig) superfamily CAMs, integrins, cadherins and selectins[16-19]. The Ig superfamily CAMs are calcium-independent transmembrane glyco-proteins and include the inter-cellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1)[17-19]. The selectins (LECAMs - leucocyte endothelial CAMs) are a family of carbohydrate-binding proteins which bind fucosylated carbohydrates, especially sialylated Lewis^x and mucins[16,17,19-22]. The three family members of selectins include endothelial (E)-selectin, leukocyte (L)-selectin and platelet (P)-selectin. They play vital role in the initial steps of leukocyte trafficking.

The main aim of our investigation has been to explore and compare the importance of the sialic acid derivatives and CAMs on the mononuclear cells (MNCs) of peripheral blood (PB) and bone marrow (BM) of same patients of childhood ALL at the diagnosis of disease and evaluate their status in PB and BM after successful chemotherapy. Immature haematopoietic precursor cells (HPCs) present in ALL are mostly characterized by the enhanced expression of CD34 antigen[25] and hence most of the children have very high $CD34^+$ cells (at least >10%) in their lymphoblast population. It is also reported that more than 95% of childhood B-lineage ALL express CD38[23-27]. Thus, comparative analysis of the status of sialic acids and CAMs in primitive HPCs ($CD34^+CD38^-$) and lineage-committed cells ($CD34^+CD38^+$) in PB and BM was carried out.

This is the first systematic comparative study between PB and BM in ALL, where noteworthy differential distribution pattern of sialylated macromolecules and CAMs has been observed. The aberrant expression of the some of these molecules may be the underlying factor(s) responsible for migration and mobilization of the cancerous blasts from the BM to PB. In depth knowledge of these cell surface antigens can, therefore, provide insight into the biology of the disease and be used for the detection of minimal residual disease and drug-targeted immunotherapy.

Material and Methods

Clinical samples

Clinical samples comprised of diagnostic peripheral blood (PB) and bone marrow (BM) from childhood ALL patients (n=15) of both sexes. PB and BM of same patients (n=10) at clinical remission (CR, PB_{CR} and BM_{CR}) were also included. Patients consisted of males and females in the ratio of 2:1, median age (6 yrs; range 0.8-16 yrs) and median presenting white blood cell count of 12×10^9/L, range 0.4-1,000 $\times$ 10^9/L. The diagnosis was established by cytological examination of the BM smears according to the French-American-British Group recommendations, belonging to L1 or L2 and by cytogenetic study. Each sample was immunophenotyped using antibodies against terminal deoxyneucleotidyl transferase (TdT), cytoplasmic μ (cyt-μ), surface membrane Ig, CD2, CD3, CD4, CD7, CD8, CD10, CD13, CD19, CD20, CD33, CD34 and CD45. Patients included in this study had significantly more lymphoblasts in PB compared to BM at diagnosis.

Venous blood (3-4 ml) and BM (100-1000 μl) were collected at Kothari Medical Centre, Kolkata and then sent to the Indian Institute of Chemical Biology, Kolkata. 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.

Analytical probes used to study the sialic acids and adhesion molecules

Analytical probes used for this study and their specificities are illustrated in Table 1. The lectin Achatinin-H was purified from the haemolymph of the African giant land snail *Achatina fulica* by affinity chromatography using bovine submaxillary mucin (BSM) as the affinity matrix. The preferential affinity

Table 1—Analytical probes for studying cell surface macromolecules on lymphoblasts present in PB and BM of children with ALL

A. Lectins used for studying the sialoglycoconjugates	
Lectins	Binding glycotope
Achatinin-H	Neu5,9Ac$_2$α2-6β-D-GalNAc
Limulus polyphemus agglutinin (LPA)	Neu5Ac
Maackia amurensis agglutinin (MAA)	Neu5Acα2-3Gal
Sambucus nigra agglutinin (SNA)	Neu5Acα2-6Gal/GalNAc
B. Monoclonal antibodies used for studying the adhesion molecules	
Monoclonal antibodies (mAbs)	Binding epitope
Anti-CD62L	L-selectin/LECAM-1/CD62L
Anti-CD62E	E-selectin/LECAM-2/CD62E
Anti-CD62P	P-selectin/LECAM-3/CD62P
Anti-CD54	ICAM-1/CD54
Anti-CD106	VCAM-1/CD106

of achatinin-H towards 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac$_2$GPs) with terminal Neu5,9Ac$_2$α2-6β-D-GalNAc was established[28,29]. The remaining lectins, namely, LPA, SNA and MAA were purchased from Vector Laboratories. The adhesion molecules were studied using five different probes namely anti-CD62L, anti-CD62E, anti-CD62P, anti-CD54 and anti-CD106 monoclonal antibodies (mAbs).

Achatinin-H and LPA were conjugated with fluorescein isothiocyanate (FITC)[3]. SNA, MAA, CD62E and CD54 (ICAM-1) were biotinylated and the signals generated were captured by FITC-streptavidin. CD62P, CD62L and CD106 (VCAM-1) were used in purified form and the signals were captivated with FITC-anti-murine IgG. All adhesion molecules and the secondary reagents, namely, FITC-streptavidin and FITC-anti-murine IgG and other mAbs were purchased from BD Biosciences (Pharmingen, San Jose, CA, USA).

Flow cytometry

Optimization of dose of different analytical probes

Optimized dose of FITC-Achatinin-H was initially standardized using MNCs from normal and diagnostic ALL patients. Different doses of FITC-achatinin-H (0.02-0.2 μg/10 μl in TBS-Ca^{2+}) were separately incubated with 1×10^6 cells at 4°C in dark for 1 h. The cells were washed and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA). The optimum doses for the remaining analytical probes namely, LPA, SNA, MAA, CD62L, CD62E, CD62P, CD54, CD106, FITC-streptavidin and FITC-anti-murine IgG were either optimized in a similar manner or used as per manufacturers' instructions.

Assessment and comparison of expression of different lectins and adhesion molecules between patients at diagnosis (PB and BM) and CR (PB_{CR} and BM_{CR})

The MNCs from the clinical samples (PB or BM) were separated using Ficoll gradient centrifugation. The cells were separated and washed twice in phosphate buffered saline (PBS, *p*H 7.2) and suspended in RPMI-1640 supplemented with 2 mm glutamine, gentamycin and 2% foetal calf serum (FCS) for 1 h on ice. Cells (1×10^6/100 μl) were individually incubated with PE-CD34, PECy5-CD38 and a FITC conjugated or biotinylated or unconjugated analytical probe (lectin/mAb), along with appropriate isotype controls in ice for 1 h in dark. This was followed by washing and incubation with FITC-streptavidin for biotinylated probes or FITC-anti-murine IgG for the unconjugated probes. Each tube contained three different fluorescent-tagged probes. The cells were washed thrice in PBS to remove unbound antibodies, fixed in paraformaldehyde (1%) and the binding was assessed. MNCs were gated for lymphocytes by staining with anti-CD45 mAb and >98.0% positive cells were present, thus reassuring their being haematopoietic cells. All the samples were processed, fixed and acquired within 24 h from the time of collection and all the conditions were rigidly maintained during the assay. Binding was analyzed and calculations were performed using CellQuestPro software (B.D. Biosciences, San Jose, CA).

The status of sialic acids and adhesion molecules was initially studied and compared between the MNCs present in PB and BM of diagnostic ALL patients using only FITC tagged probes. Subsequently, these children were monitored for these macromolecules both in PB and BM during CR. This comparative study was further extended with lineage-uncommitted, primitive HPCs ($CD34^+CD38^-$) and in the lineage-committed ($CD34^+CD38^+$) cells in diagnostic PB and BM using triple colour flow-cytometric analysis.

Acquisition and analysis of samples

At the time of acquisition, the rate of flow of the cells was kept below 300 events/s (in RUN-LO mode), so that all the cells were properly analyzed. Forward scatter (FSC) and side scatter (SSC) were kept in linear scale. Cells in the region 400-600 of FSC were gated as the lymphoblasts (Region R1) and 10,000 events in R1 were recorded. During analysis, cells in the R1 gating were plotted in FL1 histogram to assess the status of sialic acids and adhesion molecules in MNCs. The events in R1 gating were further dissected in a second dot-plot with PE-CD34 *versus* PECy5-CD38 (FL2 *versus* FL3). In this dot-plot, $CD34^+CD38^-$ and $CD34^+CD38^-$ populations were gated as R2 and R3 respectively. For assessing the status of sialic acids and adhesion molecules in $CD34^+CD38^-$ and $CD34^+CD38^+$ populations, the cells in the R2 and R3 regions were separately plotted in FL1 histograms.

Statistical analysis

Statistical analysis was performed using the Graph-Pad Prism statistics software program (Graph-Pad Software Inc., CA). Results were expressed as mean ± S.D. for individual set of experiments. Two-tailed *p* value for unpaired *t* test (p) < 0.05 was considered significant.

Results

Optimization of doses of lectins and adhesion molecules

About 90% MNCs from diagnostic patients were positive for FITC-Achatinin-H (0.04 µg). This dose of lectin gave negligible binding with MNCs from normal individuals, thus was selected for all subsequent experiments. The optimum doses of MAA, anti-CD62E, anti-CD62L, anti-CD62P anti-CD106, FITC-streptavidin and FITC-anti-murine IgG were 1 µg each, while that of SNA, LPA and anti-CD54 was 0.25 µg, 0.5 µg and 20 µl, respectively.

Differential distribution of sialylation and adhesion molecules on the MNCs between patients at diagnosis (PB and BM) and CR (PB_{CR} and BM_{CR})

Status of sialic acids

The percentage of $Neu5,9Ac_2$-GPs^+ cells as detected by FITC-achatinin-H was about two-times higher in diagnostic PB than BM (p = 0.0002, Fig. 1a). However, there was minimal presence of $Neu5,9Ac_2$-GPs^+ cells in PB_{CR} and BM_{CR} (p = 0.25); expression being eight and three-fold more in diagnostic PB and BM respectively, when compared to patients at CR (Table 2A). The dissimilarity

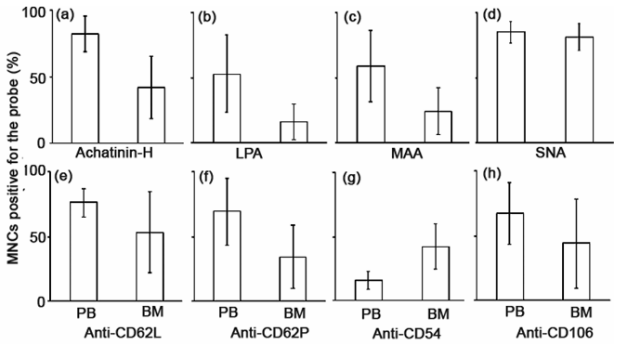

Fig. 1—Comparison of status of sialic acid and its derivatives along with adhesion molecules on MNCs present in diagnostic peripheral blood (PB) and bone marrow (BM) of same individual suffering from childhood ALL by single colour FACS analysis [Binding Achatinin-H, LPA, SNA, MAA, CD62L, CD62P, CD54, CD106 to MNCs (1 x 10^6 cells) of PB and BM, paired samples obtained from individual children (n=15) determined as described in Material and Methods. Bar depicts the mean ± SD of the percentage of cells expressing sialoglycoproteins and adhesion molecules as evidenced by binding with fluorescence tagged lectins (**a-d**) and specific mAbs against adhesion molecules (**e-h**)]

between the percentages of $Neu5Ac^+$ cells as detected by FITC-LPA was considerable between diagnostic PB and BM (p = 0.0025, Fig. 1b), being about four-fold higher in the former. Insignificant variation between PB_{CR} and BM_{CR} was observed (p = 0.23, Table 2A), while a two-fold increase in $Neu5Ac^+$ cells was found in BM_{CR} when compared to diagnostic BM.

About two-fold difference existed between diagnostic PB and BM with respect to the Neu5Acα2-$3Gal^+$ cells (PB>BM) (p=0.004, Fig. 1c), as detected by FITC-MAA. PB_{CR} and BM_{CR} had more of Neu5Acα2-$3Gal^+$ cells compared to diagnostic samples, about three-fold more Neu5Acα2-$3Gal^+$ cells found in BM_{CR} compared to diagnostic BM. However, insignificant variation was found between PB_{CR} and BM_{CR} (p = 0.33, Table 2A). The percentage of cells having cell surface sialoglycoproteins with terminal Neu5Acα2-6Gal/GalNAc as observed using FITC-SNA did not exhibit much variation between PB *versus* BM, irrespective of the diagnostic (p=0.072) or CR (p=0.46) status of the patients (Fig. 1d, Table 2A).

Status of selectins

L-$selectin^+$ cells did not show considerable discrepancy between PB *versus* BM at diagnosis (p = 0.09, Fig. 1e) and in samples collected during CR (PB_{CR} *versus* BM_{CR}, p = 0.089). However, three-fold enhanced expression of L-$selectin^+$ cells was found on diagnostic PB as compared to PB_{CR} (Table 2B).

Table 2—Status of cell surface macromolecules on lymphoblasts in PB and BM at diagnosis and clinical remission (CR) of childhood ALL

A. Differential distribution of sialoglycoproteins

Lectins	Percent positive cells (Mean ± SD)			
	*PB	*BM	#PB_{CR}	#BM_{CR}
Achatinin-H	82.16 ± 14.23	42.52 ± 24.68	9.93 ± 4.41	14.68 ± 5.29
LPA	53.11 ± 30.15	16.56 ± 14.09	34.59 ± 11.49	46.62 ± 10.34
MAA	58.42 ± 27.44	24.24 ± 17.64	77.44 ± 10.56	85.88 ± 8.92
SNA	81.33 ± 3.90	89.45 ± 8.67	79.99 ± 5.34	77.54 ± 3.80

B. Status of adhesion molecules

mAbs against adhesion molecules	Percent positive cells (Mean ± SD)			
	*PB	*BM	#PB_{CR}	#BM_{CR}
Anti-CD62L	76.4 ± 28	52.6 ± 31	26.8 ± 5.58	47.1 ± 14.18
Anti-CD62E	No binding			
Anti-CD62P	69 ± 25.5	34 ± 24.2	18.07 ± 4.8	42.49 ± 10.01
Anti-CD54	15.75 ± 16.9	41.73 ± 17.6	10.02 ± 4.15	14.55 ± 4.56
Anti-CD106	66.52 ± 33.5	43.65 ± 10.9	18.74 ± 6.13	39.17 ± 13.33

*Paired samples of peripheral blood (PB) and bone marrow (BM) from same individual at the diagnosis of childhood ALL (n = 15)
#PB_{CR} and BM_{CR}: PB and BM of patients at clinical remission (CR) (n = 10)

E-selectin$^+$ cells could not be detected in MNCs at diagnosis or during CR. Therefore, cell surface carbohydrate molecules specific to L- and E-selectins showed no significant variation on MNCs of PB and BM in childhood ALL at diagnosis and CR. In contrast, P-selectin$^+$ cells were about 2-fold higher in diagnostic PB as compared to BM (p = 0.006, Fig. 1f). However, this expression was 3.8-fold less in PB_{CR} compared to diagnostic PB. Significant difference (~2.35-fold) was also observed between PB_{CR} and $BM_{CR,}$ being more in the latter (p = 0.02, Table 2B).

Status of immunoglobulin (Ig) superfamily CAMs

Among nine cell surface macromolecules, included in this study, CD54 (ICAM-1) was the only exception exhibiting an opposite trend in expression being significantly higher (~2.65-fold) in diagnostic BM, compared to PB (p = 0.003, Fig. 1g). After successful chemotherapy, there was a considerable reduction in CD54$^+$ cells in $BM_{CR.}$ However, a small percentage of cells expressed CD54 in PB_{CR} and BM_{CR} (p = 0.24, Table 2B). Status of CD106$^+$ cells in between diagnostic PB *versus* BM and PB_{CR} *versus* BM_{CR} exhibit insignificant variation as can be observed from the p values of 0.15 and 0.07 respectively (Fig. 1h, Table 2). However, there is more than three-fold difference between diagnostic PB and PB_{CR} being more in the former.

Status of CD34$^+$, CD34$^+$CD38$^-$ and CD34$^+$CD38+ cells in diagnostic PB and BM

At the onset of ALL in children, there was an incredible increase in the percentage of CD34$^+$ cell population being 86.5 ± 8.6 and 42 ± 24 in MNCs present in PB and BM respectively (p = 0.0001, Fig. 2a, b). The primitive HPCs (CD34$^+$CD38$^-$) population in PB and BM was significantly higher in these patients. Fig. 2c illustrates one such representative patient with very high CD34$^+$CD38$^-$ population. However, a large variation in CD34$^+$CD38$^-$ population was often encountered among patients. This intrigued us to compare the pattern of sialylation and adhesion molecules in CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ population in diagnostic PB and BM.

Comparative analysis of sialylation and adhesion molecules on CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ population of PB and BM at commencement of ALL

The pattern of sialylation and adhesion molecules on CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ population present in MNCs of both diagnostic PB and BM has been shown in terms of mean fluorescence intensity (MFI, Fig. 3, Table 3). The MFIs of all the probes indicated the average number of receptors on the cell surface of primitive HPCs and in the lineage-committed cells. There was a conspicuous variation in the number of Neu5,9Ac_2-GPs molecules, being higher in the CD34$^+$CD38$^+$ and lower in the CD34$^+$CD38$^-$

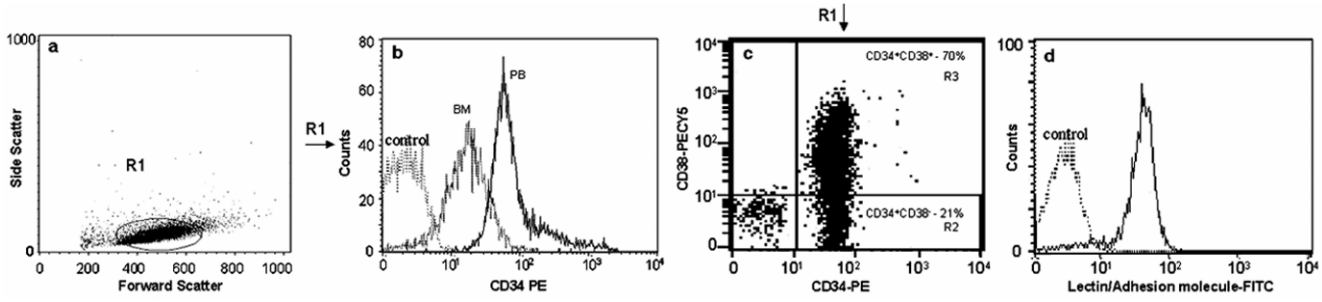

Fig. 2—Comparative analysis of $CD34^+$, $CD34^+CD38^-$ and $CD34^+CD38^+$ cells in MNCs of diagnostic PB and BM of same patient with childhood ALL by FACS analysis. A representative dot plot with forward scatter *versus* side scatter showing the region gated for the lymphoblasts revealed as R1 (**a**). Binding of PE-anti-CD34 mAb to MNCs from paired samples (PB and BM) of same children (n=15) was determined as described in Material and Methods. A representative histogram overlay plot showing the percent of $CD34^+$ cells in PB and BM from an individual patient (**b**). A double colour FACS analysis demonstrating the comparison between $CD34^+CD38^-$ and $CD34^+CD38^+$ population in MNCs of diagnostic PB and BM of same patient. Lymphoblasts in R1 region, as shown in a, was analyzed using PE-CD34 and PECy5-CD38 mAbs for all the patients included in this study. A representative dot plot showing the percent of cells which are $CD34^+CD38^-$ and $CD34^+CD38^+$ gated as R2 and R3 regions respectively (**c**). Status of sialic acids and adhesion molecules on $CD34^+CD38^-$ and $CD34^+CD38^+$ population by triple colour FACS analysis. Gated lymphoblasts, as shown in R2 or R3 region, as shown in c, was further analyzed by using FITC-lectin or mAbs specific for adhesion molecules. A representative histogram plot presenting the log fluorescence intensity of FITC-Achatinin-H on $CD34^+CD38^+$ cells present in diagnostic PB of an individual has been shown (**d**).

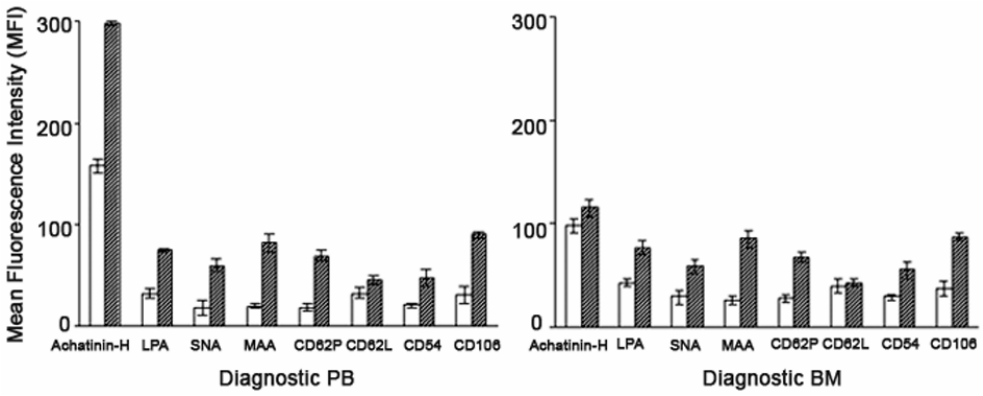

Fig. 3—Comparison of mean fluorescence intensities (MFIs) of sialoglycoproteins and adhesion molecules in $CD34^+CD38^-$ and $CD34^+CD38$ population present in MNCs of diagnostic PB and BM by triple colour FACS analysis using FITC-lectin/mAb, PE-CD34 and PECy5-CD38 [Blank and shaded bars represent primitive ($CD34^+CD38^-$) and lineage-committed ($CD34^+CD38^+$) populations respectively]

population and this phenomenon was irrespective of PB or BM. A significant increase in the number of Neu5,9Ac_2-GPs molecules on the $CD34^+CD38^-$ population present in PB is observed as compared to that of BM (p=0.004). Similar trend is observed on $CD34^+CD38^+$ population between PB and BM (p = 0.0001).

The number of other cell surface macromolecules was higher in the $CD34^+CD38^+$ as compared to $CD34^+CD38^-$ populations (Fig. 3, Table 3). However, no significant variation between PB and BM was observed. In contrast, number of ICAM-1 molecules was more in immature $CD34^+CD38^-$ than $CD34^+CD38^+$ population both in PB and BM. The number of L-selectin remained the same in both populations, irrespective of PB and BM.

Discussion

A methodical and comparative analytical study of the paired samples at the diagnosis of childhood ALL has revealed a distinct differential pattern of sialylation and adhesion molecules on MNCs of PB and BM obtained from individual patients. Upon monitoring these children at clinical remission, the

Table 3—Aberration in cell surface macromolecules on $CD34^+CD38^-$ and $CD34^+CD38^+$ populations present in PB and BM of diagnostic ALL patients

Cell surface macromolecules	Mean fluorescence intensity (MFI)			
	PB		BM	
	$CD34^+CD38^-$	$CD34^+CD38^+$	$CD34^+CD38^-$	$CD34^+CD38^+$
Sialoglycoproteins with terminal Neu5,9Ac_2α2-6β-D-GalNAc	158±12.76	298±2.3	98±11.32	115±4.5
Sialoglycoconjugates	33±5.47	75±5.44	43±6.28	77.61±3.89
Sialoglycoconjugates with terminal Neu5Ac2α2-3β-D-Gal	20.16±4.85	82.04±4.12	26.03±2.71	85.44±1.67
Sialoglycoconjugates with terminal Neu5Ac2α2-6β-D-GalNAc	19±3.25	60.11±3.79	24.11±1.34	59.33±6.38
L-Selectin	33.49±7.52	45.94±5.89	40.54±5.87	43.33±5.67
P-Selectin	19.45±6.76	70.66±2.15	28.65±6.46	68.22±7.41
VCAM-1	31.82±5.03	90.45±8.34	37.33±7.23	88.11±8.47
ICAM-1	48.29±9.27	21.55±5.87	55.92±4.56	29.85±4.95

$CD34^+CD38^-$ represents the most primitive haematopoietic precursor cell population and $CD34^+CD38^+$ represents the lineage-committed population

expression of these cell surface macromolecules alters drastically. Further analysis of these paired samples exhibits discrete variation in the content of sialic acid and their derivatives along with other adhesion molecules in primitive $CD34^+CD38^-$ and lineage-committed $CD34^+CD38^+$ populations both in diagnostic PB as well as BM.

Neu5,9Ac_2-GPs are disease-associated antigens with enhanced expression on lymphoblasts and increased anti-Neu5,9Ac_2-GPs antibodies at the commencement of childhood ALL[3,30-35]. However, there is nominal presence of this derivative on normal MNCs. Interestingly, childhood ALL patients show significantly lower Neu5,9Ac_2-GPs at CR, which is indicative of successful chemotherapy[30-31]. The status of total or linkage specific sialic acids on MNCs is poorly addressed in ALL. Present investigation has revealed a general trend of increase in these macromolecules on the MNCs after successful treatment, in contrast to Neu5,9Ac_2-GPs. At CR, there is a down-regulation of 9-*O*-acetylated sialic acids with a simultaneous upregulation of the Neu5Ac and Neu5Acα2-3Gal on the MNCs, indicating that these molecules have become 9-*O*-acetylated at the onset of the disease. This variation triggered us to further investigate and compare the status of these sialic acids between paired sample of PB and BM at diagnosis as well as in CR.

At the onset of disease, there is a significant difference in the percentages of Neu5,9Ac_2-GPs^+ cells between PB and BM, suggesting successful diagnosis even with PB. Additionally, the MFI of Neu5,9Ac_2-GPs is always higher in the lineage-committed ($CD34^+CD38^+$) population than the primitive HPCs ($CD34^+CD38^-$), suggesting that the number of Neu5,9Ac_2-GPs increases with the lineage commitment of the MNCs. Interestingly, the number of these molecules is higher on $CD34^+CD38^-$ population of diagnostic PB compared to BM, indicating that Neu5,9Ac_2-GPs expression is more on the primitive HPCs of diagnostic PB. The $CD34^+CD38^+$ cells residing in the BM compartment have lower MFI than their PB counterparts and may be the reason behind their staying in the BM compartment. Hence, this observation opens up a new avenue for exploring the role of 9-*O*-acetylation of cell surface sialic acids for possible investigation of understanding the phenomenon of maturation, mobilization, even isolation of primitive HPCs in childhood ALL.

Although same individuals show prominent difference in the percentage of $Neu5Ac^+$ and Neu5Acα2-3Gal^+ cells between diagnostic PB and BM (PB >BM), no significant difference is observed when they are in CR i.e., between PB_{CR} and BM_{CR}. This phenomenon may be due to the presence of more blasts in diagnostic PB as observed by immunotyping using standard CD antigens.

The enhanced expression (as determined by MFI) of the total sialoglycoproteins (two-fold), sialoglycoprotein with terminal Neu5Acα2-3Gal (four-fold) and terminal Neu5Acα2-6Gal (three-fold)

is observed on lineage-committed ($CD34^+CD38^+$) cells, compared to the HPCs ($CD34^+CD38^-$), irrespective of diagnostic PB and BM, indicating a general upregulation of sialylation during lineage commitment of the MNCs. However, there is lack of significant variation of these three sialoglycoproteins in $CD34^+CD38^+$ and $CD34^+CD38^-$ populations, irrespective of PB and BM at diagnosis.

In accordance with the variable pattern of sialylation of the MNCs, other macromolecules like selectins and Ig superfamily CAMs exhibit a wide divergence in their appearance on the lymphoblasts of PB and BM at diagnosis. This pattern is quite different when the MNCs of these patients are monitored in CR. This variable expression may play a role in angiogenesis, which is an upcoming aspect of the disease biology of leukaemia, and therefore, needs further investigation and monitoring.

The Ig superfamily CAMs bind to β2 integrin and have profound role in migration of lymphocytes[18-20]. In contrast to other macromolecules, ICAM-1^+ ($CD54^+$) cells are more in the MNCs of diagnostic BM, compared to diagnostic PB. This may be due to the fact that the CD54 antigen is cleaved-off during the commencement of ALL. This phenomenon is further attested by the MFI, which is two-fold higher in the primitive $CD34^+CD38^-$ population compared to the lineage-committed $CD34^+CD38^+$ population, indicating that almost half of the CD54 antigen is cleaved-off from the surface of the MNCs.

No significant variation in the percentage of VCAM-1^+ ($CD106^+$) cells at diagnosis and in CR suggests that the expression for this molecule is not aberrant in the MNC population in childhood ALL. Expression of CD106 antigen follows the usual trend of being higher in $CD34^+CD38^+$ than $CD34^+CD38^-$ population.

In conclusion, this study reveals anomalous expression of 9-*O*-acetylated sialoglycoproteins, sialic acids, α2-3 linked sialic acid, L- and P-selectins, ICAM-1 and VCAM-1 molecules on the cell surface of lymphoblasts at the onset of ALL. This pattern changes when patients at clinical remission are considered. The solitary or synergistic effect of one or many of these aberrantly expressed cell surface macromolecules may provide valuable information regarding the disease biology. Therefore, monitoring of one or all of these markers in untreated and treated ALL patients may probably help in minimal residual disease detection, deciphering the phenomenon of mobilization of the lymphoblasts from BM compartment to circulation, drug-targeted immunotherapy and identification of normal haematopoietic stem cells for autologous transplantation.

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