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Immunophenotype of normal and leukemic bone marrow B-precursors in a Brazilian population. A comparative analysis by quantitative fluorescence cytometry

E.M. Rego[1,2], A.B. Garcia[2], J.J. Carneiro[3] and R.P. Falcão[1,2]

[1]Fundação Hemocentro de Ribeirão Preto, Centro de Terapia Celular, and Departamentos de [2]Clínica Médica and [3]Cirurgia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Abstract

The distinction between normal and leukemic bone marrow (BM) B-precursors is essential for the diagnosis and treatment monitoring of acute lymphoblastic leukemia (ALL). In order to evaluate the potential use of quantitative fluorescence cytometry (QFC) for this distinction, we studied 21 normal individuals and 40 patients with $CD10^+$ ALL. We characterized the age-related changes of the CD10, CD19, TdT, CD34 and CD79a densities in normal and leukemic BM. Compared to normal adults, the B-precursors from normal children expressed significantly lower values of CD34-specific antibody binding capacity (SABC) (median value of 86.6 *vs* 160.2 arbitrary units (a.u.) in children and adults, respectively). No significant age-related difference was observed in the expression of the other markers in the normal BM, or in any of the markers in the leukemic BM. Based on the literature, we set the cut-off value for the normal CD10 expression at 45 x 10^3 a.u. for both age groups. For the remaining markers we established the cut-off values based on the minimum-maximum values in the normal BM in each age group. The expression of CD10 was higher than the cut-off in 30 ALL cases and in 18 of them there was a concomitant aberrant expression of other markers. In 9 of the 10 $CD10^+$ ALL with normal CD10 SABC values, the expression of at least one other marker was aberrant. In conclusion, the distinction between normal and leukemic cells by QFC was possible in 38/40 $CD10^+$ ALL cases.

Correspondence
E.M. Rego
Departamento de Clínica Médica
FMRP, USP
Av. Bandeirantes, 3900
14049-900 Ribeirão Preto, SP
Brasil
Fax: +55-16-633-1144
E-mail: edumrego@hotmail.com

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Key words
- Acute lymphoblastic leukemia
- Lymphocytes
- Quantitative fluorescence cytometry
- Flow cytometry
- Age-related changes

Introduction

The distinction between leukemic and normal hematopoietic B-precursors in the bone marrow (BM) is essential for the diagnosis and treatment monitoring of acute lymphoblastic leukemia (ALL). Previous studies have demonstrated that the detection of leukemic cells present at concentrations below the resolution by morphological analysis (minimal residual disease, MRD) may allow a better estimate of the leukemic burden and

may be correlated with clinical outcome (1-8).

The immunological methods for discriminating between normal and leukemic B-precursors rely on the fact that leukemic cells may express some antigens absent in, or rarely expressed by, their normal counterparts (1,3,9,10). Nevertheless, this approach to B-ALL precursors has been disappointing because of the frequent immunophenotypic similarities between normal and leukemic B-precursors (1,3). Moreover, there is an increase in B-precursors in infants' and children's BM and the age-related changes must be taken into account for the diagnosis of ALL and MRD detection in the disease (11,12). The introduction of quantitative fluorescence cytometry (QFC) techniques allowed quantifying antigen expression in an accurate and reproducible manner (13,14) and previous reports have suggested that leukemic cells may express antigens at densities distinct from those presented by their normal counterparts (4,15,16). This method may be useful for MRD detection, as well as for the diagnosis of ALL, especially in those cases preceded by transient pancytopenia, a presentation more common in children and of difficult diagnosis (17,18).

As is the case for other laboratory parameters, the interpretation of flow cytometry immunophenotyping depends on the relationship of the patient's data to normal reference ranges for the same population. However, the distribution of the antigen densities in the normal BM and the factors influencing it are largely unknown. In the present study, we analyzed the age-related quantitative changes in CD10, CD19, CD34, TdT and CD79a expression in 21 BM biopsies from normal subjects and in 40 patients with B-ALL precursors from the region of Ribeirão Preto, SP, Brazil. Finally, by comparing the combined quantitative expression of these markers in normal and leukemic B-precursors we were able to discriminate between them in more than 90% of the studied ALL cases.

Material and Methods

Normal bone marrow

We studied BM fragments obtained from the sternum of 12 children (<15 years) aged 2 months to 15 years (median, 2 years) who had undergone cardiac surgery for the correction of congenital cardiopathies, and 9 adults (≥15 years) aged 15 to 72 years (median, 35 years), who were operated upon for valvular or ischemic heart diseases. Only patients with normal hematological counts, who exhibited no evidence of infections, autoimmune disorders or renal or hepatic dysfunction, were considered eligible. All samples were collected after obtaining informed consent from the donors or the person legally responsible for them and this investigation was approved by the Ethics Committee of the University Hospital of the Medical School of Ribeirão Preto, University of São Paulo.

Leukemic bone marrow

We studied BM aspirates from 40 patients (29 children and 11 adults) with ALL, whose immunophenotypic studies were performed by the Hematology Laboratory of the University Hospital of the Medical School of Ribeirão Preto, University of São Paulo. All cases were classified as L1 or L2 according to the FAB proposal (19), and the immunophenotypic subset was determined using a large panel of monoclonal antibodies, as previously described (20). In all cases, the leukemic cells were $CD19^+$ and/or $CD79a^+$ and/or $CD22^+$ (on the membrane or in the cytoplasm), and none of them had detectable myeloperoxidase either by cytochemistry or by immunophenotypic analysis. The T markers CD3, TCRα/ß and TCRγ/δ were absent in all cases. In 18% of cases the expression of CD13, CD33 or CD15 was detectable; however, cases of biphenotypic leukemia were excluded based on the scoring system

proposed by the European Group for the Immunological Characterization of Leukemias (21). Since the detection of cytoplasmic immunoglobulin was not performed in all cases, both B-II and B-III cases were included in the analysis, and the cases were designated as $CD10^+$ ALL.

Triple-color immunofluorescence

The following monoclonal antibodies (MoAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridin chlorophyll protein (PerCP) were used: HLe-1-PerCP (CD45-PerCP), Leu12-FITC (CD19-FITC), HPCA2-FITC (CD34-FITC) (Becton Dickinson, San Jose, CA, USA), SS2/36-PE (CD10-PE) (Dako A/S, Glostrup, Denmark), and HT1,HT3,HT4-FITC (anti-TdT-FITC) (Seralab, Crawley Down, UK). JCB 117 (CD79a) was purchased unconjugated from Dako. All antibodies were used in saturating amounts (except CD45), as determined by titration assays. The samples were analyzed for the following antigen combinations: 1) CD10/CD19/CD45, 2) CD10/CD34/CD45, 3) CD10/TdT/CD45, and 4) CD10/CD79a/CD45.

Intracellular staining. The mononuclear cells were isolated by density gradient centrifugation and washed twice in PBS and the cell concentration was adjusted to 0.5-1 x 10^6 cells/ml. The cells were permeabilized/fixed by incubating for 10 min at room temperature with 2 ml of a mixture of equal volumes of 4% paraformaldehyde (PFA) in PBS and a 1:10 dilution of Becton Dickinson's FACS Lysing solution in distilled water. The cells were then washed twice with 2 ml of 0.5% (v/v) Tween 20 in PBS and incubated with 10 µl of anti-CD79a or with 10 µl of anti-TdT-FITC MoAb for 60 min at room temperature. Mouse immunoglobulin of irrelevant specificity was used as a negative control (Becton Dickinson). After washing twice with 0.5% Tween 20, the samples which had been incubated with the anti-TdT MoAb were ready to be stained for the membrane markers. After the washing step, samples which had been incubated with the anti-CD79a MoAb (indirect technique) were incubated with a goat anti-mouse antibody conjugated with FITC (Dako) for 60 min at room temperature, washed twice in the same solution, and incubated with a pool of heat-inactivated serum from normal mice for 30 min at room temperature. After washing as before, the cells were vortexed and stained for membrane antigens.

Membrane staining. In the samples to be stained only for membrane markers, the cells were initially incubated with heat-inactivated rabbit serum and then washed twice with 2 ml PBS. All samples were then incubated for 30 min at 4°C with the MoAbs CD45, CD10 and CD19 or CD34. The negative controls were incubated with the corresponding PE/FITC/PerCP-conjugated mouse immunoglobulins of irrelevant specificity (Becton Dickinson). After incubation, the cells were washed once with 2 ml PBS with 0.1% azide and 2% FCS and resuspended in 1 ml PBS with 0.1% azide and 1% PFA.

The results obtained for the membrane markers were identical when the labeling was performed in fresh material or when the cells were first permeabilized and stained for the intracellular markers (data not shown). When we compared the results obtained by CD79a and TdT staining in 9 normal BM samples with those obtained using the Fix&Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA, USA) no significant differences were observed (data not shown).

Quantitative fluorescence cytometry

Antigen densities were determined by the calibrated measurement of fluorescence intensity from labeled microbeads. For the antigens analyzed by direct immunofluorescence we used the Quantum Simply Cellular

Microbeads Kit (Sigma Chemical Co., St. Louis, MO, USA), and for the CD79a density determination we used the Dako QIFKIT Microbeads for indirect immunofluorescence staining. These kits contain a series of beads with well-defined quantities of goat anti-mouse polyclonal antibodies (Quantum Simply Cellular Microbeads) or mouse MoAb (QIFKIT Microbeads) that allow the calibration of the fluorescence scale. Three sets of microbeads were employed: one for calibration of the measurements by direct immunofluorescence (membrane staining), one for indirect immunofluorescence staining (CD79a staining), and one for nuclear staining (TdT staining). The beads were incubated and washed in the same way as described for the cells in the respective techniques, and the antibodies were used as recommended by the manufacturer.

FACS analysis. All samples were analyzed on a FACScan flow cytometer (Becton Dickinson) equipped with an argon ion laser with a wavelength of 488 nm using the Cell Quest software (Becton Dickinson). Fluorescence intensity was measured with FL1 and FL2 detectors and amplifiers set on a logarithmic scale. The cytometer was set up using standard operating procedures and the quality control was performed using the Calibrite Beads Kit (Becton Dickinson) and by CD3/CD4/CD8 triple staining of peripheral blood lymphocytes (22). The mean channel number of the fluorescence intensity (MFI) determined for each population of beads was plotted versus the corresponding antibody binding capacity (ABC) values, and a best fit line was calculated by linear regression using the QuickCal software (Becton Dickinson). Figure 1 shows an example of the gate used for data analysis. The gating procedure was based on CD45 staining and sideward light scatter (SSC) and the area containing the lymphoid cells and most of the B-progenitors was selected (23). The percentage of $CD33^+$ cells was lower than 1% in the selected gate (data not shown). Ten thousand gated events were analyzed. The MFI value observed in the positive population was converted to ABC values by interpolation in the calibration curve. In the same way, the background antibody equivalent (BAE) was calculated by determining the apparent ABC of the negative control. The specific antibody binding capacity (SABC) is the number of primary mouse MoAbs per cell after correction for background fluorescence (SABC = ABC - BAE) and was expressed in arbitrary units (a.u.).

Similar CD10 SABC values were detected in B-precursors obtained from the aspirate and the biopsy samples of a normal subject (data not shown), suggesting that the method for BM collection did not affect the QFC analysis.

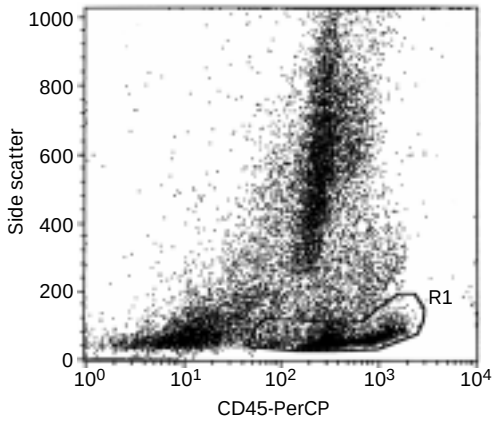

Figure 1 - Representative cytogram showing the gate used for the analyses of bone marrow cells. The dot plot shows the CD45-peridin chlorophyll protein (PerCP) staining on the X-axis and the side scatter scan on the Y-axis. The analysis of a bone marrow biopsy from a normal 2-year-old boy is shown. The area enclosed by the solid line indicates the region (R1) containing the bone marrow B-precursors.

Statistical methods

To determine if the data distribution was Gaussian, we applied the Kolmogorov-Smirnov test (24). Since the data were distributed in a non-Gaussian pattern, we reported the percentages of positive cells and the SABC values by using the median and minimum-maximum values. The Mann-Whitney U-test was applied to determine whether the distribution of the variables differed significantly ($P<0.05$) between groups (24). The association between age and intensity of marker expression and the association between the expression of two markers were

tested by regression analysis and the Spearman rank correlation coefficient was used to determine the probability of correlation between the two variables.

Results

Age-related changes of CD10, CD19, TdT, CD34 and CD79a SABC values in normal and leukemic bone marrow

Table 1 shows the distribution of the median and range of CD10, CD19, CD34, TdT and CD79a SABC values in normal and leukemic BM according to age-group. Figure 2 shows the age-related changes and the relationship between the marker SABC values in normal (crosses) and leukemic B-precursors (squares). The relationship between the intensity of the CD10 and CD19 expression is shown in Figure 2A, whereas the relationship between CD10 and TdT, CD10 and CD34, and CD10 and CD79a expression is represented in Figure 2B, 2C and 2D, respectively. Compared to normal adults' BM, the precursor cells in normal children's BM expressed significantly ($P = 0.001$) lower values of CD34 SABC. A positive association between CD34 expression and age was detected among the normal individuals and Figure 3 shows the scatter diagram with the regression line of the function of CD34 SABC against age ($R = 0.66$; standard error of the estimate (SEE) = 50.5×10^3 a.u.; $P = 0.004$). Similarly, the median of the TdT SABC among the normal population was lower in children and increased with age (Figure 2B). However, the regression analysis of TdT expression against age did not demonstrate a significant positive association ($R = 0.42$; SEE = 58.8×10^3 a.u.; $P = 0.056$). On the other hand, no significant age-related differences in SABC values were detected among the patients with $CD10^+$ ALL (Table 1).

We also analyzed the correlation between the expression levels of the different markers for normal BM. The only significant association detected was between the expression of CD19 and TdT ($R = -0.5$; $P = 0.03$).

Among normal individuals, the children's BM presented significantly higher percentages of $CD10^+$, $CD19^+$, TdT^+ and $CD34^+$ cells in relation to the adults' BM (Table 2). There was also a higher percentage of cells expressing the phenotypes $CD19^+/CD10^+$, $CD10^+/CD34^+$, and $CD10^+/TdT^+$ in children's BM (data not shown). In the patients with ALL, on the other hand, no significant differences were detected, except for the percentage of leukemic cells expressing TdT which was significantly higher in adults (Table 2).

Comparison between normal and leukemic bone marrow

The leukemic B-precursors expressed significantly higher values of CD10 SABC than their normal counterparts both in children and in adults. In addition, significantly lower values of TdT and CD34 SABCs were detected in the leukemic adults (Table 3 and Figure 2). Based on the study by Lavabre-Bertrand et al. (16), we set the cut-off value for normal CD10 expression at 45×10^3 a.u. for both age groups. We established the cut-off values based on the minimum-maximum values in the normal BM in each age group for the remaining markers (Table 4). For the CD79a expression analysis only a maximum value was adopted as cut-off, since we did not detect CD79a SABC values in leukemic cells lower than those obtained from normal individuals. The expression of CD10 in the leukemic cells was higher than the cut-off value in about 80% of the $CD10^+$ ALL cases among children and in more than 60% of the adult cases (Table 5). In addition, among these cases, the concomitant aberrant expression of one or two of the other markers was detected in all 7 adult patients and in 11/23 (47.8%) children (Table 5). Finally, we

Table 1 - Analysis of the age-related changes in CD10, TdT, CD34, CD19 and CD79a SABC values in normal and leukemic bone marrow precursors of children and adults.

Data are reported as median (range). The Mann-Whitney U-test was used for statistical analysis.

Group	CD10 SABC ($\times 10^3$)	TdT SABC ($\times 10^3$)	CD34 SABC ($\times 10^3$)	CD19 SABC ($\times 10^3$)	CD79a SABC ($\times 10^3$)
Normal					
Children (N = 12)	23	140	86.6	32.8	5
	(10-34.1)	(46.8-251.1)	(39.5-155.5)	(18.1-74.3)	(0.9-14.9)
Adults (N = 9)	18.4	213.7	160.2	29.7	6.5
	(8.1-23.4)	(90.7-314.8)	(99.7-280.8)	(20.6-33.2)	(5.9-7.8)
	P = 0.07	P = 0.06	P = 0.001	P = 0.15	P = 0.27
Leukemic					
Children (N = 29)	129.7	49.3	73.7	25.7	12.4
	(13.7-460.9)	(1.8-408)	(0.9-159.8)	(5-125.8)	(1.8-111.6)
Adults (N = 11)	98.6	61.4	73	22.1	14.3
	(17.1-211.7)	(20.8-582)	(1.9-423.5)	(8.2-85.8)	(1.7-37.5)
	P = 0.5	P = 0.73	P = 0.56	P = 0.88	P = 0.64

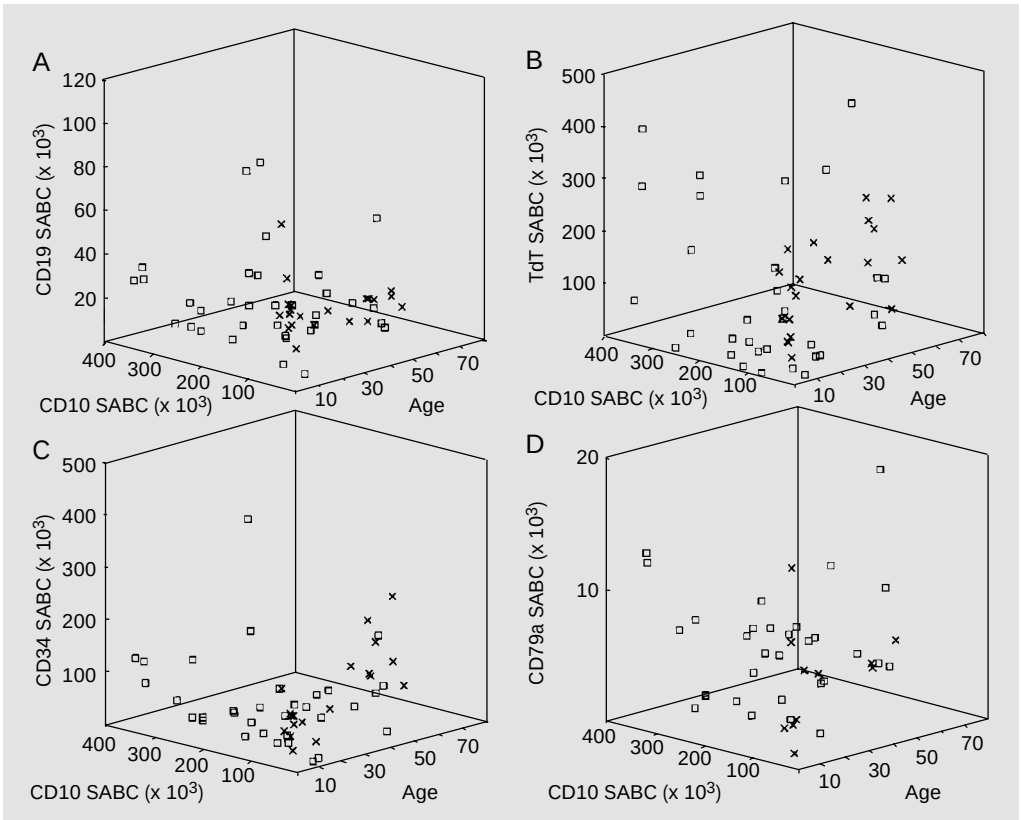

Figure 2 - Two-dimension plot showing the age-related changes in the expression of CD10, CD19, TdT, CD34 and CD79a. The age in years is represented on the Z-axis and the specific antibody binding capacity (SABC) values of CD10/CD19 (A), CD10/TdT (B), CD10/CD34 (C) and CD10/CD79a (D) markers are shown on the X- and Y-axis, respectively. The SABC values of the normal subjects are represented by crosses and those of the $CD10^+$ acute lymphoblastic leukemia cases by squares.

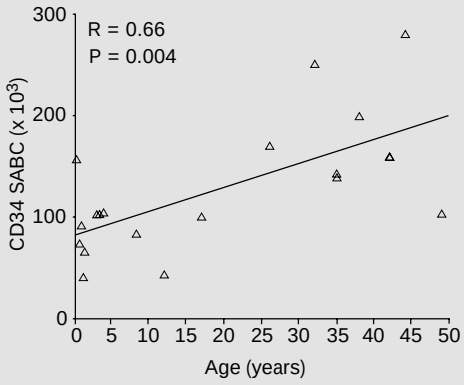

Figure 3 - Linear regression analysis of the CD34 expression (reported as specific antibody binding capacity (SABC) values in arbitrary units (a.u.)) against age. The correlation coefficient R = 0.66, standard error of the estimate = 50.5 x 10^3 a.u. and the P value were determined by the Spearman rank correlation test.

Table 2 - Frequency of CD10, TdT, CD34, CD19 and CD79a positive cells in the bone marrow of normal and leukemic children and adults.

Data are reported as median (range). P values were calculated by the Mann-Whitney U-test. ALL, Acute lymphoblastic leukemia.

Group	%CD10$^+$ cells	%TdT$^+$ cells	%CD34$^+$ cells	%CD19$^+$ cells	%CD79a$^+$ cells
Normal					
Children (N = 12)	62.5	3	4	69.5	34
	(9-83)	(0.8-12)	(2.2-11)	(29-97)	(1-50)
Adults (N = 9)	10	0.4	2	18	6
	(4-27)	(0-4)	(0.3-7)	(11-30)	(0.9-8)
	P = 0.0006	P = 0.004	P = 0.003	P = 0.0002	P = 0.07
CD10$^+$ ALL					
Children (N = 23)	92	62	53	80	72.5
	(26-99)	(1-93)	(1-99)	(19-98)	(1-97)
Adults (N = 11)	91	80	81	82	70
	(64-98)	(61-95)	(1-98)	(21-98)	(16-92)
	P = 0.98	P = 0.02	P = 0.3	P = 0.75	P = 0.8

Table 3 - Analysis of CD10, TdT, CD34, CD19 and CD79a SABC values in normal and leukemic bone marrow precursors of children and adults.

Data are reported as median (1st-99th percentiles). P values were calculated by the Mann-Whitney U-test. ALL, Acute lymphoblastic leukemia.

Group	CD10 SABC (x 10^3)	TdT SABC (x 10^3)	CD34 SABC (x 10^3)	CD19 SABC (x 10^3)	CD79a SABC (x 10^3)
Children					
Normal (N = 12)	23	140	86.6	32.8	5
	(10-34.1)	(46.8-251.1)	(39.5-155.5)	(18.1-74.3)	(0.9-14.9)
CD10$^+$ ALL	129.7	49.3	73.7	25.7	12.4
(N = 29)	(13.7-460.9)	(1.8-408)	(0.9-159.8)	(5-125.8)	(1.8-111.6)
	P<0.0001	P = 0.11	P = 0.56	P = 0.085	P = 0.22
Adults					
Normal (N = 9)	18.4	213.7	160.2	29.7	6.5
	(8.1-23.4)	(90.7-314.8)	(99.7-280.8)	(20.6-33.2)	(5.9-7.8)
CD10$^+$ ALL	98.6	61.4	73	22.1	14.3
(N = 11)	(17.1-211.7)	(20.8-582)	(1.9-423.5)	(8.2-85.8)	(1.7-37.5)
	P<0.0001	P = 0.03	P = 0.02	P = 0.26	P = 0.29

were able to distinguish between normal and leukemic cells in 9 of the 10 $CD10^+$ ALL cases in which the expression of CD10 was within the 'normal' range by analyzing the combined expression of CD19/TdT, CD19/CD34 or TdT/CD34 (Table 5 and Figure 4).

Table 4 - Cut-off values for CD19, CD34, TdT and CD79a expression in normal bone marrow precursors.

Values are reported in arbitrary units (a.u.). *Only the maximum specific antibody binding capacity (SABC) value was adopted as cut-off.

Group	Marker	Minimum SABC value (x 10^3)	Maximum SABC value (x 10^3)
Children	CD19	18	75
	CD34	38	160
	TdT	45	260
	CD79a	*	16
Adults	CD19	19	38
	CD34	95	290
	TdT	90	320
	CD79a	*	10

Table 5 - Frequency of $CD10^+$ acute lymphoblastic leukemia cases expressing marker SABC values higher or lower than the cut-off values established for normal bone marrow.

*Cases expressing CD10 SABC values >45 x 10^3 arbitrary units (a.u.) **Cases expressing CD10 SABC values ≤45 x 10^3 a.u.

Immunophenotype of the leukemic B-precursors	Children (N = 29)	Adults (N = 11)
CD10 >45 x 10^3 a.u.	23 (79.3%)	7 (63.7%)
+ CD19<cut-off*	2 (6.9%)	2 (18.2%)
+ CD19>cut-off*	1 (3.4%)	0
+ TdT<cut-off*	2 (6.9%)	1 (9.1%)
+ TdT>cut-off*	3 (10.3%)	1 (9.1%)
+ CD79a>cut-off*	0	1 (9.1%)
+ TdT<cut-off + CD19<cut-off*	1 (3.4%)	0
+ TdT>cut-off + CD19<cut-off*	1 (3.4%)	0
+ CD34>cut-off + CD19<cut-off*	1 (3.4%)	0
+ CD34>cut-off + CD19>cut-off*	0	1 (9.1%)
+ CD19>cut-off + CD79a>cut-off*	0	1 (9.1%)
CD10 ≤45 x 10^3 a.u.	6 (20.7%)	4 (36.4%)
+ CD19<cut-off**	1 (3.4%)	0
+ CD34<cut-off**	1 (3.4%)	1 (9.1%)
+ TdT<cut-off**	1 (3.4%)	0
+ CD19<cut-off + CD79a>cut-off**	0	1 (9%)
+ TdT>cut-off + CD34>cut-off + CD19>cut-off**	1 (3.4%)	0
+ TdT<cut-off + CD34<cut-off + CD19>cut-off**	0	1 (9%)
+ CD34<cut-off + CD19<cut-off**	0	1 (9%)
+ TdT<cut-off + CD19<cut-off**	1 (3.4%)	0

Thus, in 38/40 $CD10^+$ ALL cases, aberrant expression of at least one of the antigens by the leukemic cells was detected.

Discussion

QFC is a method based on the calibrated measurement of fluorescence intensity from labeled particles so as to determine the actual number of fluorescent ligands labeling each particle (25), thus allowing an objective and reproducible measurement of the intensity of expression of a given antigen. Previous studies have demonstrated that differences in the intensity of B-antigen expression are related to B-maturation and to cell proliferation (26-29). Accordingly, normal and leukemic cells expressing higher CD10 intensities are more immature and have higher proliferating rates than CD10 weak cells (26-29). Although the intensity of CD10 expression may be related to cell cycle and maturation, it is not an independent prognostic factor in ALL (30,31). Moreover, the factors that can affect the intensity of expression of an antigen are unknown. Here we report the age-related changes observed in normal and leukemic individuals from the region of Ribeirão Preto, in the Southeast of Brazil. Both Caucasians and Africans have contributed to the ethnic background of the population and the characterization of the ALL subtypes in the same population has been previously reported (20).

An important concern in analyzing the B-antigen densities in normal and aberrant hematopoiesis is the collection of reliable data concerning the normal B-progenitors in BM. Previous studies have analyzed samples obtained from BM aspirates of healthy adults, patients treated for malignancies or fetuses (4,15,16), and it has been previously demonstrated that there are significant differences in lymphoid subset distribution between BM samples from infants, children and adults (11,12,32,33). Although immunophenotypic studies for the diagnosis and MRD detection

in ALL are routinely performed in BM aspirates, we opted for the study of normal BM biopsies because it facilitates a better isolation of the normal precursor cells, considerably reducing the percentage of contaminating mature B-lymphocytes from peripheral blood (11,12,34,35). In addition, in order to better select the normal B-precursors we set up the gate of analysis based on SSC combined with CD45 fluorescence (Figure 1), which minimizes the contamination by other irrelevant and non-lymphoid events (23).

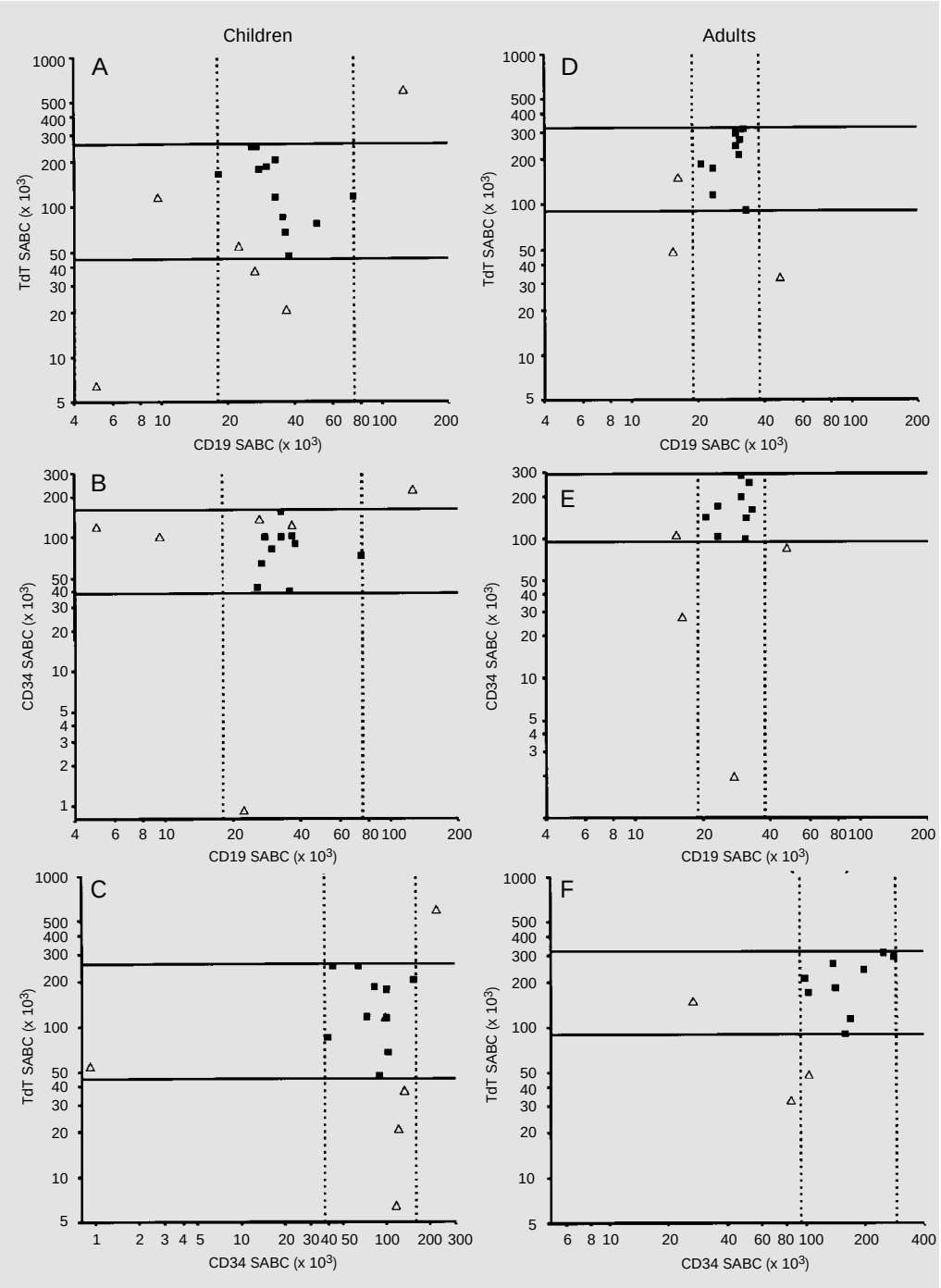

Figure 4 - Analysis of the expression of the CD19 x TdT (A and D), CD19 x CD34 (B and E) and CD34 x TdT (C and F) markers in normal and leukemic children (left panel) and in normal and leukemic adults (right panel). The specific antibody binding capacity (SABC) values are represented in log scale. Only those patients with $CD10^+$ acute lymphoblastic leukemia (ALL), whose leukemic cells expressed CD10 $\leq 45 \times 10^3$ a.u. (within the normal range) are represented. The SABC values of the normal subjects are represented by filled squares and those of the $CD10^+$ ALL cases by open triangles. The discontinuous lines show the cut-off values for the marker represented on the X-axis and the continuous lines represent the cut-off values for the marker on the Y-axis.

There is a lack of data in the literature concerning the age-related changes in antigen expression. The CD10 SABC values described here in the BM of normal children are higher than those reported by Lavabre-Bertrand et al. (16) in fetal BM and liver. The comparison between the CD10 and CD19 SABC values described here and those reported by Farahat et al. (15) in adults is hampered by the differences in the selection of the analyzed cell population. While in the present study the analysis was done on the whole lymphoid population, Farahat et al. (15) analyzed TdT^+ and TdT^- cells separately. Nevertheless, the TdT SABC values observed by these authors in healthy adults and in patients with regenerating BM were similar to those determined here in the BM from normal adults.

Nevertheless, the most interesting finding concerning the age-related changes in normal BM was the demonstration that the lymphoid precursors in children's BM expressed significantly lower levels of CD34 than their counterparts in adults' BM, and that the expression of CD34 increases with age. Precursor cells expressing low levels of CD34 have been described (36) and the precise potential of these cells for hematopoietic reconstitution is unclear. Terstappen et al. (37) demonstrated that the $CD34^{dim}$ subpopulation may include precursors committed to the erythroid, myeloid, and lymphoid lineages.

Regardless of age, an inverse correlation between the level of expression of CD19 and TdT was detected in normal BM, which may be correlated to the different pattern of expression of these markers during B-cell maturation (26).

In the present study, analysis of the distribution of the immunophenotypic subsets in normal BM biopsies showed the predominance of immature cells in children's BM in agreement with previous studies (11,12). Most cells in children's BM are $CD19^+$ $CD10^+$ B-cell precursors and the percentage of $CD34^+$ and TdT^+ cells was also higher in children.

No age-related differences were detected in the SABC values of the markers amongst $CD10^+$ B-ALL precursor samples. Although a higher percentage of TdT^+ cells in the BM from adults was observed, the remaining immunophenotypic profile was similar for the two age groups and their leukemic cells may be considered to be in similar stages of B-cell development (21,26).

Compared to their normal counterparts, the leukemic $CD10^+$ B-progenitors expressed significantly higher CD10 SABC values both in children and in adults. Farahat et al. (15) and Lavabre-Bertrand et al. (16) have previously reported higher CD10 densities in leukemic cells compared to their normal counterparts. In the three studies the maximal CD10 expression in normal B-precursors ranged from 35-50 x 10^3 a.u. We adopted the same cut-off value for CD10 expression (45 x 10^3 a.u.) as Lavabre-Bertrand et al. (16), since the cell population selected by these authors was closer to the one described in the present study, thus permitting a comparison. Based exclusively on CD10 expression, normal and leukemic B-precursors could be distinguished in 43.5% of the B-cell neoplasias in the study mentioned above (16) and in 79.3% of the children and in 63.7% of the adults with $CD10^+$ ALL in the present study. This discrepancy in the frequencies is possibly due to the fact that different B-cell neoplasias were analyzed by Lavabre-Bertrand et al. (16), whereas only $CD10^+$ ALL cases were included in our study. However, one cannot rule out that differences in the flow cytometer setting may affect the SABC values, but the use of calibrated beads as internal controls should prevent any arbitrariness in the settings from affecting the general conclusions, which were similar in the three available studies (present study, and 15,16).

In adults, the leukemic B-precursors expressed significantly lower levels of TdT and CD34 than the normal counterparts, re-

inforcing the need for normal age-matched controls for QFC analysis. Nevertheless, in addition to 6 ALL cases expressing TdT SABC values below the lower cut-off, we also detected 6 ALL cases in which the expression of TdT was above the higher cut-off value for normal BM, suggesting that an upper and a lower normal cut-off value for TdT expression should be used. Contrary to the data reported by Farahat et al. (15), we did not detect significant differences in CD19 SABC values between normal and leukemic samples but, as mentioned above, this difference may be due to the cell population selected.

There is a lack of information in the literature comparing the CD79a and the CD34 densities in normal and leukemic cells. In the present study we detected 7 and 3 ALL cases in which CD34 and CD79a expression was beyond the normal boundaries, respectively, suggesting that analysis of these markers may be useful for the distinction between normal and leukemic B-precursors. However, we detected lower percentages of $CD79a^+$ cells in normal BM than those reported by Dworzak et al. (38). Nevertheless, these authors studied only TdT^+ precursor cells in children's BM. Moreover, we cannot rule out the possibility that the indirect immunofluorescence technique used here was less sensitive in detecting CD79a expression.

By our approach, we found that at least one of the 5 markers was expressed at aberrant intensity in 38/40 (95%) $CD10^+$ ALL cases. In addition, the concomitant aberrant expression of both CD10 and another marker was detected in all the 7 adult cases and in 11/23 of the children's $CD10^+$ ALL cases. By analyzing the expression of CD19/TdT, CD19/CD34 or TdT/CD34 we could distinguish between normal and leukemic cells in the 9/10 $CD10^+$ ALL cases in which the expression of CD10 was within the 'normal' range. This suggests that the use of the combined assessment of these markers by QFC may distinguish more precisely between normal and leukemic B-precursors than the non-quantitative methods of flow cytometry, especially in the analysis of samples from children, who present a high percentage of normal B-precursors in BM that mimic the immunophenotypic features of ALL (11,32). Moreover, the approach described here may be useful in those cases of ALL presenting with pancytopenia, a condition of difficult diagnosis by morphological analysis alone (17,18).

In conclusion, we demonstrated that there are significant age-related changes in CD34 density in B-precursors of normal BM, which were not detected amongst leukemic samples. In addition, we demonstrated that the combined assessment of the expression of B-related markers by QFC is a reliable method for the distinction between normal and leukemic B-precursors in $CD10^+$ ALL and that the application of this method to MRD detection deserves future studies.

References

1. Campana D & Pui CH (1995). Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance. *Blood*, 85: 1416-1434.
2. Roberts WM, Estrov Z, Ouspenskaia MV, Johnston DA, McClain KL & Zipf TF (1997). Measurement of residual leukemia during remission in childhood acute lymphoblastic leukemia. *New England Journal of Medicine*, 336: 317-323.
3. Coustan-Smith E, Behm FG, Sanchez J, Boyett JM, Hancock ML, Raimondi SC, Rubnitz JE, Rivera GK, Sandlund JT, Pui C-H & Campana D (1998). Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. *Lancet*, 351: 550-554.
4. Farahat N, Morilla A, Owusu-Ankomah K, Morilla R, Pinkerton CR, Treleaven JG, Matutes E, Powles RL & Catovsky D (1998). Detection of minimal residual disease in B-lineage acute lymphoblastic leukaemia by quantitative flow cytometry. *British Journal of Haematology*, 101: 158-164.
5. Cave H, van der Werfften BJ, Suciu S, Guidal C, Waterkeyn C, Otten J, Bakkus M, Thielemans K, Grandchamp B & Vilmer E (1998). Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer - Childhood Leukemia Cooperative

Group. *New England Journal of Medicine*, 339: 591-598.
6. Yamada M, Wasserman R, Lange B, Reichard BA, Womer RB & Rovera G (1990). Minimal residual disease in childhood B-lineage lymphoblastic leukemia: persistence of leukemic cells during the first 18 months of treatment. *New England Journal of Medicine*, 323: 448-455.
7. Nizet Y, Van Daele S, Lewalle P, Vaerman JL, Philippe M, Vermylen C, Cornu G, Ferrant A, Michaux JL & Martiat P (1993). Long-term follow-up of residual disease in acute lymphoblastic leukemia patients in complete remission using clonogenic IgH probes and the polymerase chain reaction. *Blood*, 82: 1618-1625.
8. Brisco MJ, Condon J, Hughes E, Neoh SH, Sykes PJ, Seshadri R, Toogood I, Waters K, Tauro G & Ekert H (1994). Outcome prediction in childhood acute lymphoblastic leukemia by molecular quantification of residual disease at the end of induction. *Lancet*, 343: 196-200.
9. Drach J, Drach D, Glassl H, Gattringer C & Huber H (1992). Flow cytometric determination of atypical antigen expression in acute leukemia for the study of minimal residual disease. *Cytometry*, 13: 893-901.
10. Babusikova O, Glasova M, Kusenda J, Konikova E & Mesarova A (1998). Flow cytometric determination of leukemia-associated marker combinations for the study of minimal residual disease. *Neoplasma*, 41: 305-313.
11. Rego EM, Garcia AB, Viana SR & Falcão RP (1998). Age-related changes in lymphocyte subsets in normal bone marrow biopsies. *Cytometry*, 34: 22-29.
12. Clark P, Normansell DE, Innes DJ & Hess CE (1986). Lymphocyte subsets in normal bone marrow. *Blood*, 67: 1600-1606.
13. Poncelet P & Carayon P (1985). Cytometric quantification of cell-surface antigens by indirect immunofluorescence using monoclonal antibodies. *Journal of Immunological Methods*, 85: 65-74.
14. Vogt Jr RF, Cross GD, Henderson LO & Phillips DL (1989). Model system evaluating fluorescein-labeled microbeads as internal standards to calibrate fluorescence intensity on flow cytometers. *Cytometry*, 10: 294-302.
15. Farahat N, Lens D, Zomas A, Morilla R, Matutes E & Catovsky D (1995). Quantitative flow cytometry can distinguish between normal and leukaemic B-cell precursors. *British Journal of Haematology*, 91: 640-646.
16. Lavabre-Bertrand T, Janossy G, Ivory K, Peters R, Secker-Walker L & Porwit-MacDonald A (1994). Leukemia-associated changes identified by quantitative flow cytometry: I. CD10 expression. *Cytometry*, 18: 209-217.
17. Breatnach F, Chessels JM & Greaves MF (1981). The aplastic presentation of childhood leukaemia: a feature of common ALL. *British Journal of Haematology*, 49: 387-393.
18. Atra A, Abboudi Z, Farahat N & Catovsky D (1997). Quantitative flow cytometry can predict childhood acute lymphoblastic leukaemia presenting with aplasia. *Leukemia and Lymphoma*, 27: 173-177.
19. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR & Sultan C (FAB Cooperative Group) (1981). The morphological classification of the acute lymphoblastic leukemia: concordance among observers and clinical correlation. *British Journal of Haematology*, 47: 553-561.
20. Rego EM, Garcia AB, Viana SR & Falcão RP (1996). Characterization of acute lymphoblastic leukemia subtypes in Brazilian patients. *Leukemia Research*, 20: 349-355.
21. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A & van't Veer MB (1995). Proposal for the immunological classification of acute leukemias. *Leukemia*, 9: 1783-1786.
22. Rothe G, Schmitz G for the Working Group on Flow Cytometry and Image Analysis, Members of the Editorial Committee: Adorf D, Barlage S, Gramatzki M, Hanenberg H, Höffkes HG, Janossy G, Knüchel R, Ludwig WD, Nebe T, Nerl C, Orfao A, Serke S, Sonnen R, Tichelli A & Wörmann B (1996). Consensus protocol for the flow cytometric immunophenotyping of hematopoietic malignancies. *Leukemia*, 10: 877-895.
23. Syrjälä M, Ruutu T & Jansson S-E (1994). A flow cytometric assay of CD34-positive cell populations in the bone marrow. *British Journal of Haematology*, 88: 679-684.
24. Armitage P & Berry G (1994). *Statistical Methods in Medical Research*. 3rd edn. Blackwell Scientific Publications, Oxford, UK.
25. Henderson LO, Marti GE, Gaigalas A, Hannon WH & Vogt Jr RF (1998). Terminology and nomenclature for standardization in quantitative fluorescence cytometry. *Cytometry*, 33: 97-105.
26. Loken MR, Shah VO, Dattilio KL & Civin CI (1987). Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development. *Blood*, 70: 1316-1324.
27. Hollander Z, Shah VO, Civin CI & Loken MR (1988). Assessment of proliferation during maturation of the B lymphoid lineage in normal human bone marrow. *Blood*, 71: 528-531.
28. Look AT, Melvin SL, Brown K, Dockter ME, Roberson PK & Murphy SB (1984). Quantitative variation of the common acute lymphoblastic leukemia antigen (gp 100) on leukemic marrow blasts. *Journal of Clinical Investigation*, 73: 1617-1628.
29. LeBien TW & McCormack RT (1989). The common acute lymphoblastic leukemia antigen (CD10) - Emancipation from a functional enigma. *Blood*, 73: 625-635.
30. Rego EM, Tone LG, Garcia AB & Falcão RP (1999). CD10 and CD19 fluorescence intensity of B-precursors in normal and leukemic bone marrow. Clinical characterization of $CD10^{+strong}$ and $CD10^{+weak}$ common acute lymphoblastic leukemia. *Leukemia Research*, 23: 441-450.
31. Pui C-H, Behm FG & Crist WM (1993). Clinical and biological relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. *Blood*, 82: 343-362.
32. Caldwell CW, Poje E & Helikson MA (1991). B-cell precursors in normal pediatric bone marrow. *American Journal of Clinical Pathology*, 95: 815-823.
33. Hirsch K, Zaontz M, Marchildon M, Blumstein L, McCoy Jr JP & Donaldson MH (1993). Immunophenotyping of pediatric bone marrow. *Annals of the New York Academy of Sciences*, 677: 410-412.
34. Fauci AS (1975). Human bone marrow lymphocytes. I. Distribution of lymphocyte subpopulations in the bone marrow of normal individuals. *Journal of Clinical Investigation*, 56: 98-110.
35. Gale RP, Opelz G, Kiuchi M & Golde DW (1975). Thymus-dependent lymphocytes in human bone marrow. *Journal of Clinical Investigation*, 56: 1491-1498.
36. Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria MA, Paradis G, Grupp SA, Sieff CA, Mulligan RC & Johnson RP (1997). Dye efflux studies suggest that hemopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nature Medicine*, 3: 1337-1345.
37. Terstappen LWMM, Huang S, Safford M, Landsdorp PM & Loken MR (1991). Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^{+}CD38^{-}$ progenitor cells. *Blood*, 77: 1218-1227.
38. Dworzak MN, Fritsch G, Fröschl G, Printz D & Gadner H (1998). Four-color cytometric investigation of terminal deoxynucleotidyl transferase-positive lymphoid precursors in pediatric bone marrow: CD79a expression precedes CD19 in early B-cell ontogeny. *Blood*, 9: 3203-3209.