



BIOMED-1 concerted action report: flow cytometric immunophenotyping of precursor B-ALL with standardized triple-stainings

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The flow cytometric detection of minimal residual disease (MRD) in precursor-B-acute lymphoblastic leukemias (precursor-B-ALL) mainly relies on the identification of minor leukemic cell populations that can be discriminated from their normal counterparts on the basis of phenotypic aberrancies observed at diagnosis. This technique is not very complex, but discordancies are frequently observed between laboratories, due to the lack of standardized methodological procedures and technical conditions. To develop standardized flow cytometric techniques for MRD detection, a European BIOMED-1 Concerted Action was initiated with the participation of laboratories from six different countries. The goal of this concerted action was to define aberrant phenotypic profiles in a series of 264 consecutive *de novo* precursor-B-ALL cases, systematically studied with one to five triple-labelings (TdT/CD10/CD19, CD10/CD20/CD19, CD34/CD38/CD19, CD34/CD22/CD19 and CD19/CD34/CD45) using common flow cytometric protocols in all participating laboratories. The use of four or five triple-stainings allowed the identification of aberrant phenotypes in virtually all cases tested (127 out of 130, 98%). These phenotypic aberrancies could be identified in at least two and often three triple-labelings per case. When the analysis was based on two or three triple-stainings, lower incidences of aberrancies were identified (75% and 81% of cases, respectively) that could be detected in one and sometimes two triple-stainings per case. The most informative triple staining was the TdT/CD10/CD19 combination, which enabled the identification of aberrancies in 78% of cases. The frequencies of phenotypic aberrations detected with the other four triple-stainings were 64% for CD10/CD20/CD19, 56% for CD34/CD38/CD19, 46% for CD34/CD22/CD19, and 22% for CD19/CD34/CD45. In addition, cross-lineage antigen expression was detected in 45% of cases, mainly coexpression of the myeloid antigens CD13 and/or CD33 (40%). Parallel flow cytometric studies in different laboratories finally resulted in highly concordant results (>90%) for all five antibody combinations, indicating the high reproducibility of our approach. In conclusion, the technique presented here with triple-labelings forms an excellent basis for standardized flow cytometric MRD studies in multicenter international treatment protocols for precursor-B-ALL patients. *Leukemia* (2001) 15, 1185–1192.

Keywords: minimal residual disease; acute lymphoblastic leukemia; immunophenotype; flow cytometry

Introduction

Currently available strategies for the treatment of patients with precursor-B-ALL result in high complete remission rates. Nevertheless, a significant number of these patients will

eventually relapse during follow-up, due to the persistence of low numbers of leukemic cells, which are undetectable by conventional cytomorphology. In recent years, highly sensitive techniques have been developed, that allow the distinction between normal and leukemic residual cells, even when they occur in numbers below the cytomorphological detection limit. For this purpose, flow cytometric immunophenotyping of leukemic cells has generally been accepted as one of the most useful approaches, because of its speed, simplicity and sensitivity. Moreover, from the clinical point of view, immunophenotypic detection of MRD was shown to contribute both to predict relapses and to discriminate high-risk patients.^{1–3}

In most studies, the immunological detection of MRD was based on the expression by leukemic cells of uncommon or aberrant phenotypes, which differ from those exhibited by normal cells.^{4,5} However, the reported incidence of phenotypic aberrancies in precursor-B-ALL greatly varies in the literature, from 4% to 99% of cases.^{2,3,6–22} Such discrepancies probably result from differences in the methodological approaches used by different groups in the definition of phenotypic aberrations, such as different panels of monoclonal antibody (MAB) combinations, different sample preparation procedures, and particularly different analytical methods. Moreover, the investigation of MRD classically depends on the design of patient-specific MAB combinations, according to the aberrant phenotypic profiles detected at diagnosis, that are subsequently used during follow-up studies. Although of potential clinical relevance, this diagnostic strategy becomes increasingly difficult to standardize between laboratories and is associated with increasing costs, because of the need for large panels of MAB combinations and experienced personnel.

The European BIOMED-1 Concerted Action 'Investigation of Minimal Residual Disease in Acute Leukemia: International Standardization and Clinical Evaluation' aimed at the development of standardized flow cytometric and PCR-based MRD techniques.^{23–26} This included the design of a simple and reproducible flow cytometric approach for the immunophenotypic analysis of precursor-B-ALL. The first part of this study aimed at the precise characterization of the phenotypic profiles exhibited by normal bone marrow (BM) precursor-B-cell subpopulations, according to well-defined triple-staining MAB combinations. These profiles could then be used as a frame of reference for the diagnosis of precursor-B-ALL patients, in order to identify the presence of aberrant phenotypes. We have shown that the standardized use of five triple-MAB combinations easily allows the definition of distinct subpopulations within normal BM B-lineage cells.²³ Here we report on the second part of this multicentric cooperative study, by

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presenting the results of the analysis of 264 consecutive precursor-B-ALL patients, carried out under standardized and optimized conditions, including the use of the five common triple-stainings previously tested in normal BM samples.

Materials and methods

Patients and samples

BM samples were collected at diagnosis from 264 consecutive patients with *de novo* precursor-B-ALL, by the six participating European laboratories involved in the European BIOMED-1 Concerted Action: Department of Hematology, Portuguese Institute of Oncology, Lisbon, Portugal ($n = 56$); Department of Pathology, Karolinska Hospital, Stockholm, Sweden ($n = 64$); Clinica Pediatrica, University of Milan, Ospedale San Gerardo, Monza, Italy ($n = 43$); Department of Hematology, University of Salamanca, Salamanca, Spain ($n = 36$); Department of Immunology, University Hospital Dijkzigt/Erasmus University Rotterdam, Rotterdam, The Netherlands ($n = 35$); Dutch Childhood Leukemia Study Group, The Hague, The Netherlands ($n = 30$).

The diagnosis of precursor-B-ALL was established according to conventional FAB and immunological criteria.^{27,28} The age of the 264 patients included in this study varied from 8 months to 83 years, 197 were children aged less than 15 and 67 were adults (median ages were 5 and 41, respectively). The male/female ratio was 1.1.

Informed consent to participate in the study was obtained from all patients and parents in the case of children, according to the local or national guidelines of the Medical Ethics Committees.

Samples were obtained in either heparin or EDTA_{K3} and processed within 24 h after collection.

Immunophenotypic studies

BM samples were processed according to previously described procedures.²³ Briefly, immediately after collection of the BM sample, 2×10^6 nucleated unseparated cells were incubated for 10 min in the dark with saturating amounts of the relevant fluorochrome-conjugated mouse anti-human MAb (room temperature); subsequently, erythrocyte lysis was carried out using FACS Lysing solution, according to the manufacturer's instructions (Becton Dickinson, San Jose, CA, USA). Nuclear TdT was detected after staining for surface markers (as above) and subsequent fixation/permeabilization with Permeafix (Ortho, Raritan, NY, USA), Fix&Perm (An der Grub, Vienna, Austria), Intraprep (Immunotech, Marseille, France) or FACS Lysing solution (Becton Dickinson). The reagents were used following the manufacturer's recommendations. Previous comparative studies showed that all four intracellular staining procedures yielded similar results in what concerns the pattern of TdT expression in both normal and leukemic cells,²⁹ although it should be noted that the latter technique provided slightly higher TdT-associated mean fluorescence intensity than the former three.³⁰ Finally, the cells were resuspended in 1 ml/tube of PBS until the analysis was performed.

For standardization purposes, all groups used the same set of MAb clones and fluorochrome-conjugated reagents, which were carefully selected on the basis of their reactivity patterns and the absence of background staining. All reagents were identical to those previously used for staining the BM samples

Table 1 Source and characteristics of the fluorochrome conjugated MAb used in this study

Specificity	MAb clone	Fluorochrome	Source
CD2	S5.2	FITC	BD
CD2	S5.2	PE	BD
CD3	Leu-4	FITC	BD
CD4	Leu-3	PE	BD
CD7	4H9	FITC	BD
CD7	M-T701	PE	BD
CD8	3B5	PECy5	Caltag
CD10	W8E7	FITC	BD
CD10	J5	PE	Coulter
CD13	L138	PE	BD
CD15	MMA	FITC	BD
CD19	Leu-12	FITC	BD
CD19	SJ25-C1	PECy5	Caltag
CD20	Leu-16	PE	BD
CD22	Leu-14	PE	BD
CD33	P67.6	PE	BD
CD34	HPCA-2	FITC	BD
CD34	HPCA-2	PE	BD
CD38	Leu-17	PE	BD
CD45	HLe-1	PERCP	BD
CD65	VIM2	FITC	An der Grub
TdT	HT6	FITC	Supertechs

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridin chlorophyll protein; PECy5, phycoerythrin + cyanine 5; BD, Becton Dickinson (San Jose, CA, USA); Coulter (Hialeah, FL, USA); Caltag, Caltag Laboratories (South San Francisco, CA, USA); An der Grub, An der Grub Bioresearch (Austria); Supertechs (Bethesda, MD, USA).

from healthy volunteers,²³ as shown in Table 1. Accordingly, the following triple-stainings: fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/PE-cyanin 5 (PECy5) or peridin chlorophyll protein (PerCP) were used: (1) TdT/CD10/CD19; (2) CD10/CD20/CD19; (3) CD34/CD38/CD19; (4) CD34/CD22/CD19; and (5) CD19/CD34/CD45.

For each MAb combination the analysis was restricted to the B-lineage, by gating on CD19-positive cells. Due to the limited number of cells obtained in some patients, it was not possible to perform all five triple-stainings in each case (Table 2). Additional multiple-stainings were performed in order to investigate the expression of both T-lineage (CD2 and CD7) and myeloid associated markers (CD13, CD15, CD33 and CD65) on CD19⁺ BM leukemic B cells (Table 1). Only those cases with unequivocally abnormally high expression of these markers were considered to show cross-lineage antigen expression.³¹

Data acquisition was performed by all laboratories in FAC-Scan flow cytometers (Becton Dickinson), using either the

Table 2 Incidence of aberrant phenotypes in precursor-B-ALL patients according to the number of MAb combinations tested

	Number of MAb combination tested				
	1 <i>n</i> = 11	2 <i>n</i> = 44	3 <i>n</i> = 79	4 <i>n</i> = 73	5 <i>n</i> = 57
Number of patients with aberrancies	6	33	64	70	57
%	(55%)	(75%)	(81%)	(96%)	(100%)

Lysis II (Becton Dickinson) or the CellQuest (Becton Dickinson) software programs. For the standardized calibration of flow cytometers, normal peripheral blood lymphocytes stained with CD3FITC (Leu-4), CD4PE (Leu-3) and CD8 PECy5 were used, PMT settings being checked out with CALIBRITE beads (Becton Dickinson) in order to assess interlaboratory variability; additionally, a normal peripheral blood sample simultaneously stained for CD3FITC/CD4PE/CD8PerCP was always analyzed in parallel with patient samples. The data analysis was performed on Hewlett-Packard or Macintosh computers using the Paint-A-Gate software (Becton Dickinson).

Standardization of the procedures

One of the primary goals of this concerted action was to fully standardize the analytical procedure, in order to make it easily reproducible among different laboratories. For that purpose, standardized common protocols for instrument set-up and calibration, specimen manipulation, sample preparation, and data acquisition were used in all the participating institutions, as previously reported by our group.^{23,26} In addition, the same MAb reagents were used in all centers in identical triple-staining combinations that were previously tested in normal BM samples.²³

To support the standardization process, practical workshops took place twice a year during a total period of 3 years and written protocols were distributed to all participants. Standardized procedures were also used for data analysis. Additionally, each data file in this study was independently analyzed locally as well as by a reference laboratory for each specific triple combination: Rotterdam for TdT/CD10/CD19, The Hague for CD10/CD20/CD19, Salamanca for CD34/CD38/CD19, Monza for CD34/CD22/CD19, and Lisbon for CD19/CD34/CD45.

In order to easily identify phenotypic aberrations in precursor-B-ALL cells, we defined so-called 'empty spaces', ie dot-plot regions devoid of normal BM cells, where malignant cells could localize because of their aberrant antigenic expression. For each triple staining the empty spaces were coded as patterns A to F (Figure 1). Accordingly, each laboratory recorded the aberrancies found in each triple-staining of its local cases. The flow cytometry data files were then blindly reviewed by the reference laboratory and the reproducibility of these two evaluations was obtained matching both records. For those cases in which leukemic cells were found in more than one 'empty space', we agreed that they should be considered within the empty space where most of the cells were included. Reference laboratories have also reviewed their own files blindly and significant differences were not found between intralaboratory or interlaboratory analysis.

Results

Aberrant phenotypes involving B-lineage associated markers

Table 2 depicts the incidence of aberrant phenotypes involving B-lineage associated antigens observed in the analysis of 264 precursor-B-ALL patients, according to the number of common triple-stainings used per patient. The presence of aberrant phenotypes exhibited by leukemic cells could be easily visualized in the dot plots of each individual combination,

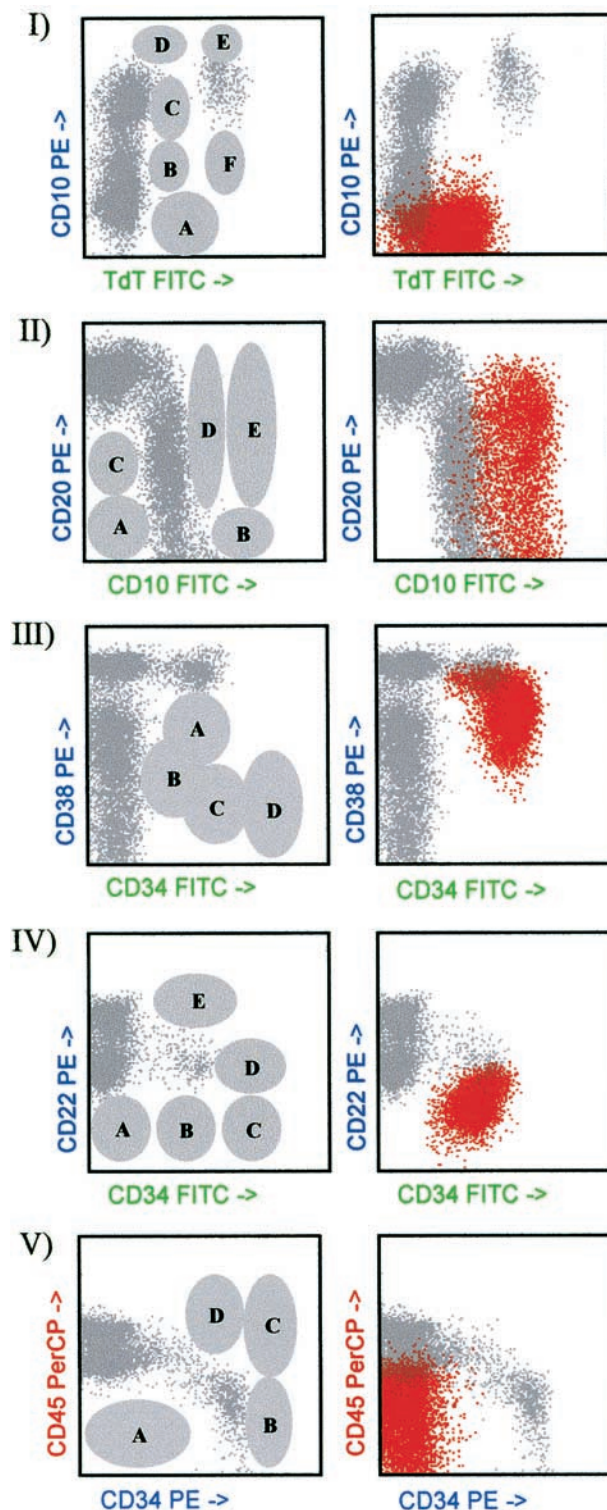


Figure 1 Left column, localization of the 'empty-spaces' corresponding to aberrant phenotypes in the following triple-stainings: (I) TdT/CD10/CD19; (II) CD10/CD20/CD19; (III) CD34/CD38/CD19; (IV) CD34/CD22/CD19; and (V) CD19/CD34/CD45. The distribution of normal precursor-B-cells is represented as grey dots and the areas of 'empty space', devoid of normal BM cells, are depicted as circles identified by letters (from A to F). Right column, examples of the abnormal distribution of leukemic B cells (depicted in red) in the 'empty spaces' defined for each of the triple combinations listed above (I to V).

because of their localization in the 'empty spaces' that we previously defined. Overall, the use of four or five triple-stainings allowed the identification of aberrant phenotypes in virtually all cases tested (127 out of 130, 98%), while the use of fewer triple-stainings per patient (one to three) resulted in a lower incidence of identifiable aberrant profiles (55 to 81%, respectively). Furthermore, when four or five triple combinations were used, the presence of two or even three distinct aberrant phenotypic patterns could frequently be identified in each individual patient, depending on the triple-stainings used. By contrast, if the analysis was based on less than four triple-stainings, fewer patients exhibited more than one aberrant profile. The results observed in children did not differ from those in adult patients.

The proportion of aberrant phenotypes identifiable by each individual triple-staining showed important variations, ranging from as high as 78% with the TdT/CD10/CD19 combination to 22% with the CD19/CD34/CD45 triple-staining. When using the combinations CD10/CD20/CD19, CD34/CD38/CD19 and CD34/CD22/CD19 aberrant phenotypes could be disclosed in 64%, 56% and 46% of cases, respectively (Table 3).

Table 3 summarizes the particular phenotypic aberrant profiles that we observed localizing in the different empty spaces of each dot plot, as we previously defined. Six different empty spaces were considered when using the TdT/CD10/CD19 triple-staining, named I.A to I.F (Table 3 and Figure 1). The most frequent aberrancy observed with this combination was the abnormally low expression of TdT by CD19⁺ leukemic cells (patterns I.A to I.D: 60.4%), followed by the dim expression of CD10 by TdT⁺/CD19⁺ cells (patterns I.A to I.C and I.F: 41.1%) that contrasts with the overexpression of the CD10 antigen in the two other remaining empty spaces (patterns I.D and I.E: 36.4%).

With the CD10/CD20/CD19 combination we observed five different aberrant profiles corresponding to the empty spaces defined as II.A to II.E (Table 3 and Figure 1). This triple-staining disclosed the overexpression of CD10 by CD20⁻ cells (pattern II.B), as well as by CD20⁺ cells (pattern II.E), corresponding to 20.9% and 11.4% of cases, respectively. Additionally, the asynchronous CD10^{strong+} expression by CD20^{strong+} cells was observed in 13.3% of cases (pattern II.D). Lack of CD10 expression in the absence of a strong reactivity for CD20 was detected in 18.3% of cases (patterns II.A plus II.C).

The triple-staining CD34/CD38/CD19 showed that four different profiles of phenotypic aberrations could be considered within the corresponding empty spaces (patterns III.A to III.D) (Table 3, Figure 1). In all the aberrant cases, the asynchronous expression of CD38 (abnormally low) was observed in CD34⁺ cells. Moreover, the overexpression of CD34 (pattern III.D) was detected in 4.8% of cases.

The combination CD34/CD22/CD19 disclosed five different patterns of phenotypic aberrations (patterns IV.A to IV.E) (Table 3, Figure 1). Dim membrane CD22 expression in both CD34⁻ (pattern IV.A) and CD34⁺ CD19⁺ B cells (patterns IV.B and IV.C) was the most consistent aberration (25.7%). Furthermore, overexpression of CD34 by CD22^{dim} (pattern IV.C) or CD22⁺ (pattern IV.D) cells was observed in 7.7% of cases. An abnormally high CD22 expression (asynchronous) on CD34⁺/CD19⁺ blast cells (pattern IV.E) was also detected in 15.4% (Table 3).

Finally, the CD19/CD34/CD45 MAb combination was less useful. Four different patterns of phenotypic aberrations were detected, corresponding either to an abnormally low CD45 expression by CD34⁻ or CD34^{dim+} cells (pattern V.A: 9.9%) or to an overexpression of CD34 by CD45^{dim} (pattern V.B: 5.2%) or CD45⁺ B cells (patterns V.C: 4.2% and V.D: 2.8%).

The overall relative distribution of the different types of phenotypic aberrations is summarized in Table 4.

Aberrant phenotypes involving cross-lineage antigen expression

Cross-lineage antigen expression was detected in a relatively high proportion of cases, as shown in Table 4. Aberrant expression of either CD13 or CD33 antigens was detected in 31.9% and 17.9% of cases, respectively. Co-expression of these two myeloid-associated markers was found in a significant number of precursor-B-ALL cases (10.2%), while the isolated expression of CD13 or CD33 was found in 21.7% and 7.7% of cases, respectively. The overall incidence of CD13 and/or CD33 expression was 39.6% of all precursor-B-ALL cases. In contrast, aberrant expression of other myeloid-associated (CD15 and CD65) and T-related (CD2 and CD7) antigens was only detected in a minority of precursor-B-ALL cases (less than 5% for each of these markers) (Table 4).

Table 3 Number of cases identified by each MAb triple combination as displaying an aberrant phenotype and the relative frequency of each empty space

Empty space	% of aberrant cases (No. cases)				
	I TdT/CD10/CD19 (n = 129)	II CD10/CD20/CD19 (n = 158)	III CD34/CD38/CD19 (n = 124)	IV CD34/CD22/CD19 (n = 39)	V CD19/CD34/CD45 (n = 212)
A	6.2 (8)	17.7 (28)	33.1 (41)	7.7 (3)	9.9 (21)
B	8.5 (11)	20.9 (33)	6.5 (8)	15.4 (6)	5.2 (11)
C	24.8 (32)	0.6 (1)	11.3 (14)	2.6 (1)	4.2 (9)
D	20.9 (27)	13.3 (21)	4.8 (6)	5.1 (2)	2.8 (6)
E	15.5 (20)	11.4 (18)	—	15.4 (6)	—
F	1.6 (2)	—	—	—	—
Total	77.5% (100)	63.9% (101)	55.6% (69)	46.2% (18)	22.2% (47)

Results expressed as percentage of cases and their number in brackets; for an explanation of A–F, see Figure 1.

Table 4 Precursor-B-ALL: classification and relative distribution of the different phenotypic aberrations detected in CD19⁺ blast cells

Type of phenotypic aberrations	Relative frequency ^a
Asynchronous antigen expression	
CD10 ^{strong+} /TdT ^{dim+}	45.7%
CD10 ^{-dim+} /TdT ^{dim+}	16.3%
CD10 ^{dim+} /CD34 ⁺	55.6%
CD10 ^{strong+} /CD20 ^{strong+}	13.3%
CD10 ⁻ /CD20 ^{-dim+} /CD34 ⁺	18.3%
CD22 ^{strong+} /CD34 ⁺	15.4%
CD45 ^{-dim+} /CD34 ^{-dim+}	9.9%
Low antigen expression	
CD22 ^{-dim+}	25.7%
Antigen overexpression	
CD10	32.3%
CD34	9.4%
Cross-lineage antigen expression	
CD2	4.0%
CD7	0.8%
CD13	31.9%
CD33	17.9%
CD15	4.7%
CD65	0.7%

*Results expressed as percentage of cases from the total number of patients analysed.

Concordance of immunophenotyping results between laboratories

The concordance between different laboratories was high (90%) with respect to the characterization of the phenotypic patterns as detected with the five common triple-stainings (range: 90.9% to 95.9%) (Table 5). The percentage of discrepant cases for each individual triple-staining was as follows: 4.1% for TdT/CD10/CD19; 9.1% for CD10/CD20/CD19; 6.5% for CD34/CD38/CD19; 6.7% for CD34/CD22/CD19 and 5.2% for CD19/CD34/CD45.

Discussion

Multiparameter flow cytometric immunophenotyping has been widely used for the diagnosis of precursor-B-ALL. Generally, the phenotypic similarities between the neoplastic cells and their normal counterparts were the basis for the classification of precursor-B-ALL. However, more recent studies

Table 5 Evaluation of the concordance between centers of the classification of phenotypic patterns for their assignment to a specific empty space

Triple combination	% of cases with concordant results between laboratories
(I) TdT/CD10/CD19	95.9%
(II) CD10/CD20/CD19	90.9%
(III) CD34/CD38/CD19	93.5%
(IV) CD34/CD22/CD19	93.3%
(V) CD19/CD34/CD45	94.8%
Mean ± s.d.	93.7% ± 1.9%

have shown that once compared with their normal counterparts, leukemic cells display immunophenotypic discrepancies that may be used to monitor MRD in precursor-B-ALL patients who have achieved morphological complete remission. The MRD levels were shown to be of relevance, both for a better definition of the cytomorphologic remission and for predicting which patients are at a risk of relapse.^{1-3,32-39}

Most studies aiming at the detection of aberrant phenotypes for the investigation of MRD, have been based on large panels of MAb combinations, in order to design patient-specific triple-labelings at diagnosis, that could be used for follow-up studies. From a practical point of view, such an approach is time-consuming and expensive, due to the large number of reagents required and the need for experienced personnel in the identification of the most informative phenotypic aberrations for each patient. Furthermore, this heterogeneous approach becomes difficult to standardize among laboratories. These limitations have restricted the use of immunophenotyping to experienced laboratories, thus limiting the large-scale application of flow cytometric MRD studies.

In the present study we investigated the occurrence of aberrant phenotypes in a large series of *de novo* precursor-B-ALL, using a limited number of five common well-defined triple-stainings. Our results show that distinct aberrant phenotypic profiles can be identified in virtually all precursor-B-ALL cases (98%), if four or five of the well-defined triple-stainings are tested simultaneously.

The proportion of phenotypic aberrancies observed in the present series is clearly higher than that reported in most of previous publications.^{1-3,7,11,15,20-22} Moreover, our strategy permitted the characterization of more than one phenotypic aberration in most of the individual cases, in particular when the analysis was based on four or five triple-stainings. However, it should be noted that several aberrancies herein reported are redundant due to the use of some monoclonal antibodies in more than one combination, for example, the overall CD10 overexpression was identified in 45.6% of all the precursor-B-ALL cases, identified as empty spaces II.B, II.D and II.E in CD10/CD20/CD19 triple combination; part of these cells were redundantly considered aberrant in the empty spaces I.D and I.E (20.9% and 15.5%, respectively) from TdT/CD10/CD19 combination.

The use of these triple-stainings improves the immunophenotypic discrimination between normal hematopoietic differentiation patterns and leukemic B cell populations, because of their localization in the different 'empty spaces' of the dot plots for each Mab combination. It represents a reproducible strategy based on a small panel of MAb that is easy to standardize and easy to handle, also by personnel with moderate experience. We believe that apart from its value for the precise immunophenotypic diagnosis of precursor-B-ALL, it may be of great utility for MRD monitoring in follow-up studies, considering that preliminary results published by our group⁴⁰ and others⁴¹ suggest that similar patterns of antigen expression are found in normal as compared to regenerating BM. The differences between these two groups are restricted to the relative distribution of cells at distinct maturation stages, supporting the assumption that the MRD detection methodology described in the present study might also be appropriate for regenerating BM samples.

Among the individual markers tested in the five combinations, CD10 was most frequently abnormally expressed, followed by CD22, CD38 and TdT. This was reflected by the higher frequency of phenotypic aberrations observed with the triple-stainings that contained these antigens. According to our

results, CD10 is probably the most informative of these four markers. During B cell maturation CD10 is one of the earliest antigens to become detectable, its expression being down-regulated once B cell precursors differentiate into mature $\text{slg}^+/\text{CD20}^{\text{strong}+}$ B-lymphocytes. CD10 overexpression has been reported to be one of the most frequent phenotypic aberrations in precursor-B-ALL,⁴² as confirmed in the present study. In addition to cases with CD10 overexpression, a relatively high proportion of cases exhibited the $\text{CD10}^{\text{strong}+}/\text{TdT}^{\text{dim}+}$ and $\text{CD10}^{\text{strong}+}/\text{CD20}^{\text{strong}+}$ phenotypes, suggesting absent or diminished down-regulation of CD10 expression, in contrast to normal B cell differentiation.

The phosphoglycoprotein CD22 is a pan B cell adhesion molecule that is present on the cell surface of virtually all precursor-B-ALL cells. As this glycoprotein is associated with B cell proliferation,^{43,44} abnormalities of the expression of CD22, as well as those of CD10, might therefore be associated with the abnormal differentiation and/or proliferation of neoplastic B cells.

CD38 is also related to cell activation and proliferation.⁴⁵ In this series the CD38 expression in CD34^+ leukemic B cells was abnormally weak in approximately half of the precursor B-ALL cases. The existence of profound changes in CD38 expression during B cell development is in line with its regulatory role in hematopoiesis.⁴⁶

TdT is an intranuclear enzyme that catalyzes the addition of deoxynucleotides triphosphates to the 3'-ends of DNA breaks without need for a template strand.^{47,48} The high frequency of TdT⁺ precursor-B-ALL suggests that TdT expression is not down-regulated and thereby provides a useful marker for the identification of abnormal B cell maturation.

As previously suggested, the expression of aberrant phenotypes could indirectly reflect the genetic abnormalities underlying the disease, as different genetic lesions might be translated into distinct and specific patterns of aberrant protein expression. So far it is still not clear whether there is any relationship between the aberrant expression patterns of CD10, CD22, CD38 and TdT and specific genetic abnormalities,⁴⁹ except for the abnormally low expression of CD38, that was recently associated with *BCR/ABL* gene rearrangement.⁵⁰ Further studies are necessary to elucidate this question.

As shown in the present study, the simultaneous assessment of abnormal phenotypic patterns during B cell maturation and cross-lineage antigen expression as reflected by the reactivity for the CD13 and CD33 pan-myeloid-associated markers on leukemic B cells would contribute to the identification of a higher number of phenotypic aberrations per case in around half of the patients. Although the reported incidence of these myeloid-associated antigens in precursor-B-ALL varies widely in the literature,^{2,3,6-11,17,18} it is generally agreed that these markers are the most representative examples of cross-lineage antigen expression in this particular group of ALL patients. The utility of other myeloid associated markers (CD15 and CD65) and T cell-related antigens (CD2 and CD7) for the identification of aberrant phenotypes would be restricted to a minority of cases, in accordance with previous publications.

In summary, our results show that in spite of the low frequency of tumor-specific proteins in precursor-B-ALL, abnormal patterns of antigen expression are found in virtually all cases. Our earlier report on the immunophenotypic patterns during normal B cell differentiation provides a frame of reference for the detection of phenotypic aberrancies in precursor-B-ALL. We also show that this approach can easily be standardized between laboratories.

The next question to be addressed concerns the sensitivity

levels of the flow cytometry immunophenotypic approach for the identification of MRD in precursor-B-ALL described herein and the lowest number of events to be acquired for the accurate measurement of the residual leukemic cells. Further follow-up studies, which are already in progress by our group, will hopefully provide relevant information regarding the clinical impact of this methodology in monitoring the response to treatment and in the assessment of MRD in patients who are in cytomorphologic complete remission.

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