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Immunophenotypic analysis of CD19+ precursors in normal human adult bone marrow: implications for minimal residual disease detection

Juana Ciudad,* Alberto Orfao,* Belén Vidriales, Ana Macedo, Agustin Martínez, Marcos González, Maria Consuelo López-Berges, Berta Valverde, Jesus F. San Miguel
*Servicio de Hematología, Hospital Clínico Universitario, *Servicio General de Citometría and Department of Medicine, University of Salamanca, Spain*

Abstract

Background and Objective. **Normal B-cell differentiation has been characterized extensively, but discrepancies persist regarding the exact sequence of antigen expression. Few systematic studies focusing on identification of the minor or undetectable B-cell subsets in normal human bone marrow (BM) which are frequently found in leukemic cells have been performed. Such studies could help to monitor minimal residual disease (MRD) in precursor-B-acute lymphoblastic leukemia (precursor-B-ALL). The aim of the present study was to analyze the sequence of antigen expression among normal human CD19+ B cells from adult BM. Our major goal was to identify infrequent and undetectable B-cell phenotypes that could be used for the detection of MRD in patients with precursor-B-ALL.**

Design and Methods. **Adult BM samples from a total of 33 healthy volunteers were analyzed using triple stainings, and measured by flow cytometry. A sensitive method based on the two-step acquisition procedure was used for the identification and characterization of cells present at very low frequencies.**

Results. **Five different subsets of CD19+ cells were identified in normal BM samples according to their degree of maturation: 1) CD19+/CD34+/CD10–/CD20–/CD22dim+ (0.5±0.4% B cells); 2) CD19+/CD34–/CD10++ /CD20–/CD22dim+ (3.4±2.7%); 3) CD19+/CD34–/CD10+/CD20–/CD22dim+ (3.5±2.2%); 4) CD19+/ CD34–/CD10+/CD20+,++ /CD22dim+ (21±11%), and 5) CD19+ /CD34–/CD10–/CD20++ /CD22+ (73±19%). We observed that several B-cell phenotypes are frequent among precursor-B-ALL, but are infrequent or undetectable in normal human B cell differentiation. Accordingly, in all normal BM samples analyzed, less than 4×10^{-5} cells co-expressed CD19 and CD117; CD20strong+/CD34+ and CD22strong+/CD34+ events were found at frequencies less than 5×10^{-4}, while CD20+/CD34+ phenotypes were found in less than 1×10^{-3} BM cells. Although both CD19+/CD13+ and CD19+/CD33+ events were found at frequencies of up to 3×10^{-3}, they never formed a well-defined population of cells and therefore these latter phenotypic patterns could also be of use for MRD investigation in CD13+ and/or CD33+ precursor-B-ALL cases.**

Interpretation and Conclusions. **Our results show that in adult BM normal B-cells display constant patterns of maturation as regards both their phenotypic characteristics and their relative distribution. Abnormalities in these patterns provide a potentially useful tool for monitoring MRD in precursor-B-ALL patients who achieve cytomorphologic complete remission.**

Key words: B cells, normal human bone marrow, minimal residual disease, immunophenotype, flow cytometry

Correspondence: Professor Alberto Orfao de Matos, Servicio de Citometría, Hospital Universitario, Paseo de San Vicente, 37007 Salamanca, Spain.
Fax: international +34-923-294624 • Phone: international + 34-923-291375 • E-mail: orfao@gugu.usal.es

Human B-cell maturation from early committed CD34+ B-cell progenitors to functionally mature sIg+μ/μδ+ B-lymphocytes occurs in adults in the bone marrow (BM).[1-3] Although information on this subject has existed for more than a decade, only in recent years have the availability of an increasingly high numbers of B-cell-associated monoclonal antibody reagents and the development of multiparametric flow cytometry made possible a detailed analysis of the discrete stages of both normal and leukemic B-cell differentiation in BM.[4-8] Moreover, in spite of the large body of information generated in this area some controversial points remain to be elucidated. Among these discrepancies, one of the most controversial is related to the sequence of expression of the CD10 and CD19 antigens on CD34+ cells. Nadler *et al.*[9] proposed that CD19 precedes CD10 expression. In contrast, Uckun *et al.* reported on the existence of CD34+/CD10+/ CD19- BM cells[10] in fetal tissues suggesting that CD10 expression precedes that of CD19. However, in a study based on *in vitro* cell cultures, Pontvert-Delucq *et al.*[11] showed that these CD10+/CD19- BM cells gave rise to macrophage colonies but not B-cells.

In parallel, the analysis of the immunophenotype of blast cells in ALL showed that leukemic cells display, with a high frequency, phenotypic characteristics which had not been reported in normal human BM, the so-called *leukemic-associated* phenotypes or *phenotypic aberrations.*[12-16] However, on many occasions, later studies showed that these aberrant phenotypes, rather than being leukemic-specific, had just gone undetected in normal samples due to the low sensi-

tivity of the approaches used for their detection in preliminary reports.[10,12,17-19] In this sense, the CD22$^+$s/CD34$^+$ phenotype, which is frequently (80%) observed in precursor-B-ALL, was initially referred by Hurwitz *et al.*[13] as being aberrant. More recently, it has been clearly demonstrated that all CD19$^+$ B cells from normal human BM, including the CD34$^+$/CD19$^+$ subset, express surface CD22.[20] Altogether, results show that, at present, there is still need for a systematic study in which the B-cell phenotypes present in normal human BM are investigated using sensitive multiparametric approaches specifically devoted to the identification of rare or infrequent B-cell phenotypes. This could be of help for the investigation of minimal residual disease (MRD) in precursor-B-ALL once cytomorphologic complete remission (CR) is achieved.

Taking into account that the great majority of precursor-B-ALL patients display a CD19$^+$ phenotype,[21-23] the aim of the present study was to analyze the sequence of antigen expression among human CD19$^+$ B-cells in normal BM from adult control subjects. Our major goal was to identify the absence/presence of minor phenotypes which have been claimed to be leukemic-associated and which could be used for the investigation of MRD in precursor B-ALL patients.

Materials and Methods

Sample collection

BM samples from a total of 33 adults were included in this study. In all cases BM samples were obtained after informed consent, according to the Ethical Committee of the University Hospital of Salamanca (Spain), from healthy volunteers undergoing orthopedic surgery (mean age 46±16 years, median of 41, range from 18 to 67 years). Samples were collected in EDTA anticoagulant and immediately diluted in PBS, the final cell concentration being adjusted to 1×10^7/mL.

Immunologic studies

For the immunophenotypic analysis of antigen expression of BM CD19$^+$ B-cells a stain and then lyse technique was used. Briefly, a 100 μL diluted BM sample was incubated for 10 minutes in the dark (room temperature) with saturating amounts of the fluorochrome-conjugated monoclonal antibody reagents listed below. Once this incubation period was finished 2 mL of FACS lysing solution (Becton Dickinson, San José, CA, USA) diluted 1/10 vol/vol in distilled water were added to each tube and another incubation for 10 minutes in the dark (room temperature) was performed. Afterwards, cells were centrifuged (5 minutes at 500g) and the cell pellet washed (5 min at 500g) in 2 mL of PBS/tube. In all cases antigen expression was analyzed using triple combinations of the following monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and either peridinchlorophyll protein (PerCP) or the PE/cyanin5 (Cy5) fluorochrome tandem: 1) CD19 (Leu 12-FITC or Leu12-PE), CD34 (HPCA-2-PE or HPCA-1-FITC), CD33 (leuM-9-PE), CD10 (anti-cALLA-FITC), CD13 (leuM7-PE) and CD22 (Leu14-FITC) purchased from Becton Dickinson; 2) CD19 (SJ25-C1-PE/Cy5) and CD45 (GAP8.3-PE/Cy5) obtained from Caltag Laboratories (San Francisco, CA, USA) and 3) CD117 (95C3-PE) and CD34 (QBEND10-FITC) purchased from Immunotech (Marseille, France). CD19 was systematically used in all the monoclonal antibody combinations tested. In all cases an isotype-matched negative control with no reactivity against BM cells and the CD4-FITC/CD8-PE/CD3-PerCP combination (positive control) were used. The selection of PE conjugated monoclonal antibodies helped the detection of antigens expressed at low intensity on the CD19-labeled cells. In order to assess the relationship between different B-cell subsets identified by two different monoclonal antibody combinations, appropriate four-color stainings were performed.

Measurements were performed on a FACScan and a FACScalibur flow cytometer (Becton Dickinson) for triple and quadruple-stainings, respectively. Both instruments were equipped with an argon ion laser tuned at 488 nm and 15 mWatts. In addition, the FACScalibur flow cytometer was equipped with a HeNe laser diode. Calibration of the instruments was performed prior to data acquisition using previously well-established protocols.[24] For data acquisition the LYSYS II and CellQuest software programs (Becton Dickinson) were used and a two-step acquisition was performed. In the first step, acquisition of at least 10,000 cells was performed and information stored for all these events. Afterwards, in a second step, a minimum of 10^5 cells were measured, information being stored only on CD19$^+$ cells which were acquired through a pre-established SSC/CD19 live gate, as previously described.[25] The Paint-A-Gate PRO software program (Becton Dickinson) with the polynomial SSC transformation capability was used for further data analysis.[26] The analysis process was performed in four sequential steps: 1) selection of the CD19$^+$ population from the files in which data on all bone marrow cells was collected, after exclusion of cell debris and cell aggregates 2) calculation of the percentage of the CD19$^+$ cells on the above mentioned files, 3) identification and phenotypic characterization of the different CD19 populations acquired in the second step through the SSC/CD19 live gate and, 4) calculation of the percentage of each CD19$^+$ cell subset within both the total bone marrow cells and the whole CD19$^+$ cell population.

Results

The overall proportion of CD19$^+$ cells in the normal bone marrow samples analyzed was 3.23%±1.89%, (range: 0.6% to 7.9%). The overall mean percentage of CD34-PE$^+$ precursor cells was 0.79%± 0.39% (range: 0.2% to 1.6%).

Table 1. Subpopulations of CD19+ cells in normal human adult BM established according to their reactivity for the CD10 and CD20 antigens.

Cell subset	*% of CD19+ BM cells*		*% of total BM cells*	
CD19+/CD10-/CD20-	0.54±0.40	(<0.01-1.3)	0.023±0.026	(0.0004-0.091)
CD19+/CD10++/CD20-	3.38±2.73	(0.1-8.3)	0.137±0.134	(0.0043-0.406)
CD19+/CD10+/CD20-	3.52±2.23	(<0.01-7.1)	0.141±0.123	(0.0002-0.406)
CD19+/CD10+/CD20+/++	19.4±11.5	(1-44)	0.77±0.78	(0.03-3.1)
CD19+/CD10-/CD20++	73.2±19.1	(45.7-98.7)	2.497±0.701	(1.70-4.244)

Results expressed as mean percentage of positive cells (± SD) and range in brackets.

The use of multicolor stainings allowed the identification of different maturation stages within the CD19+ cells from the more immature CD34+/CD19+ B-cell precursors to mature CD34- B lymphocytes. Table 1 and Figure 1B show the distribution of different B-cell subsets as defined by the reactivity of CD19+ cells for either CD10 or CD20 antigens. Five sequential B-cell differentiation stages were defined from immature B-cell precursors to mature B-lymphocytes: 1) CD19+ /CD10- /CD20-; 2) CD19+ /CD10++ /CD20-; 3) CD19+ /CD10+ /CD20-; 4) CD19++ /CD10+ /CD20+/++ and 5) CD19++ /CD10- /CD20++. In all normal bone marrow samples analyzed, the more mature CD19++/CD10- /CD20++ B-lymphocytes represented the majority of the CD19+ B-cells followed by the CD19+/CD10+ /CD20+,++ subset. The CD19+/CD10+/CD20-, and CD19+ /CD10++ /CD20- cells represented only a small proportion of the total CD19+ BM cells. The CD19+ /CD10- /CD20- population was the least represented CD19+ subset. The relative values of each of these cell subsets from both total CD19+ cells and the whole BM cellularity are shown in Table 1.

The combined use of CD19/CD10/CD34 (Figure 1C) showed the existence of four subsets of B-cell precursors clearly defined according to their degree of maturation: CD19+/CD34+/CD10-, CD19+/CD34+ /CD10++, CD19+/CD34-/CD10+ and CD19+/CD34- /CD10-. As shown by four color immunophenotyping, the CD19+/CD34+/CD10- B-cell precursors corresponded to the CD19+/CD10-/CD20- cells identified in the previous tube and they were the least represented subset. In contrast, the more mature CD19+ /CD34- /CD10- B-cells corresponded to the CD19+ /CD10- /CD20++ subset which, in all bone marrow samples analyzed, was the most frequently occurring B-cell subset. Reactivity for CD34 allowed the discrimination of two cell populations among CD10+/ CD20- B cells: CD10++/CD34+ and CD34-/CD10+ (both representing around 3.5% of all CD19+ BM B-cells).

Simultaneous staining for CD45, CD34 and CD19 showed that CD34+/CD19+ B cells display a much lower expression of CD45 which ranges from negative to dim positive (Figure 1D) than the remaining CD34-/CD19+ subsets. Moreover, assessment of CD22 expression on CD34 cells showed that all CD19+ cells coexpress CD22 although the reactivity found was weak (Figure 1E). In addition, two CD34- /CD19+ B-cell subsets were distinguished on the basis of CD22 expression: $CD22^{dim+}$ and $CD22^{strong+}$ which represented 23.79±17.52% and 71.96±19.94% of the CD19+ B-cells. Four color-stainings confirmed that $CD22^{strong+}$/CD19+ B-cells corresponded to the CD34- /CD20++ and CD10-/CD20++ B-cell precursor subsets.

Upon combining the information provided by all the monoclonal antibody combinations mentioned above, the following B-cell differentiation stages were identified in normal BM samples according to their degree of maturation:

1) CD19+ /CD34+ /CD10- /CD20- /$CD22^{dim+}$/ $CD45^{-/dim+}$ (0.54±0.40%); 2) CD19+ /CD34+ /CD10++ /CD20- /$CD22^{dim+}$/ $CD45^{-/dim+}$ (3.38±2.70%); 3) CD19+ /CD34- /CD10+ /CD20-/$CD22^{dim+}$ /CD45+ (3.52±2.30%); 4) CD19+ /CD34- /CD10+ /CD20+,++ /$CD22^{dim+}$/CD45+ (19.4±11.50%), 5) CD19+ /CD34- /CD10- /CD20++ /CD22+ /CD45++ (73.20±19.10%).

Minor B-cell phenotypes

Table 2 summarizes the incidence of the minor B-cell BM subsets investigated in the present study and Figures 1D and 2 illustrate some of these populations. As may be seen CD19+ /CD22++ /CD34+ and CD19+/ CD20++ /CD34+ events, in addition to the CD19+/ CD117+ phenotype, were found to be present at frequencies lower than 5×10^{-4} cells, in all the normal bone marrow samples analyzed. In addition, other infrequent B-cell phenotypes found at slightly higher levels included: CD19+ /$CD20^{dim+}$ /CD34+, CD19+/ CD13+ and CD19+ /CD33+ cell subsets which were found at frequencies of 0.024±0.033% (range 0.01% to 0.13%), 0.058±0.045% (range 0.0033% to 0.152%) and 0.059±0.079% (range 0.00025 to 0.32%), respectively. The pattern of positivity found for all these three phenotypes was never defined as a clear population, as illustrated in Figure 2.

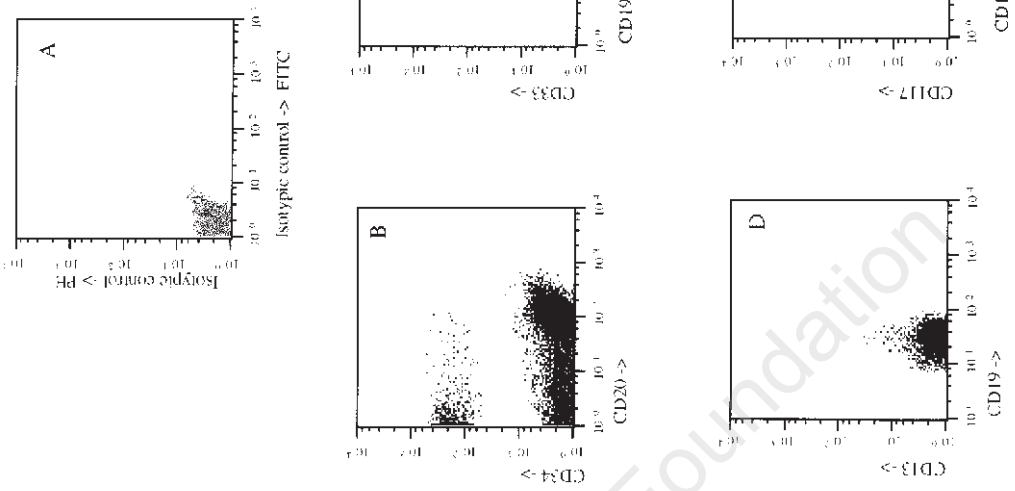

Figure 2. Minor subsets of CD19+ cells in normal adult BM: representative dot plots of $CD34^+/CD20^+$ (B), $CD19^+/CD33^+$ (C), $CD19^+/CD13^+$ (D) and $CD19^+/CD117^+$ (E). Dot plot A shows the isotype-matched negative control.

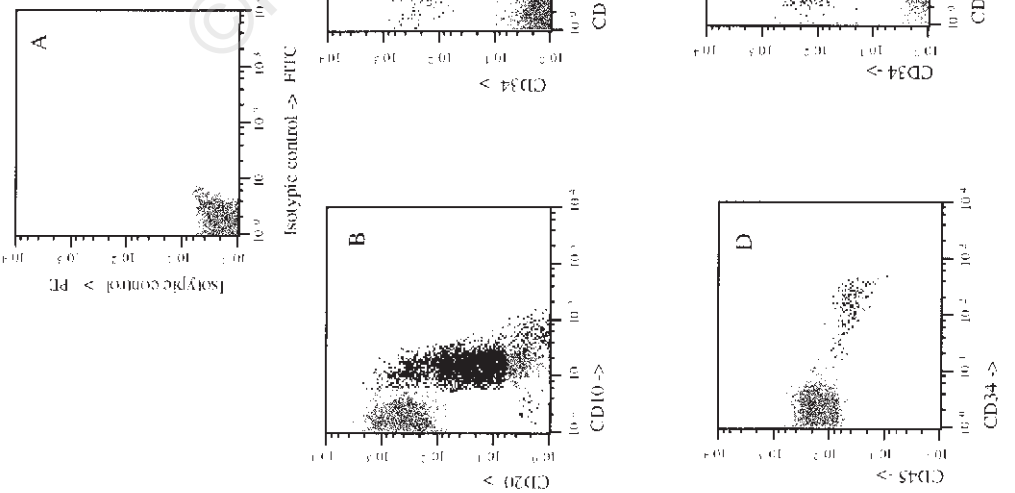

Figure 1. $CD19^+$ gated BM cells: representative dot plots of their reactivity for CD10/CD20 (B), CD10/CD34 (C), CD45/CD34 (D) and CD34/CD22 (E). Dot plot A shows the isotype-matched negative control.

Table 2. Normal adult BM: Incidence of minor phenotypes of CD19+ cells.

Cell subset	*% of total BM cells*	
CD19+/CD10-/CD20-	0.023±0.026	(0.0004-0.091)
CD19+/CD20++/CD34+	0.013±0.011	(0.001-0.034)
CD19+/CD22++/CD34+	0.027±0.013	(0.011-0.050)
CD19+/CD20+/CD34+	0.024±0.033	(0.010-0.130)
CD19+/CD13+	0.058±0.045	(0.003-0.152)
CD19+/CD33+	0.059±0.079	(0.0002-0.320)
CD19+/CD117+	0.001±0.001	(0.0002-0.004)

Results expressed as mean percentage of positive cells (± SD) and range in brackets.

Discussion

In the present study we analyzed the phenotype of CD19+ B-lymphoid precursors in a large series of normal adult BM samples. Overall, our results confirm the existence of discrete stages of B-cell differentiation characterized by different patterns of antigen expression.[12,17,27,28] Accordingly, combined assessment of the CD10 and CD20 antigens showed the existence of five clearly defined differentiation stages among CD19+ BM B-cells. Immature CD19+ /CD10- /CD20- B-cell precursors differentiate into CD10+, CD10+/CD20+/++ and later on into CD10- /CD20++ B-lymphocytes. Interestingly, the relative number of each B-cell subset increased from the more immature to the more differentiated B-cells indicating that differentiation of normal BM B-cells goes in parallel with proliferation.[29] To the best of our knowledge, this is the first study in which a CD10-/CD20- subset of normal CD19+ B-cells is reported. The existence of this B-cell subset in normal BM would support the model proposed by Nadler *et al.*[9] for the sequence of antigen expression in normal B-cell differentiation. In addition, it indicates that the Pro B/BI phenotype, frequently found in precursor-B-ALL, probably reflects, at least in part, a phenotype which corresponds to an early stage of B-cell differentiation present at very low frequencies (< 0.03% of the total BM cellularity) in normal adult BM.

The use of further monoclonal antibody combinations confirmed previous findings[11,17,20,30-32] regarding the expression of CD34, CD45 and CD22 antigens along the BM B-cell maturational process. Accordingly, reactivity for CD34 was almost exclusively present on the CD10-/CD20- and CD10strong+ /CD20 subsets. The CD45 and CD22 antigens were found to be expressed at a low intensity in the CD34+/CD19+ B-cell subsets, their reactivity becoming stronger in the more mature CD34- BM B-cell subsets.

As has been previously suggested by others[33] the identical phenotypic patterns found among different normal individuals provide the basis for the identification of leukemic cells as aberrant whenever, under the same technical conditions, they fall into dot plot areas in which normal B-cells are never found – *empty spaces* – with the monoclonal antibody combinations used. Moreover, an abnormal distribution of the different B-cell subsets identified in the present study as corresponding to distinct differentiation stages with increased numbers of immature cells would indicate an alteration in the normal hemopoietic pattern and would be highly suggestive of residual disease. In any case, further studies in which the pattern of B-cell differentiation in regenerating BM is analyzed are necessary to confirm the potential clinical utility of these parameters in the follow-up of precursor B-ALL patients who achieve cytomorphologic complete remission.

In this regard, the major goal of the present study was to identify minor and undetectable immunophenotypic patterns that could be used for the detection of MRD in precursor B-ALL patients. Preliminary reports[12,13,17] suggested that both the CD34+/CD22+ and CD20+/CD34+ B-cell phenotypes might be leukemia-associated phenotypes. Nevertheless, more recent studies[20,34,35] have shown that a small proportion of normal BM B-cells display these phenotypic profiles. In agreement with these reports, in the present study we have found that in normal adult BM all CD19+ B-cells which are CD34+ display reactivity for surface CD22 and that a small proportion of CD19+ B-cells coexpress CD34 and CD20. The existence of both B-cell populations may limit the use of these phenotypes for monitoring MRD in precursor-B-ALL patients who are in cytomorphologic complete remission. However, most of these CD34+/CD19+ B-cells are weakly positive for CD22 and for CD20; by contrast, the proportion of cells displaying a strong reactivity for these two antigens was always lower than 5×10^{-4} in normal adult BM indicating that follow-up of CD34+/CD19+/CD22strong+ or CD34+/CD19+ /CD20strong+ B-cells may be of great help in monitoring MRD in precursor-B-ALL cases which display these phenotypes at diagnosis.

CD13 and CD33 have been classically assumed to be myeloid associated.[36-39] Therefore, their expression in precursor-B-ALL has been considered to be aberrant. In a similar way, CD117, a marker which is present in a high proportion of CD34+ BM cells, has been reported as being associated with the myeloid lineage, results on its expression on normal BM B-cells being controversial.[40,41] Interestingly, in the present study we show that while no CD19+/CD117+ cells are usually detected (< 4×10^{-5} cells) in normal adult BM both CD19+/CD13+ and CD19+/CD33+ events were found in almost all BM samples analyzed.

These latter findings are in line with those reported by Uckun *et al.*[10] and Campana *et al.*[14,18] who reported on the existence of CD13 and/or CD33 expression on B-cell precursors from fetal liver and bone marrow, and from fetal bone marrow samples, respectively. However, based on our experience, it should be noted that neither $CD19^+/CD13^+$ nor $CD19^+/CD33^+$ events found in normal adult BM correspond to well-defined cell subpopulations. In this sense, if a clearly defined homogeneous expression of CD13 and/or CD33 on leukemic B-cells from ALL patients is detected at diagnosis it may be of use in confirming or excluding the presence of residual leukemic cells later on during follow-up.

In summary, our results show that in adult BM, normal B-cells display constant patterns of maturation as regards both their phenotypic characteristics and their relative distribution. Abnormalities in these patterns provide a potentially useful tool for monitoring MRD in precursor-B-ALL patients who achieve cytomorphologic CR.

Contributions and Acknowledgments

JC, AO and JFSM were the principal investigators, designed the study and wrote the paper. JC carried out the cytometric studies and handled the data. BVid, AM, BVal and AM contributed to carrying out the flow cytometric studies. MCLB and MG were involved in the design of the study and in critically revising the intellectual content.

The criteria for the order in which the authors appear are based on the amount of work engaged in the study except Dr. San Miguel who is the last name since he is the head of the department in which the study was performed.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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References

1. Quesenberry P, Levitt L. Hematopoietic stem cells (first of three parts). N Engl J Med 1979; 301:775-60.
2. Quesenberry P, Levitt L. Hematopoietic stem cells (second of three parts). N Engl J Med 1979; 301:819-23.
3. Quesenberry P, Levitt L. Hematopoietic stem cells (third of three parts). N Engl J Med 1979; 301:868-72.
4. Lovett EJ, Schnitzer B, Keren DF, Flint A, Hudson JL, McClatchey KD. Application of flow cytometry to diagnostic pathology. Lab Invest 1984; 50:115-40.
5. Krause JR, Penchansky L, Contis L, Kaplan SS. Flow cytometry in the diagnosis of acute leukemia. Am J Clin Pathol 1988; 89:341-6.
6. Raimondi SC, Privitera E, Williams DL, et al. New recurring chromosomal translocations in childhood acute lymphoblastic leukemia. Blood 1991; 77:2016-22.
7. Orfao A, González M, Ciudad J, et al. Aplicaciones de la citometría de flujo en el diagnóstico hematológico. Biol Clin Hematol 1992; 14:193-203.
8. Terstappen LW, Safford M, Konemann S, et al. Flow cytometric characterization of acute myeloid leukemia. Part II. Phenotypic heterogeneity at diagnosis. Leukemia 1992; 6:70-80.
9. Nadler LM. B cell/leukemia panel workshop. In: Reinherz EL, Haynes BF, Nadler LM, Berstein ID, eds. Leukocyte Typing II. New York: Springer-Verlag: 1986:3-43.
10. Uckun FM. Regulation of human B-cell ontogeny. Blood 1990; 76:1908-23.
11. Pontvert-Delucq S, Breton-Gorius J, Schmitt C, et al. Characterization and functional analysis of adult human bone marrow cell subsets in relation to B-lymphoid development. Blood 1993; 82:417-29.
12. Ryan DH, Chapple C, Kossover SA, Sandberg AA, Cohen HJ. Phenotypic similarities and differences between cALLA-positive acute lymphoblastic leukemia cells and normal marrow cALLA-positive B cell precursors. Blood 1987; 70:814-21.
13. Hurwitz CA, Loken MR, Graham ML, et al. Asynchronous antigen expression in B lineage acute lymphoblastic leukemia. Blood 1988; 72:299-307.
14. Campana D, Coustan-Smith E, Janossy G. The immunologic detection of minimal residual disease in acute leukemia. Blood 1990; 76:163-71.
15. van Dongen JJ, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H. Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. Leukemia 1992; 6 (Suppl 1):47-59.
16. Campana D, Freitas RO, Coustan-Smith E. Detection of residual leukemia with immunologic methods: technical developments and clinical implications. Leuk Lymphoma 1994; 13 (Suppl 1):31-4.
17. Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: II. Normal B lymphocyte development. Blood 1987; 70:1316-24.
18. Campana D, Pui C-H. Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance. Blood 1995; 85:1416-34.
19. Tjonnfjord GE, Steen R, Veiby OP, Morkrid L, Egeland T. Haemopoietic progenitor cell differentiation: flow cytometric assessment in bone marrow and thymus. Br J Haematol 1995; 91:1006-16.
20. Hurwitz CA, Gore SD, Stone KD, Civin CI. Flow cytometric detection of rare normal human marrow cells with immunophenotypes characteristic of acute lymphoblastic leukemia cells. Leukemia 1992; 6:233-9.
21. Ross CW, Stoolman LM, Schnitzer B, Schlegelmilch JA, Hanson CA. Immunophenotypic aberrancy in adult acute lymphoblastic leukemia. Am J Clin Pathol 1990; 94:590-9.
22. Lamkin T, Brooks J, Annett G, Roberts W, Weinberg K. Immunophenotypic differences between putative hematopoietic stem cells and childhood B-cell precursor acute lymphoblastic leukemia cells. Leukemia 1994; 8:1871-8.
23. Ludwig WD, Reiter A, Loffler H, et al. Immunophenotypic features of childhood and adult acute lymphoblastic leukemia (ALL): experience of the German Multicentre Trials ALL-BFM and GMALL. Leuk Lymphoma 1994; 13 (Suppl 1):71-6.
24. Macedo A, Orfao A, González M, et al. Immunological detection of blast cell subpopulations in acute myeloblastic leukemia at diagnosis:implications for minimal residual disease studies. Leukemia 1995; 9:993-8.
25. Orfao A, Ciudad J, Lopez-Berges MC, et al. Acute lymphoblastic leukemia (ALL): Detection of minimal residual disease (MRD) at flow cytometry. Leuk Lym-

phoma 1994; 13 (Suppl. 1):87-90.
26. Terstappen LW, Mickaels RA, Dost R, Loken MR. Increased light scattering resolution facilitates multidimensional flow cytometric analysis. Cytometry 1990; 11:506-12.
27. Look AT, Melvin SL, Brown LK, Dockter ME, Roberson PK, Murphy SB. Quantitative variation of the common acute lymphoblastic leukemia antigen (gp100) on leukemic marrow blasts. J Clin Invest 1984; 73:1617-28.
28. Ryan D, Kossover S, Mitchell S, Frantz C, Hennessy L, Cohen H. Subpopulations of common acute lymphoblastic leukemia antigen-positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. Blood 1986; 68:417-25.
29. Hollander Z, Shah VO, Civin CI, Loken MR. Assessment of proliferation during maturation of the B lymphoid lineage in normal human bone marrow. Blood 1988; 71:528-31.
30. Shah VO, Civin CI, Loken MR. Flow cytometric analysis of human bone marrow. IV. Differential quantitative expression of T-200 common leukocyte antigen during normal hemopoiesis. J Immunol 1988; 140: 1861-7.
31. Uckun FM, Ledbetter JA. Immunobiologic differences between normal and leukemic human B-cell precursors. Proc Natl Acad Sci USA 1988; 85:8603.
32. LeBien TW, Villablanca JG. Ontogeny of normal human B-cell and T-cell precursors and its relation to leukemogenesis. Hematol Oncol Clin North Am 1990; 4:835-47.
33. Lúcio P, Parreira A, Van den Beemd MWM, et al. Flow cytometric analysis of normal and leukemic bone marrow B-cell differentiation: a frame of reference for the detection of minimal residual disease in precursor-B-ALL patients. Proceedings of the International Symposium on Minimal Residual Disease. Salamanca, Spain 1998; 18-20.
34. Gore SD, Kastan MB, Civin CI. Normal human bone marrow precursors that express terminal deoxynucleotidyl transferase include T-cell precursors and possible lymphoid stem cells. Blood 1991; 77:1681-90.
35. Dworzak M, Fritsch G, Fleischer C, et al. Multiparameter phenotype mapping of normal and post-chemotherapy B lymphopoiesis in pediatric bone marrow. Leukemia 1997; 11:1266-73.
36. Griffin JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 1981; 68:932-41.
37. Andrews RG, Torok-Storb B, Bernstein ID. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood 1983; 62:124-32.
38. Griffin JD, Sullivan R, Beveridge RP, Larcom P, Schlossman SF. Expression of My7 antigen on myeloid precursor cells. J Cell Cloning 1984; 1:33-8.
39. Terstappen LW, Loken MR. Myeloid cell differentiation in normal bone marrow and acute myeloid leukemia assessed by multi-dimensional flow cytometry. Anal Cell Pathol 1990; 2:229-40.
40. Strobl H, Takimoto M, Majdic O, Hocker P, Knapp W. Antigenic analysis of human hemopoietic progenitor cells expressing the growth factor receptor c-kit. Br J Haematol 1992; 82:287-94.
41. Escribano L, Ocqueteau M, Almeida J, Orfao A, San Miguel JF. Expression of the C-Kit (CD117) molecule in normal and malignant hematopoiesis. Leuk Lymphoma 1998; in press.