Incidence, Sensitivity, and Specificity of Leukemia-Associated Phenotypes in Acute Myeloid Leukemia Using Specific Five-Color Multiparameter Flow Cytometry

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Abstract

We assessed the usefulness of 5-color multiparameter flow cytometry to detect leukemia-associated phenotypes (LAPs) in the bone marrow of patients with newly diagnosed acute myeloid leukemia (AML) and determined its usefulness for detection of minimal residual disease (MRD). Overall, 94% of patients (51/54) with AML had LAPs at diagnosis. The frequency of leukemic bone marrow/median frequency of LAPs in normal or regenerating bone marrow samples using maximum log difference statistics revealed that CD2, CD56, CD11b, CD7, and CD19 expression on AML blasts represented the most sensitive and reliable markers for detection of MRD. Serial dilutional experiments showed that the sensitivity level of immunophenotyping was between 10^{-4} and 10^{-5} and that the approach was highly reproducible.

Immunophenotypic analysis using a CD45 gating strategy, 5-color staining, and an extensive panel of monoclonal antibodies allowed the identification of LAPs in 94% of AML cases, and these immunophenotypes can be used for MRD monitoring with a sensitivity limit of 10^{-4} to 10^{-5}.

Immunophenotyping of hematologic malignancies is one of the most important clinical applications of flow cytometry. In recent years, its use has extended from clinical research to diagnostic laboratories. Despite the well-established usefulness of immunophenotyping for the diagnosis, classification, prognostic stratification, and monitoring of minimal residual disease (MRD) in acute lymphoblastic leukemia, limited data are available on its usefulness for MRD monitoring in acute myeloid leukemia (AML).

MRD is defined as the persistence of leukemic cells after chemotherapy that cannot be identified with routine morphologic evaluation. The leukemia-associated phenotypes (LAPs) are not present or are only very infrequently present on normal blood or bone marrow (BM) cells.[1-5] For AML, previously described aberrations include the following: (1) asynchronous antigen expression (simultaneous expression of early and late markers in 1 cell, such as the coexpression of CD34 and CD15 antigens); (2) lineage infidelity, which is expression of lymphoid-associated markers, ie, CD2, CD3, CD5, CD7, CD10, and CD19, on myeloid blast cells; (3) antigen overexpression, which is abnormally increased expression of a certain antigen per cell; (4) aberrant light-scatter properties, which involves the expression of lymphoid-associated antigens in blast cells displaying relatively high forward scatter (FSC) and side scatter (SSC), corresponding to normal myeloid cells[1,6,7]; and (5) absence of lineage-specific antigens, which involves absence of expected antigen expression, such as CD13 and CD33, on myeloid blasts.[8]

The existence of LAPs may be a valuable tool for the detection of MRD because neoplastic cells can thus be distinguished from normal hematopoietic progenitors on the basis of their aberrant antigen expression.[9-11] The presence of LAPs

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has been demonstrated in AML,[2,8,12,13] but their real incidence and the most sensitive and reliable markers for MRD have not been established owing to disease heterogeneity. In addition, preliminary reports suggest that high levels of sensitivity can be reached by using multiparameter flow cytometry (MFC) when appropriate double-step acquisition and multidimensional analytic procedures are combined.[14-16]

The aim of the present study was to explore the applicability and sensitivity of 5-color MFC immunophenotyping for the study of MRD in patients with AML. To achieve this we analyzed the sensitivity of MFC, assessed specificity by determining immunophenotypic profiles of LAPs of AML vs those of regenerating and normal BM samples, and determined the incidence of these LAPs in a series of 54 consecutive patients with newly diagnosed AML.

Materials and Methods

Samples

Fresh BM samples from 54 consecutive, unselected patients with AML were obtained at diagnosis between January 2005 and April 2007. The diagnoses were based on morphologic findings, immunophenotyping, and cytogenetics.[17] Patient characteristics are shown in **Table 1**. The patients were stratified for risk according to cytogenetic abnormalities.[18] Normal BM samples from healthy volunteers (n = 10) and regenerating BM samples (n = 4) from patients with nonmyeloid malignancies were used as control samples to determine the specificity of LAPs. Regenerating BM samples were obtained from 4 patients with lymphoproliferative disorders. Two samples were from patients recovering from standard chemotherapy and two samples were from patients who had received myeloablative chemotherapy and peripheral blood stem cell transplantation. All samples were collected on day 28.

Flow Cytometry

Immunophenotypic analysis was performed on erythrocyte-lysed whole BM samples with directly conjugated monoclonal antibodies (MoAbs) **Table 2**. Antigen expression was analyzed by using 5-color combinations of MoAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin–Texas red (ECD), phycoerythrin-cyanin (PC)-5, and PC-7 at diagnosis. Two MoAb panels were used in the study: screening and secondary panels. The screening panel is our standard diagnostic panel used to distinguish acute lymphoblastic leukemia from AML. Subsequently, in AML, the secondary panel was used to identify LAPs. Blasts were identified by using a CD45/SSC log gating strategy. Back-gating strategies using CD34 and CD117 were used to better define the blast population. CD45/CD34/CD117 were used in combination with different myeloid and lymphoid markers in a 5-color combination to increase the sensitivity of LAP detection. This was done to obtain a maximum advantage, incorporating various permutations and combinations, of 5-color MFC in defining the LAPs.

Table 1
Characteristics of 54 Patients With Newly Diagnosed AML by Using 5-Color Staining*

Characteristic	Total
No. of M/F	37/17
Mean (range) age at diagnosis (y)	58 (18-85)
Median (range) WBC count at diagnosis (× 10^9/L)	5.6 (0.26-179)
Median (range) hemoglobin level at diagnosis (g/L)	94 (52-143)
Median (range) platelet count at diagnosis (× 10^9/L)	61 (11-241)
Median (range) bone marrow blasts (%)	
By morphologic examination	42 (19-96)
By multiparametric flow cytometry	36 (7-86)
AML cases	
De novo	42 (78)
Secondary	12 (22)
French-American-British classification	
M0	0 (0)
M1	9 (17)
M2	11 (20)
M3	2 (4)
M4	9 (17)
M5	5 (9)
M6	1 (2)
M7	1 (2)
Not classified	16 (30)
Cytogenetic risk group	
Favorable	9 (17)
Intermediate	25 (46)
Poor	17 (31)
No metaphases	3 (6)
World Health Organization classification	
AML with recurrent genetic abnormalities	11 (20)
AML with multilineage dysplasia	10 (19)
AML and myelodysplastic syndromes, therapy-related	0 (0)
AML, not otherwise categorized	33 (61)

AML, acute myeloid leukemia.
* Data are given as number (percentage) unless otherwise indicated. WBC, hemoglobin, and platelet values are given as Système International units; conversions to conventional units are as follows: WBC count (/µL), divide by 0.001; hemoglobin (g/dL), divide by 10; and platelet count (× 10^3/µL), divide by 1.0.

The screening panel was as follows: CD7-FITC/CD13-PE/CD45-PC-5, CD19-FITC/CD10-PE/CD45-PC-5, CD34-FITC/CD33-PE/CD45-PC-5, CD14-FITC/CD56-PE/CD45-PC-5, HLA-DR-FITC/CD45-PC-5, CD15-FITC/CD117-PE/CD45-PC-5, myeloperoxidase/CD45-PC-5, terminal deoxynucleotidyl transferase (TdT)/CD45-PC-5, and CD45-PC-5/Simultest Control γ_1/γ_2 (a combination of FITC-IgG1 and PE-IgG2b) (Becton Dickinson, San Jose, CA). The secondary panel is shown in **Table 3**.

Appropriate isotype-matched negative controls were used in the panel of MoAbs to assess background fluorescence intensity. The respective combinations of antibodies were added to approximately 10^6 mononuclear cells and incubated

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Table 2
Monoclonal Antibodies Used for Immunostaining 54 Cases of Acute Myeloid Leukemia*

Antibody	Clone	Isotype	Conjugation	Source
CD2	SFCI3Pt2H9	IgG1 κ	FITC	Coulter
CD7	3A1E-12H7	IgG2b κ	FITC	Coulter
CD10	SS2/36	IgG1 κ	PE	DAKO
CD11b	Bear1	IgG1 κ	PE	Coulter
CD13	L138	IgG1 κ	PE	BD
CD14	MfP9	IgG2b κ	FITC	BD
CD15	MMA	IgM κ	FITC	BD
CD19	HD37	IgG1 κ	FITC	DAKO
CD33	P67.6	IgG1 κ	PE	BD
CD33	P67.6	IgG1 κ	FITC	BD
CD34	8G12	IgG1 κ	FITC	BD
CD34	581	IgG1	PC-5	ImmunoTech
CD38	T16	IgG1	FITC	Coulter
CD45	Immu19.2	IgG1 κ	PC-5	ImmunoTech
CD45	J33	IgG1	ECD	ImmunoTech
CD56	N901 (NKH-1)	IgG1 κ	PE	Coulter
CD64	22	IgG1	FITC	ImmunoTech
CD65	88H7	IgM	FITC	ImmunoTech
CD117	104D2D1	IgG1	PE	ImmunoTech
CD117	104D2D1	IgG1	PC-7	Coulter
CD123	9F5	IgG1 κ	PE	BD
CD235a (Glyco A)	JC159	IgG1 κ	FITC	DAKO
HLA-DR	L243	IgG2a	FITC	BioDesign
MPO	MPO-7	IgG1 κ	FITC	DAKO
TdT	HT-6	IgG1 κ	FITC	DAKO
Simultest γ_1/γ_{2a}†	—	IgG1 κ and IgG2b κ	FITC/PE	BD
Control ECD	679.1MC7	IgG1	ECD	ImmunoTech
Control PC-5	679.1Mc7	IgG1	PC-5	ImmunoTech
Control PC-7	679.1Mc7	IgG1	PC-7	Coulter

ECD, phycoerythrin–Texas red; FITC, fluorescein isothiocyanate; MPO, myeloperoxidase; PC, phycoerythrin-cyanin; PE, phycoerythrin; TdT, terminal deoxynucleotidyl transferase.
*BioDesign, Saco, ME; BD, San Jose, CA; Coulter, Fullerton, CA; DAKO, Glostrup, Denmark; ImmunoTech, Marseille, France.
†BD Simultest Control γ_1/γ_{2a} (IgG1-FITC/IgG2b-PE) is a 2-color direct immunofluorescent for use as a negative control.

for 10 minutes. RBC lysis was carried out using a Beckman Coulter Multi Q-Prep (semiautomated procedure; Beckman Coulter, Fullerton, CA).

Data Acquisition and Analysis

Data acquisition was performed on a Coulter FC500 flow cytometer. Analysis of list mode data was performed using CD45/SSC log by CXP Software (Coulter). Thresholds for positivity were based on isotype and internal negative control samples. The positivity threshold was 20% for all markers. Asynchronous antigen expression, lineage infidelities, and absence of lineage-specific antigens were studied. For all samples, at least 250,000 events were acquired.

Dilutional Experiments

Serial dilutional experiments were performed by adding increasingly lower numbers of LAP-positive cells obtained from selected samples from 8 patients with AML to normal BM samples obtained from 5 healthy subjects and from 3 subjects with regenerating BM to determine the level of sensitivity of MFC immunophenotyping for the detection of leukemic cells. For each experiment, the dilution factor ranged from $1{:}10^1$ to $1{:}10^5$ and in each tube, at least 250,000 events were collected.

Table 3
Secondary Panel for Studying 54 Cases of Acute Myeloid Leukemia

FITC	PE	ECD	PC-5	PC-7
Control FITC	Control PE	CD45	Control PC-5	Control PC-7
CD2	CD56	CD45	CD34	CD117
CD7	CD33	CD45	CD34	CD117
CD14	CD11b	CD45	CD34	CD117
CD15	CD33	CD45	CD34	CD117
CD65	CD33	CD45	CD34	CD117
CD33	CD13	CD45	CD34	CD117
CD64	CD33	CD45	CD34	CD117
CD19	CD10	CD45	CD34	CD117
CD38	CD123	CD45	CD34	CD117

ECD, phycoerythrin–Texas red; FITC, fluorescein isothiocyanate; PC, phycoerythrin-cyanin; PE, phycoerythrin.

The specificity of these analyses was ensured by back-gating on CD34 or CD117 and looking at the coexpression of different categories of LAPs with CD34 and/or CD117 and using the same gate for the subsequent dilutions and by establishing the normal ranges for normal and regenerating BM. Diluted specimens were considered positive only if the percentage of AML cells with the specific LAP exceeded the normal range.

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Quantification of Differences in Cells With Aberrant Immunophenotypes Between AML Samples and Normal and Regenerating BM Samples

The frequencies of cells within the normal and regenerating BM samples with the respective LAPs were determined for each individual LAP as defined in cases with AML. To estimate the degree of the reduction in leukemic cell mass that is detectable by the present approach for each individual LAP, the log difference "frequency in leukemic BM/median frequency of LAP in normal and regenerating BM" was calculated; for example, the log difference would have been 3.00 in a case in which LAP-positive cells were present in 50% of the leukemic BM and a median of 0.05% in normal BM. In case of a median frequency of 0.00% of LAP-positive cells in normal or regenerating BM, this frequency was set to 0.001% to allow the calculation of log difference (0.001% is the highest frequency displayed as 0.00% and was chosen as worst case possible). If more than 1 LAP was defined in 1 patient, the most sensitive LAP was selected for the respective evaluation for MRD as indicated on the basis of the maximum log difference in comparison with other LAPs in the same patient.

Study Approval

Before therapy, all patients gave informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study and potential risks. The study design was approved by the Royal Adelaide Hospital Research Ethics Committee before its initiation.

Statistical Analysis

The Student *t* test was used to determine any statistical difference between normal and regenerating BM. The statistical method proposed by Reed et al[19] was used to assess the precision in sensitivity experiments as measured by the coefficient of variation (CV). This analysis was performed by using SAS, version 9.1 (SAS Institute, Cary, NC). A *P* value of .05 or less was required for statistical significance.

Results

Clustering of BM Lineages Using CD45 Gating Strategy

First, the recommended routine use of CD45/SSC vs FSC/SSC as the preferred gating strategy for leukemia was validated. CD34 and CD117 back-gating was performed in every case when present. CD14 back-gating was also performed to exclude any monocytes, so unless falling within the dim CD45/SSC gate with coexpression of CD34 or CD117, these cells were excluded. In some cases, there were 2 heterogeneous blast populations; in those cases, each blast gate was defined separately, and, consequently, the LAPs were defined accordingly in each blast subpopulation.

In M4 and M5 cases (French-American-British classification) when the blast population formed a continuum to the monocytic population and a clear-cut population of blasts was not obvious, CD34 and/or CD117 back-gating was used. The gating strategy used to identify LAPs is shown in **Image 1**. At diagnosis, there was strong positive correlation between morphologic findings and MFC immunophenotyping in terms of the blast percentage ($r = 0.8$).

Identification of LAPs

The baseline clinical and laboratory characteristics of 54 patients with AML are given in Table 1. LAPs were observed in 51 cases (94%). By considering the data using the standard panel used at our institution with 3-color staining, LAPs were observed in only 43 cases (80%; $P < .001$).

Application of the extensive panel of MoAbs resulted in the identification of 200 LAPs in 51 LAP-positive cases. Only 1 aberrant immunophenotype was identified in 11 cases (22%), but in the other 40 cases (78%), 2 to 10 aberrant immunophenotypes were identified (2 LAPs, 7; 3 LAPs, 3; 4 LAPs, 12; 5 LAPs, 5; 6 LAPs, 6; 7 LAPs, 1; 8 LAPs, 3; 9 LAPs, 1; and 10 LAPs, 2). The most frequent LAP identified was asynchronous antigen expression of progenitor cell markers and differentiation markers (LAPs = 146), followed by lineage infidelity (LAPs = 40). The number of aberrant immunophenotypes identified by the application of the various combinations of antibodies is detailed in **Table 4**.

The percentage of AML cells carrying specific LAPs was assessed in each case and ranged from 10% to 100% (median, 52%). The distributions of these frequencies among the different classes of aberrant immunophenotypes and among the respective combinations of antibodies are shown in **Table 5**. Antigen overexpression and aberrant light scatter properties were shown to have insufficient reproducibility and, so, were not examined in this study.

The most common LAPs identified were CD117+/CD15+, CD117+/CD65+, CD34+/CD15+, and CD34+/CD65+ and these were present in 25 (49%), 22 (43%), 20 (39%), and 15 (29%) of 51 cases, respectively.

Lineage Infidelity

Lineage infidelity was detected 40 times. The most frequent lymphoid antigen detected was CD7 in 12 (24%) of 51 cases, followed by CD2 in 5 (10%) cases, CD19 in 2 (4%) cases, and CD10 in 1 (2%) case (Table 4).

Asynchronous Antigen Expression

Asynchronous antigen expression was detected 146 times. The most common asynchronous antigen expression and most common marker in our cohort was CD117+/CD15+ (25/51 [49%]), followed by CD117+/CD65+ (22/51 [43%]) (Table 4).

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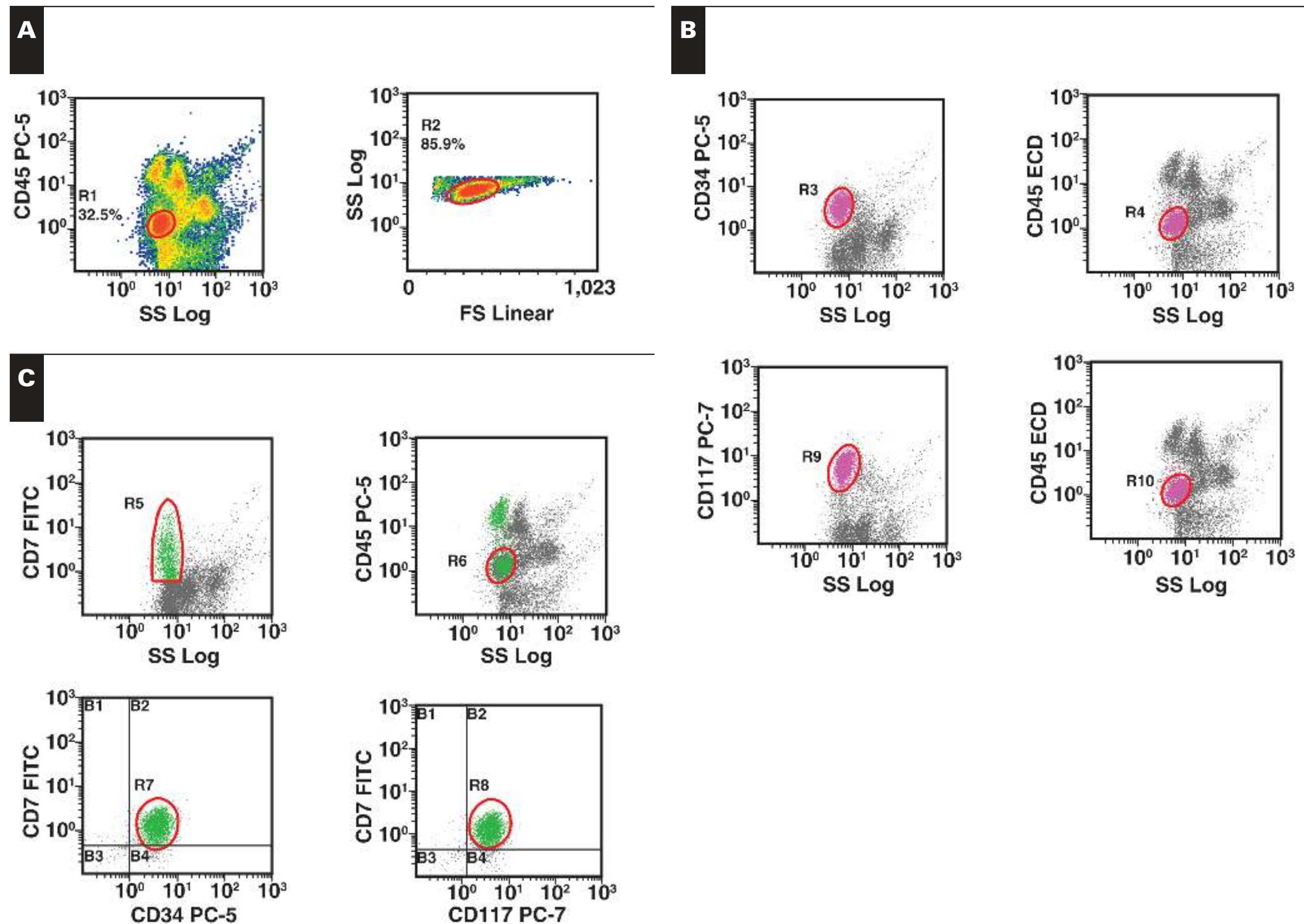

Image 1 Establishment of a leukemia-associated phenotype (LAP). **A**, Defining acute myeloid leukemia (AML) blasts in the WBC compartment. No gate: WBC compartment is gated based on CD45 expression and side scatter (SSC) (R1). Gated on R1: cell fragments and/or dead cells can be further excluded in forward scatter (FSC)/SSC plot (R2) and to ensure homogeneity of blast populations. **B**, Back-gating steps of cells showing CD34+/CD117+/CD45dim. Back-gating on CD34+ cells (R3). Part of the AML blasts show CD34 expression (R4). These CD34+ cells show dim CD45 expression. Back-gating on CD117+ cells (R9). Part of the AML blasts show CD117 expression (R10). These CD117+ cells show dim CD45 expression relative to lymphocytes (~10^0-10^1 log fluorescence). **C**, LAP expression on AML blasts. Back-gating on CD7+ cells (R5). Gated on R2, showing CD7+ cell position on CD45+ cells; some of these cells are T lymphocytes and some are in the blast population, gate R6, including only cells in the blast population. Gated on R6, showing cells coexpressing CD34+/CD7+. The CD34+ cells that show dim CD45 expression show CD7 expression, which defines the LAP (R7). Gated on R6, showing cells coexpressing CD117+/CD7+. The CD117+ cells that show dim CD45 expression show CD7 expression, which defines the LAP (R8). ECD, phycoerythrin–Texas red; FITC, fluorescein isothiocyanate; PC-5, phycoerythrin-cyanin-5.

Absence of Lineage-Specific Antigens

At least 1 myeloid marker was absent 14 times, with CD33 the most frequent marker absent (9/51 [18%]; Table 4).

Aberrant Marker Expression in Normal and Regenerating BM

The median percentage of LAP-positive cells in the normal and regenerating BM samples was calculated for each LAP. This median percentage ranged from 0.002% to 0.067% for normal BM and 0.0038% to 0.0864% for regenerating BM. Restricting these analyses to the most sensitive LAP in each case resulted in a range from 0.000% to 0.067% for the median percentage of LAP-positive cells in normal BM (median, 0.002%) and from 0.0005% to 0.0864% in regenerating BM (median, 0.00386%). The distributions of these frequencies among the different classes of LAP and among the respective combinations of antibodies in normal BM and regenerating BM are shown in Table 5. MFC analysis showed no differences in expression of immunophenotypic aberrancies in 10 normal and 5 regenerating BM samples ($P = .2$).

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❚Table 4❚
Frequencies of Leukemia-Associated Phenotypes in 51 Patients With Acute Myeloid Leukemia

	No. of Cases	Percentage of Positive Cells in Bone Marrow*
Lineage infidelity (n = 40)		
CD34+/CD2+	5	26-92
CD34+/CD7+	12	11-88
CD34+/CD10+	1	22
CD34+/CD19+	2	24-65
CD117+/CD2+	5	13-91
CD117+/CD7+	11	18-90
CD117+/CD10+	1	17
CD117+/CD19+	2	15-53
CD34+/CD235a+	0	0
CD117+/CD235a+	1	30
Asynchronous antigen expression (n = 146)		
CD34+/CD11b+	11	11-80
CD34+/CD14+	0	0
CD34+/CD15+	20	10-36
CD34+/CD56+	5	29-84
CD34+/CD64+	6	10-49
CD34+/CD65+	15	11-63
CD117+/CD11b+	10	12-62
CD117+/CD14+	0	0
CD117+/CD15+	25	10-55
CD117+/CD56+	6	11-80
CD117+/CD64+	9	11-70
CD117+/CD65+	22	10-62
CD33+/CD15+†	2	80-94
CD33+/CD64+†	5	18-97
CD33+/CD65+†	5	10-99
CD33+/CD11b+†	3	20-26
CD33+/CD56+†	2	10-78
Lack of lineage-specific antigen (n = 14)		
CD33++/CD13–	5	93-99
CD33–/CD13++	9	25-98

* Data are given as the range when >1 case was identified.
† These phenotypes were defined only when CD34 and/or CD117 were negative. They were defined within the blast gate defined by CD45/side scatter.

Quantification of Differences in Cells With Aberrant Immunophenotypes Between AML and Normal and Regenerating BM Samples

In normal BM samples, the median of the log difference between leukemic BM/median frequency of LAP in normal and regenerating BM samples was 3.92 (range, 2.22-4.96). In regenerating BM, the median of difference was 3.67 (range, 2.06-4.81; Table 5).

Restricting the analysis to the maximum log difference from the median frequency in normal and regenerating BMs **❚Figure 1❚** resulted in identifying the 5 most sensitive LAPs in our series: CD2, CD56, CD7, CD11b, and CD19 in normal BM and CD56, CD11b, CD2, CD7, and CD19 in regenerating BM, in order of sensitivity based on the maximum log difference, which means that these LAPs are the most sensitive markers for tracking MRD when present in AML samples.

Study of In Vitro Sensitivity of Flow Cytometric Immunophenotyping for Detection of MRD

Four LAPs in selected AML cases were used to assess sensitivity: CD34+/CD7+, CD117+/CD7+, CD34+/CD56+, and CD117+/CD56+. These AML samples with these LAPs were selected for 2 reasons: (1) These LAPs have very high sensitivity when compared with their expression level in normal and regenerating BM samples. (2) These LAPs represent 2 major groups of LAPs: lineage infidelity and asynchronous antigen expression. For each experiment, the dilutions covered a range from 50% to 0.001%.

Assessment of Linearity

As shown in **❚Table 6❚** and **❚Image 2❚**, the immunophenotypic approach allowed the detection of at least 1 aberrant leukemic cell among 10^4 to 10^6 normal hematopoietic cells. Accordingly, the in vitro sensitivity level of this approach would be at least 10^{-4}. Dilutions covering the range from 50% to 0.001% resulted in a coefficient of correlation of more than 0.9 (P = .002) in all 8 cases. Two cases are shown in **❚Figure 2❚** with CD34+/CD56+ and CD34+/CD7+.

Assessment of Reproducibility

To assess the precision and reproducibility of the sensitivity experiments, these experiments were repeated 5 times under the same conditions and dilutions using the same normal BM and AML samples on the same day to exclude the possibility of random errors and to increase the reliability of this approach. The results were calculated by measuring the CV.

We linked CV and $P(\kappa)$ (where κ can be any number greater than 1 and arbitrarily close to 1)[19] to assess whether the difference in 5 different measurements is due to random variation and to assess whether the variation in a set of replicates is larger than that implied by the assumed CV **❚Table 7❚**. The results revealed that sensitivity from level 0.01% to 0.001% dilutions is reproducible and precise using 4 LAPs mentioned (for studying in vitro sensitivity) for each LAP from 6 samples. As shown in **❚Table 8❚**, $P(\kappa)$ values in the 6 samples tested were higher than .05, indicating that there was no statistical difference in the 5 trials except as noted in samples 1, 2, and 5 at levels of 0.01%, 0.001%, and 0.001%. These experiments indicated that detection of 1 leukemic cell among 10^4 is achievable and, possibly, 10^5 is also feasible in most of the cases. Linearity was also established in all samples in the 5 replicate trials **❚Figure 3❚**.

Discussion

The monitoring of MRD may become increasingly important to guide therapy in patients with AML. Current methods used to quantify MRD are hampered by the lack of high sensitivity and applicability for each patient with AML.

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The ideal assay system for the detection of small numbers of leukemic cells that persist after chemotherapy in BM or blood samples should fulfill the following criteria: (1) applicable in most cases of the disease under study, (2) specific for neoplastic cell type, (3) sensitive, and (4) allows quantitation of tumor burden for prognostic purposes.[20]

The present study based on using 5-color flow cytometry and an extensive panel of MoAbs in AML cases showed that LAPs were present in the majority of cases (94%). Moreover,

Table 5
Frequencies of LAPs in 51 AML Samples, 10 Normal BM Samples, and 5 Regenerating BM Samples

LAP	No. of Cases With AML*	LAPs in AML (%)		Positive Cells in Normal BM (%)			Log Difference		Positive Cells in Regenerating BM (%)			Log Difference	
		Min	Max	Median	Min	Max	Min	Max	Median	Min	Max	Min	Max
CD34+/CD2+	5	26	92	0.001	0.000	0.030	4.41	4.96	0.004	0.000	0.030	3.81	4.36
CD34+/CD7+	12	11	88	0.002	0.000	0.009	3.74	4.64	0.004	0.000	0.005	3.49	4.39
CD34+/CD10+	1	22	22	0.012	0.000	0.185	3.26	3.26	0.035	0.002	0.072	2.79	2.79
CD34+/CD19+	2	24	65	0.002	0.000	0.007	4.08	4.51	0.003	0.000	0.015	3.96	4.39
CD117+/CD2+	5	13	91	0.003	0.000	0.010	3.64	4.48	0.003	0.000	0.019	3.70	4.54
CD117+/CD7+	11	18	90	0.003	0.000	0.033	3.78	4.48	0.006	0.001	0.017	3.48	4.18
CD117+/CD10+	1	17	17	0.001	0.000	0.014	4.23	4.23	0.003	0.001	0.050	3.75	3.75
CD117+/CD19+	2	15	53	0.002	0.000	0.011	3.88	4.42	0.004	0.000	0.004	3.63	4.18
CD34+/CD235+	0	0	0	0.000	0.000	0.006	NC	NC	0.053	0.002	0.237	NC	NC
CD117+/CD235+	1	30	30	0.001	0.000	0.008	4.48	4.48	0.015	0.003	0.443	3.31	3.31
CD34+/CD11b+	11	11	80	0.002	0.000	0.022	3.74	4.60	0.002	0.001	0.095	3.74	4.60
CD34+/CD14+	0	0	0	0.000	0.000	0.001	NC	NC	0.002	0.001	0.005	NC	NC
CD34+/CD15+†	20	10	36	0.004	0.000	0.024	3.40	3.95	0.012	0.002	0.014	2.92	3.48
CD34+/CD56+	5	29	84	0.001	0.000	0.039	4.46	4.92	0.001	0.000	0.024	4.35	4.81
CD34+/CD64+	6	10	49	0.051	0.000	0.160	2.29	2.98	0.086	0.000	0.105	2.06	2.75
CD34+/CD65+	15	11	63	0.013	0.005	0.047	2.93	3.69	0.057	0.003	0.106	2.29	3.05
CD117+/CD11b+	10	12	62	0.002	0.000	0.048	3.78	4.49	0.001	0.000	0.026	4.08	4.79
CD117+/CD14+	0	0	0	0.000	0.000	0.010	NC	NC	0.001	0.000	0.011	NC	NC
CD117+/CD15+	25	10	55	0.010	0.000	0.039	3.00	3.74	0.017	0.005	0.093	2.76	3.50
CD117+/CD56+	6	11	80	0.003	0.000	0.037	3.56	4.43	0.002	0.000	0.025	3.74	4.60
CD117+/CD64+	9	11	70	0.067	0.001	0.127	2.22	3.02	0.040	0.002	0.247	2.44	3.24
CD117+/CD65+	22	10	62	0.007	0.001	0.042	3.15	3.95	0.028	0.023	0.266	2.55	3.34
Median		11	62	0.002	0.000	0.027	3.74	4.43	0.004	0.001	0.028	3.48	4.18
Minimum		0	0	0	0.000	0.001	2.22	2.98	0.001	0.000	0.004	2.06	2.75
Maximum		30	92	0.067	0.005	0.185	4.48	4.96	0.086	0.023	0.443	4.35	4.81

AML, acute myeloid leukemia; BM, bone marrow; LAP, leukemia-associated phenotype; Max, maximum; Min, minimum; NC, no cases found for the defined aberrant immunophenotype.
* Some cases have more than 1 LAP.
† CD34+/CD15+/CD33^{dim} was defined.

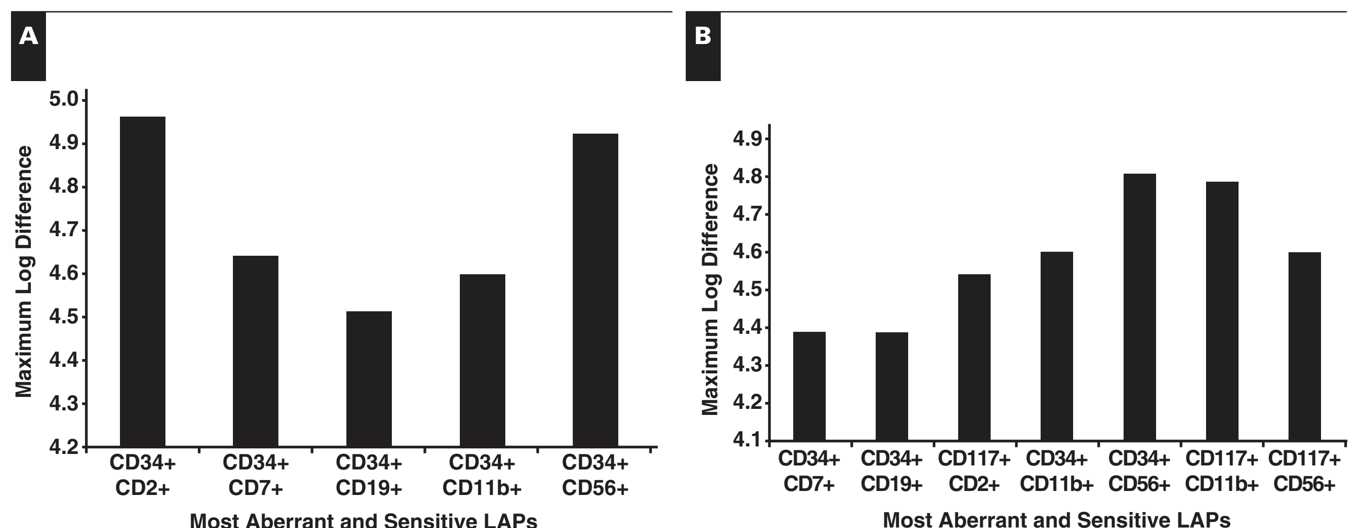

Figure 1 The most aberrant and most sensitive phenotypes based on maximum log difference in percentage of positive cells of leukemia-associated phenotypes (LAPs) in acute myeloid leukemia samples from normal bone marrow (**A**) and regenerating bone marrow (**B**).

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in 78% of cases, leukemic cells simultaneously displayed more than 1 LAP. This was important when immunophenotypic shifts occurred during treatment.[21-23] The phenomenon of immunophenotypic switch is a relatively rare but significant problem in using LAPs as a strategy for detection of MRD.[21,22] The analysis of LAPs by 5-color MFC should help address this problem because we detected more than 1 LAP in 78% of cases, which increases the probability of detection for MRD analysis. However, if we were to include a broader definition of LAP incorporating abnormal intensity of antigen expression, we would probably extend the percentage of LAP detection from 94% to 100%.

The literature demonstrates wide variability of aberrant phenotype expression in AML. Adriaansen et al[24] identified subsets of myeloblasts that expressed TdT in 75% of 45 AML cases; however, in most of these cases, TdT was positive in fewer than 20% of blasts. Reading et al[3] detected LAPs in 85% of 272 cases of AML; Macedo et al[1] found that 29 (73%) of 40 AML cases analyzed displayed the existence of at least 1 aberrant phenotype; San Miguel et al[7] reported that 46 (87%) of 53 AML cases had an aberrant phenotype; this figure was confirmed by the same authors in another report on 126 AML cases.[11] In the experience of Venditti et al,[25,26] 70% of 113 newly diagnosed AML cases had an aberrant phenotype. In addition, incidence as high as 88% has been also reported,[27] possibly because of the use of a large variety of MoAbs. The lowest incidence of aberrant immunophenotypes was reported by Drach et al,[28] who observed LAPs in 35 (51%) of 68 AML cases. The use of double but not triple-quadruple or 5-color staining assays and a limited panel of antibodies are possible explanations for this lower frequency. Altogether, these studies demonstrate different levels of LAP detection by MFC but point to potential for using LAPs as a feasible approach for

Table 6
Sensitivity Level of Multiparametric Flow Cytometric Immunophenotyping Based on Dilutional Experiments of Leukemic Cells in Normal and Regenerating Bone Marrow

Case No.	LAP	% of LAPs*	Sensitivity Level
Normal bone marrow			
1	CD34+/CD7+	0.0700	10^{-4}
	CD117+/CD7+	0.0600	10^{-4}
2	CD34+/CD56+	0.0007	10^{-6}
	CD117+/CD56+	0.0005	10^{-6}
3	CD34+/CD7+	0.0060	10^{-5}
	CD117+/CD7+	0.0060	10^{-5}
3	CD34+/CD56+	0.0304	10^{-4}
	CD117+/CD56+	0.0199	10^{-4}
4	CD34+/CD7+	0.0032	10^{-5}
	CD117+/CD7+	0.0022	10^{-5}
5	CD34+/CD7+	0.0012	10^{-5}
	CD117+/CD7+	0.0025	10^{-5}
5	CD34+/CD56+	0.0360	10^{-4}
	CD117+/CD56+	0.0540	10^{-4}
Regenerating bone marrow			
6	CD34+/CD56+	0.0176	10^{-4}
	CD117+/CD56+	0.0040	10^{-5}
7	CD34+/CD7+	0.0016	10^{-5}
	CD117+/CD7+	0.0028	10^{-5}
8	CD34+/CD7+	0.0148	10^{-4}
	CD117+/CD7+	0.0020	10^{-5}

LAP, leukemia-associated phenotype.
* Results expressed as the percentage of LAPs detected in the tube corresponding to the 1:100,000 dilutional experiments.

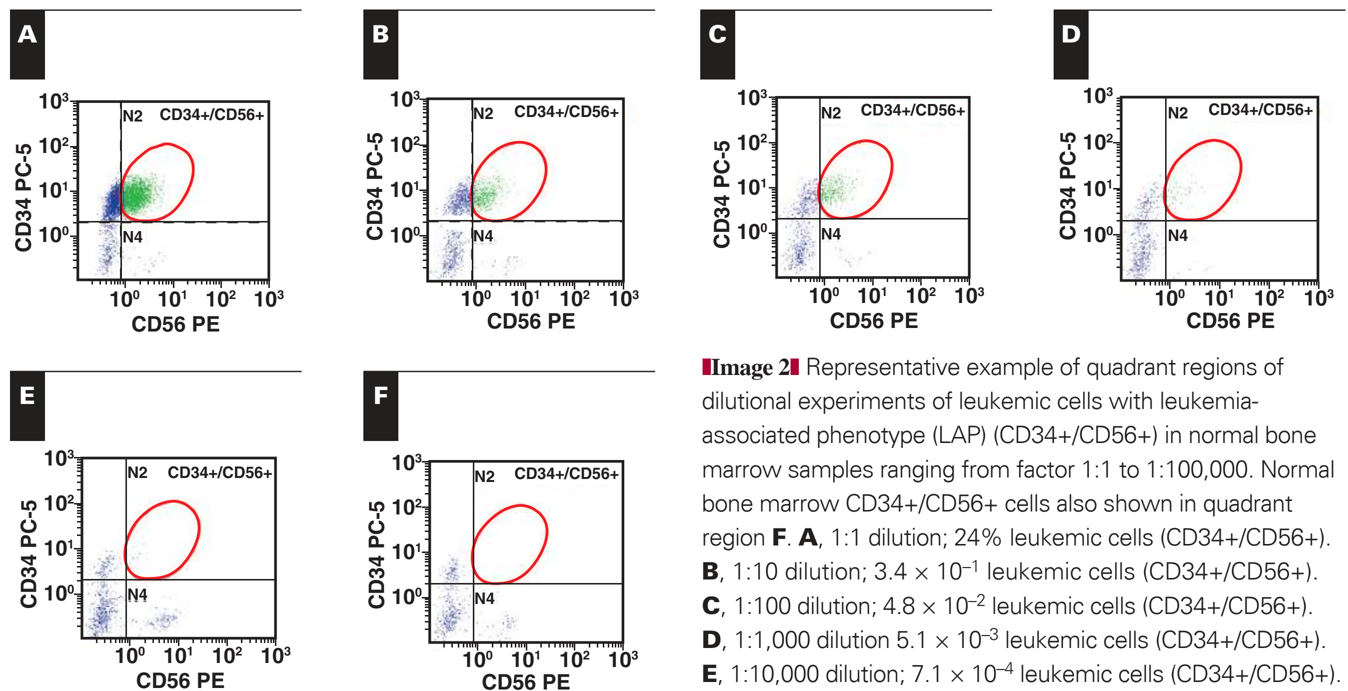

Image 2 Representative example of quadrant regions of dilutional experiments of leukemic cells with leukemia-associated phenotype (LAP) (CD34+/CD56+) in normal bone marrow samples ranging from factor 1:1 to 1:100,000. Normal bone marrow CD34+/CD56+ cells also shown in quadrant region **F**. **A**, 1:1 dilution; 24% leukemic cells (CD34+/CD56+). **B**, 1:10 dilution; 3.4×10^{-1} leukemic cells (CD34+/CD56+). **C**, 1:100 dilution; 4.8×10^{-2} leukemic cells (CD34+/CD56+). **D**, 1:1,000 dilution 5.1×10^{-3} leukemic cells (CD34+/CD56+). **E**, 1:10,000 dilution; 7.1×10^{-4} leukemic cells (CD34+/CD56+). **F**, No dilution; normal bone marrow, 3.8×10^{-5} (CD34+/CD56+ cells). PC-5, phycoerythrin-cyanin-5; PE, phycoerythrin.

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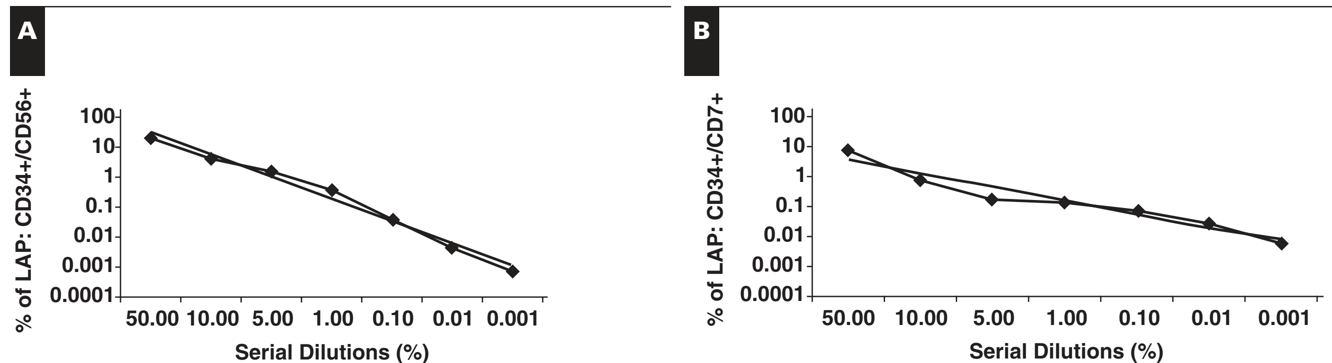

Figure 2 Assessment of linearity in sensitivity experiments. Two dilutional experiments are shown. **A**, Dilution from 50% to 0.001% of an acute myeloid leukemia (AML) sample with CD34+/CD56+. **B**, Dilution from 50% to 0.001% of an AML sample with CD34+/CD7+. The cases resulted in coefficients of correlation of 0.99 (**A**) and 0.97 (**B**). LAP, leukemia-associated phenotype.

Table 7
Reproducibility and Precision in Sensitivity Experiments*

	1.00%	0.10%	0.01%	0.001%	BM Only
Trial					
1	0.19	0.08	0.02	0.020	0.004
2	0.20	0.04	0.03	0.028	0.003
3	0.18	0.03	0.02	0.022	0.003
4	0.22	0.03	0.01	0.009	0.005
5	0.20	0.04	0.02	0.001	0.006
Lowest value	0.18	0.03	0.01	0.009	0.003
Highest value	0.22	0.08	0.03	0.028	0.006
Difference	0.05	0.05	0.02	0.018	0.003
% Increase	0.27	1.67	1.53	2.000	1.000
κ	1.27	2.67	2.53	3.000	2.000
CV	0.20	0.05	0.02	0.020	0.004
P(κ)	.95	.69	.62	.52	.210

BM, bone marrow; CV, coefficient of variation.
* An example of CD117+/CD56+ showing the results of dilutions from the 5 trials covering the range from 1.00% to 0.001%. Results from BM samples without dilution are also shown. The highest and lowest values within each dilution were tabulated, the difference was determined, the percentage difference was calculated, and the probability *P*(κ) was determined; κ is 1 + the percentage increase between the 2 values. Assuming that the actual CV is the mean of the 5 trials allowed us to calculate the *P*(κ), which is the probability of obtaining a difference in CVs just by random error. A *P*(κ) >.05 indicates random error, and a value <.05 is an indication that the difference is significant. It is clear from the *P*(κ) values, all are >.05, that nothing else is happening in the data other than random error.

detection of MRD. Some of the limitations in these studies can be overcome by the use of 5-color assays, an extensive panel of MoAbs, and a stringent assessment of blasts, as demonstrated by our data.

We confirm earlier reports suggesting that the blast cell population can be clearly identified by virtue of its low membrane expression of the CD45 antigen and its light scatter properties.[16,29-31] In addition, specific 5-color combinations of MoAbs would identify aberrant phenotypes in the majority of cases of AML, which can then be used for monitoring MRD. By using 5-color MFC, more accurate population identification can be performed with greater informational content. We also can make better use of small specimens with fewer cells and gain more information. In addition, by using MFC, we can process fewer tubes and, thus, save on reagents and instrument time. Moreover, we can collect very large numbers efficiently. Thus, MFC offers advantages in efficiency, informational content, and standardization.

Furthermore, our data show that CD2, CD56, CD7, CD19, and CD11b are the most useful markers for detection of MRD by determining their maximum log difference based

Table 8
Summary of Reproducibility for Sensitivity Experiments*

		P(κ)								
Sample No.	LAP	LAP (No Dilution)	50.000%	10.000%	5.000%	1.000%	0.100%	0.010%	0.001%	BM Only
1	CD34+/CD56+	.991	.983	.969	.971	.974	.800	.001†	.000†	ND
2	CD117+/CD56+	.991	.985	.966	.894	.977	.845	.243	.027†	ND
3	CD34+/CD56+	ND	ND	ND	ND	.893	.732	.620	.449	.033
4	CD117+/CD56+	ND	ND	ND	ND	.945	.688	.619	.515	.205
5	CD34+/CD7+	ND	ND	ND	ND	.945	.717	.740	.018†	.000
6	CD117+/CD7+	ND	ND	ND	ND	.945	.695	.719	.059	.001

BM, bone marrow; LAP, leukemia-associated phenotype; ND, not done.
* *P* values from 4 LAPs using 6 samples in 5 trials per sample. The Table demonstrates that in all samples, 1 leukemic cell among 10^4 is achievable except for sample 1. In 3 samples, up to 1 in 10^5 was also feasible.
† Likely the difference in the 5 trials was significant and not due to random error.

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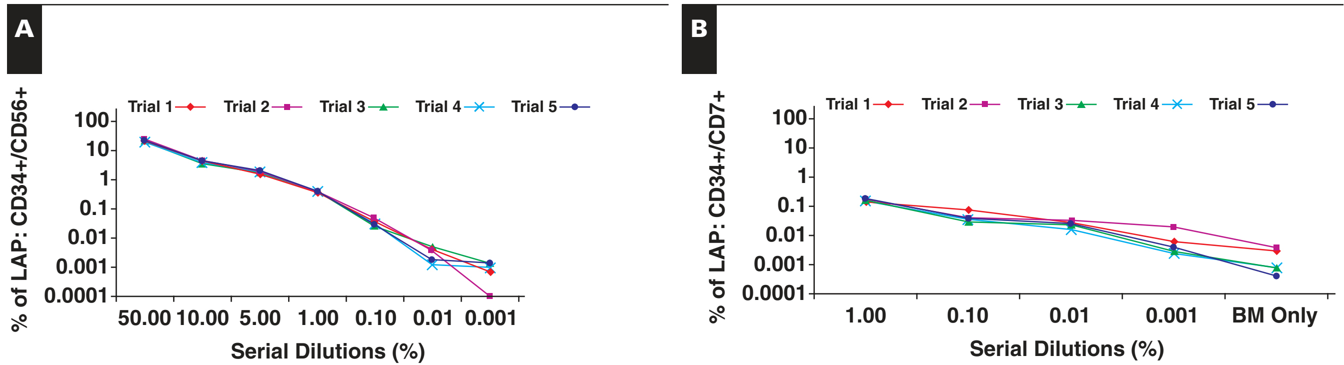

Figure 3 Assessment of reproducibility in sensitivity experiments. Two dilutional experiments are shown. **A**, Five trials of dilution from 50% to 0.001% of an acute myeloid leukemia (AML) sample with CD34+/CD56+. **B**, Five trials of dilution from 1.00% to 0.001% of an AML sample with CD34+/CD7+. Results for bone marrow only are also shown. LAP, leukemia-associated phenotype.

on the baseline of normal and regenerating BM samples. Of 54 cases, 24 (44%) expressed at least 1 of these markers, and, thus, these markers should be included in any diagnostic panel of AML for MRD monitoring.

Previous studies on the prognostic impact of MRD level in patients with AML in complete remission mainly focused on only a proportion of cases of AML expressing the highly aberrant immunophenotypes.[11,25,32,33] In our study, LAPs were able to be detected in almost every patient with AML when the comprehensive panel of MoAbs was applied. The introduction of 4-color flow cytometry and CD45 gating has been shown to improve detection of leukemic cells among normal BM cells with a log increase of sensitivity for MRD quantification over 3-color flow cytometry.[34] In our study, we demonstrated that the detection of LAPs by 5-color flow cytometry with CD45 gating can be further improved by determining the maximum log difference between LAP at diagnosis compared with normal and regenerating BM samples. The maximum log differences were found in the groups of LAPs with lineage infidelity, ie, CD2, CD7, and CD19, and asynchronous antigen expression, ie, CD11b and CD56.

The percentage of LAP-positive cells in AML samples was the same for 5- and 3-color flow cytometry. However, the frequency of LAP-positive cells in normal and regenerating BM samples was reduced by applying 5-color staining flow cytometry. This finding is most important with respect to posttherapy evaluations. The sensitivity of MFC increases in parallel to the number of aberrantly expressed antigens.

Thus, the high incidence of aberrant phenotypes detected in the present study and the marker combinations defined for their identification would represent ideal probes for the detection of MRD. However, an additional prerequisite of MRD techniques is to be sensitive enough to detect a low level of residual leukemic cells. Dilutional experiments are rarely performed in these types of studies, and important aspects of quantitative assay studies are precision and reproducibility. Our results show that, based on a 2-step acquisition procedure in which specific selection of LAPs is used in the latter step, MFC immunophenotyping is a highly sensitive technique for the detection of minimal numbers of myeloid blasts displaying aberrant phenotypes. In all experiments, a minimum sensitivity level of 10^{-4} (1 leukemic cell among 10^4 normal cells) was reached, independent of the type of aberration explored. Moreover, it should be noted that in a significant number of cases, an even higher sensitivity level (10^{-5}) was reached by using this approach. In addition, this approach is highly reproducible.

Based on the applicability and sensitivity of the MFC immunophenotyping approach shown in the present study, it could be concluded that this is a well-suited approach for the specific detection of minimal numbers of leukemic cells and, hence, could help to obtain a more precise and early evaluation of the effectiveness of new treatment strategies and to better assess complete remission status in patients with AML.[29,35-37]

Future longitudinal, prospective studies with larger samples of patients with AML to determine MRD frequency are necessary. Because of the clinical usefulness of comparing the results obtained from immunophenotypic characterization of healthy and leukemic cells, a major challenge for the near future is the possibility of performing stable, calibrated, and standardized measurements in such a way that identical cells provide identical phenotypic patterns whenever they are analyzed at different times and in different laboratories.

We showed the high applicability and sensitivity of MFC for the detection of leukemic cells in patients with AML. For this purpose, unique 5-color staining combinations of MoAbs are specifically proposed.

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