Flowcytometric Immunophenotypic Profile of Acute Leukemia: Mansoura Experience

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Abstract Acute leukemia (AL) displays characteristic patterns of antigen expression, which facilitate their identification and proper classification. The purpose of this study is to evaluate the diagnostic usefulness of commonly used immune-markers for immunophenotyping of AL and to define the best immune-markers to be used for proper diagnosis and classification of AL. Besides, to recognize the frequency of different AL subtypes and the antigen expression profile in our Egyptian patients. We retrospectively analyzed the immunophenotypic data of 164 de novo AL patients from our institution during 2009 and 2010. Among these patients, 68.9% were classified as acute myeloblastic leukemia (AML) while 31.1% classified as acute lymphoblastic leukemia (ALL). The commonest FAB subtype in AML group was AML-M4/5 (34.5%) which may differ from most published data. As regard ALL, there were 74.5% with B-ALL and 25.5% with T-ALL. It was found that combined use of HLADR and CD34 was much more helpful in distinguishing APL from non-APL AML than either of these antigens alone. It was found that cCD79a and CD19 were the most sensitive marker for B-ALL while cCD3, CD7 and CD5 were the most sensitive antigens for T-ALL. Our analysis of AL phenotypes proved that employed antibody panels are adequate for proper diagnosis and classification of AL. Flowcytometry was found to be especially useful in the identification of AML-M0 and differentiation of APL from non-APL AML. Immunophenotyping results and FAB classification of our AL patients were comparable to

internationally published studies apart from predominance of AML-M4/5 and more frequent APL.

Keywords Acute leukemia · Immunophenotyping · Flowcytometry

Introduction

Acute leukemia (AL) classification has undergone a paradigm shift with the World Health Organization (WHO) classification [1]. Acute leukemia are a heterogeneous group of malignancies with varying clinical, morphologic, immunologic, and molecular characteristics. Many distinct types are known to carry predictable prognoses and warrant specific therapy. Distinction between lymphoid and myeloid leukemia, most often made by flowcytometry, is crucially important. Acute leukemias reflect the pattern of antigen acquisition seen in normal hematopoietic differentiation, yet invariably demonstrate distinct aberrant immunophenotypic features. Detailed understanding of these phenotypic patterns of differentiation, particularly in myeloid leukemia, allows for more precise classification of leukemia than does morphology alone [2]. Multiparameter flowcytometry is a useful adjunct to morphology and cytochemistry and it is an invaluable tool in the diagnosis of AL [3]. Flowcytometry of leukemic cells plays essential role in identification of leukemia cell line, maturation stage and detection of residual disease. Several advances in flowcytometry, including availability of an expanded range of antibodies and fluorochromes, improved gating strategies, and multiparameter analytic techniques, have all dramatically improved our ability to identify different normal cell populations and recognized phenotypic aberrancies, even when present in a small proportion of the cells analyzed [4].

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Materials and methods

A total of 164 AL cases immunophenotyped at flowcytometry laboratory, Clinical Pathology Department, Faculty of Medicine, Mansoura University using 4 color flowcytometer were reviewed retrospectively. These samples were referred from OCMU during 2-year period (2009–2010). There were 56 children (34.2%) and 108 adults (65.8%) with a male to female ratio of 1.9:1. The analyzed samples were either of peripheral blood (PB) or bone marrow aspirate (BMA) according to availability and presence of blast cells in considerable percentage. Acute myeloid leukemia cases included in this study were classified according to French-American-British Cooperative Group (FAB) criteria at the time of initial review while ALL cases were classified immunologically according to the available immune markers. This study did not include relapsed or recurrent cases of acute leukemia. The clinical and laboratory findings are summarized in Table 1.

Morphological examination

For morphologic examination, all BMA/PB smears were air dried and subsequently stained with Leishman's stain to be examined microscopically.

Enzyme cytochemical analysis

All BMA/PB smears were stained using myeloperoxidase (MPO), periodic acid schiff (PAS) and alpha naphthyl acetate esterase (ANAE) cytochemical stains.

Flowcytometric analysis

Bone marrow aspirates or PB samples were collected on EDTA tubes and immediately transported to the flowcytometry laboratory. For surface antigen staining, the received samples were lysed using home made lysing solution (8 g Ammonium chloride, 1 g EDTA, and 0.1 g dihydrogen potassium phosphate in 1 l—10×—), washed with phosphate buffered saline (PBS) until complete RBCs lysis and resuspended in appropriate amount of PBS. The cells were stained with different fluorescently labeled monoclonal antibodies (mAbs) according to manufacturer recommendations (Dakocytomation, Denmark, and Beckman Coulter, France). One hundred microliters of cell suspension were mixed with 10 µl of the fluorescently labeled mAb and incubated in the dark at room temperature (RT) for 30 min. Washing with PBS containing 2% bovine serum albumin was done twice and the pellet was resuspended in PBS and analyzed immediately on flowcytometer. For detection of cytoplasmic and nuclear antigens, IntraPrep Permealization Kit was used (Beckman Coulter, France). Fifty microliters of EDTA PB/BMA sample were mixed with 100 µl of IntraPrep reagent 1 (fixative), incubated for 15 min at RT protected from light, and washed with PBS. 100 µl of IntraPrep reagent 2 (permealization) were mixed with the cells and incubated for 5 min at RT without vortexing nor shaking. The tube was shook carefully and manually for 2–3 s and then 10–20 µl of the mAb were added, vortexed, and incubated for 20 min in case of cytoplasmic antigens and for 1 h in case of nuclear

Table 1 Clinical and laboratory characteristics of AL patient group

		No.	%	No.	%
Age	Median (range)	37 (3–54)		6 (2–12)	
Sex	M/F	1.7		2.4	
Fever		47	41	43	84
Pallor		71	63	39	76
Hepatomegaly		49	43	36	70
Splenomegaly		38	34	29	57
Lymphadenopathy		22	19	13	25
Hb	Median (range)	8.4 (4.9–12.3)		7.2 (4.5–11)	
WBCs count ($\times 10^3$)	Median (range)	13.2 (1.9–178)		16.7 (0.7–47)	
Platelet count ($\times 10^3$)	Median (range)	62 (7.6–233)		43 (9.8–215)	
FAB distribution					
M0		16	14.2	–	–
M1–2		27	23.9	–	–
M3		26	23	–	–
M4–5		39	34.5	–	–
M6		2	1.8	–	–
M7		3	2.6	–	–
B-ALL		–	–	38	74.5
T-ALL		–	–	13	25.5

antigens at RT protected from light. Then, the mixture was washed and resuspended in PBS and analyzed on the flowcytometer immediately. The mAbs were used in different combinations of fluorochromes; namely fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanine5 (PeCY5). Different combination of mAb against the following antigens were used: cCD3, cCD79a, MPO, CD34, CD3, CD4, CD5, CD7, CD8, TdT, CD10, CD19, CD20, CD13, CD33, CD14, CD36, HLADR, glycophorin A, CD41, CD61 and CD117. The immunophenotyping was performed on EPICS-XL flowcytometer (Coulter, Miami, FL). The cells were analyzed with the most appropriate blast gate using the combination of forward and side scatters. An antigen was considered positive when the expression is at least 20% of the gated cells.

Results

Out of 164 cases of AL diagnosed in our laboratory over two-year period, there were 113 cases (68.9%) of AML and 51 cases (31.1%) of ALL. Acute promyelocytic leukemia (APL) accounted for 23%, while non-APL AML accounted for 77% of all AML cases. The commonest FAB subtype in AML group in our series was AML-M4/5 (34.5%) followed by AML1/2 (23.9%). AML-M0 accounted only for 14.2% of all AML cases, while AML-M6 and AML-M7 represented 1.8% and 2.6% respectively. As regard ALL, there were 38 cases (74.5%) with B-ALL and 13 cases (25.5%) with T-ALL (Fig. 1).

Flowcytometric pattern of antigen expression for determination of maturation stage as CD34 and HLA-DR were shown in Table 2 and Fig. 2. Expression of HLADR was seen in 78/87 (89.6%) of patients with non-APL AML, most of them were M4/M5 and in only 1/26 (3.8%) of patients with APL. On the other hand, expression of CD34 was seen in 54/87 (62.1%) of non-APL AML cases most of them were M1/M2 and in 4/26 (15.4%) of APL cases. The combined use of HLA-DR and CD34 was much more helpful in distinguishing cases of non-APL AML from APL cases, as none of 26 cases of APL were positive for

both CD34 and HLA-DR in contrast to 59.8% of non-APL AML cases that were positive for both markers.. On the other hand, negativity of both antigens was seen in only 6.9% of non-APL AML cases and in 80.8% of APL cases. So, negativity of both antigens does not always suggests the diagnosis of APL. As regard ALL cases, B-ALL showed HLADR and CD34 expression in 37/38 (97.4%) and 29/38 (76.3%) of cases respectively. However, none of T-ALL cases were positive for HLADR or CD34.

As regard the expression pattern of lineage demarcation markers (MPO, cCD79a and cCD3), MPO was expressed in all APL cases and expressed with some variation in non-APL AML cases. The highest percentage of MPO positivity in non-APL AML was seen in AMLM1/M2 (96.3%), followed by AMLM4/M5 (53.8%), then AMLM0 (18.8%). MPO was not seen in any of our M6 or M7 cases. As regard ALL, there was no detection of MPO in either B- or T-ALL cases. On the other hand, both cCD79a and cCD3a were negative in all AML cases in contrast to their positivity in all B-ALL and T-ALL cases respectively (Table 3).

The leukemic cells in all cases of M0 through M7 commonly express various combinations of CD34, HLA-DR, CD13, CD33 and CD117 (Tables 4; Figs. 3, 4, 5). CD33 was the myeloid marker that most commonly present in all AML subtypes, its percent was 89.4%. CD13 was the next most commonly expressed antigen showing 77.9% positivity in all AML categories, while expression of CD117 was seen in 74.3% of AML. CD14 and CD36 positivity were more commonly associated with the monocytic leukemias (64.1 and 61.5%, respectively). CD14 expression was seen with the highest percentage in M4 subtype (79.2%) but also detected in AML-M0 and AML-M1/M2 with much lower percentages (6.3 and 7.4%, respectively). CD36 expression was highest in M5 subtype (73.3%). The expression of megakaryocyte-associated Antigens, CD41 and CD61, although seen in all tested cases of AML-M7, were also seen in some cases of AML-M0, M1/2 and M4/5. Regarding glycophorin A, its expression was restricted to AML-M6 cases.

In our study, aberrant expression of lymphoid antigens in AML were seen in 11.1% of cases. CD7 was the most commonly expressed lymphoid antigen (23%) and CD19 expression was the least often seen (1.8%). Cases of MPO-positive AML that expressed lymphoid-associated (CD3, CD4, CD5, CD7, CD8, CD19 and TdT) but not lymphoid-specific (cyCD3, cyCD79a) antigens were best considered as lymphoid antigen-positive AML.

All cases of B-ALL expressed cCD79a and CD19 while CD20, CD10 and TdT were expressed in 26.3, 89.5 and 97.4%, respectively. Few cases of B-ALL expressed CD33 (10.5%) and CD13 (7.9%) without the expression of MPO or CD117 and they were classified as B-ALL with myeloid antigen aberrant expression. None of B-ALL cases

Fig. 1 FAB distribution of AL patients group

Table 2 HLADR and CD34 expression in Acute leukemia

CD	Non-APL AML (n = 87)	APL (n = 26)	ALL-B (n = 38)	T-ALL (n = 13)
+HLADR	78 (89.6%)	1 (3.8%)	37 (97.4%)	0 (0%)
CD 34+	54 (62.1%)	4 (15.4%)	29 (76.3%)	0 (0%)
CD34+/HLADR+	52 (59.8%)	0 (0%)	35 (92.1%)	0 (0%)
CD 34–/HLADR–	6 (6.9%)	21 (80.8%)	4 (10.5%)	13 (100%)

Fig. 2 Example of HLADR⁺/CD34⁺ expression in AML (this phenotype is not found in any APL case) **Table 3** Lineage demarcation markers expression in acute leukemia

CD	M0 (n = 16)	M1/2 (n = 27)	M3 (n = 26)	M4/5 (n = 39)	M6 (n = 2)	M7 (n = 3)	B-ALL (n = 38)	T-ALL (n = 13)
cCD3 ⁺	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	13/13 (100%)
cCD79a ⁺	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	38/38 (100%)	0 (0%)
MPO ⁺	3 (18.8%)	26 (96.3%)	26 (100%)	21 (53.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

expressed T-cell associated antigens (CD3, CD4, CD5, CD7, CD8).

All cases of T-ALL were positive for cCD3 while surface CD3 was positive only in 38.5%. Sensitivity of CD3 might not sound too impressive as it is primarily present in the cytoplasm. Most cases of T-ALL showed expression of CD7 (92.3%) and CD5 (84.6%). As regard TdT, CD4 and CD8 they were expressed in 76.9, 30.8 and 53.8%, respectively. Only 1 of 13 cases of T-ALL showed CD33 expression and none expressed CD13. It was observed that 23.1% of T-ALL cases showed CD10 expression, but none expressed CD19 or CD20. None of the B- or T-ALL cases showed expression of CD117.

Discussion

Flowcytometric immunophenotyping has become an important and sensitive diagnostic tool in establishing the diagnosis and classification of AL. It is also useful in the

early detection of minimal residual disease. Therefore, it has great diagnostic, prognostic and therapeutic implications. Its ability to measure multiple parameters on individual cells in a suspension at high speed is ideal for the study of leukemic cells.

Leukemia subtypes

In our AL cases, 68.9% were classified as AML while 31.1% classified as ALL. The high percentage of AML may be due to the large number of adults involved in our study (65.8%). The FAB distribution of AML has been extensively studied by many researchers all over the world [4–11]. In our study, APL accounted for 23%, while non-APL AML accounted for 77% of all AML cases. Most of the previous studies reported lower APL percentages ranging from 5 to 14% of all AML cases [7–11] while fewer investigators stated nearly similar percentage to our results (24%) [4, 5]. Rego et al. [6] found completely different percentages of APL in two different cities within

Fig. 3 Column graph representing the expression of CD34, HLADR in AL patients. Notice non of AML-M3 express both antigens simultaneously

Fig. 4 Column graph representing the expression of myeloid antigens in AL patients

Fig. 5 Column graph representing the expression of lymphoid antigens in AL patients

Brazil (7.8 and 21% of AML). Most published data indicated the predominance of M1-2 as the most common AML subtypes [5, 9–11]. In the current study, the commonest FAB AML subtype was AML-M4/5 (34.5%) and this was in concordance with other published studies who reported marked predominance of M4/5 subtypes varying between 42.2 and 73% of AML cases [4, 8]. Further studies on larger number of cases may be needed to confirm this finding and its cause.

As regard ALL, there were 38 cases (74.5%) with B-ALL and 13 cases (25.5%) with T-ALL. These results were in concordance with the most published data [6, 12,

13]. Common ALL (CD10 positive) accounted for 89.5% of B-ALL cases which is concomitant with Rego et al. and Gujral et al. [6, 13].

Immunophenotypic criteria of APL and non-APL AML

About 62% of our non-APL AML cases were CD34 positive with the highest positivity seen in AML-M0 followed by AML-M1/M2 subtypes. In most reports CD34 positivity in non-APL AML has varied between 55.8 and 69.1% [13–15]. We have found that HLA-DR is the single best marker for distinguishing APL from other

Table 4 Immunophenotypic profile of 164 de novo AL patients

CD	AML (<i>n</i> = 113)						ALL (<i>n</i> = 51)	
	M0 (<i>n</i> = 16)	M1/2 (<i>n</i> = 27)	M3 (<i>n</i> = 26)	M4/5 (<i>n</i> = 39)	M6 (<i>n</i> = 2)	M7 (<i>n</i> = 3)	B-ALL (<i>n</i> = 38)	T-ALL (<i>n</i> = 13)
CD34 ⁺	14 (87.5%)	20 (74.1%)	4 (15.4%)	18 (46.2%)	1 (50%)	1 (33.3%)	29 (76.3%)	2 (15.4%)
HLADR ⁺	15 (93.8%)	25 (92.6%)	1 (3.8%)	36 (92.3%)	1 (50%)	1 (33.3%)	37 (97.4%)	0 (0%)
CD34 ⁺ , HLADR ⁺	13 (81.3%)	19 (70.4%)	0 (0%)	18 (46.2%)	1 (50%)	1 (33.3%)	31 (81.6%)	0 (0%)
CD34 ⁺ , HLADR [−]	1 (6.3%)	2 (7.4%)	21 (80.8%)	3 (7.7%)	0 (0%)	0 (0%)	1 (2.6%)	12 (92.3%)
CD13 ⁺	10 (62.5%)	21 (77.8%)	24 (92.3%)	33 (84.6%)	0 (0%)	0 (0%)	3 (7.9%)	0 (0%)
CD33 ⁺	11 (42.3%)	24 (88.9%)	26 (100%)	39 (100%)	0 (0%)	1 (33.3%)	4 (10.5%)	1 (7.7%)
CD117 ⁺	14 (87.5%)	24 (88.9%)	21 (80.8%)	22 (56.4%)	2 (100%)	1 (33.3%)	0 (0%)	0 (0%)
CD14 ⁺	1 (6.3%)	2 (7.4%)	0 (0%)	25 (64.1%)	0 (0%)	0 (0%)	ND	ND
CD36 ⁺	1 (6.3%)	2 (7.4%)	1 (3.8%)	24 (61.5%)	0 (0%)	0 (0%)	ND	ND
CD41 ⁺	2 (12.5%)	5 (18.5%)	ND	9 (23.1%)	0 (0%)	3 (100%)	ND	ND
CD61 ⁺	1 (6.3%)	1 (3.7%)	ND	1 (2.6%)	0 (0%)	3 (100%)	ND	ND
Glyco. A ⁺	0 (0%)	0 (0%)	ND	0 (0%)	2 (100%)	0 (0%)	ND	ND
CD10 ⁺	ND	ND	ND	ND	ND	ND	34 (89.5%)	3 (23.1%)
CD19 ⁺	1 (6.3%)	1 (3.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	38 (100%)	0 (0%)
CD20 ⁺	ND	ND	ND	ND	ND	ND	10 (26.3%)	0 (0%)
TdT ⁺	5 (31.3%)	1 (3.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	37 (97.4%)	10 (76.9%)
CD3 ⁺	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (38.5%)
CD4 ⁺	1 (6.3%)	1 (3.7%)	0 (0%)	14 (35.9%)	0 (0%)	0 (0%)	0 (0%)	4 (30.8%)
CD5 ⁺	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	11 (84.6%)
CD7 ⁺	8 (50%)	9 (33.3%)	2 (7.7%)	6 (15.4%)	0 (0%)	1 (33.3%)	0 (0%)	12 (92.3%)
CD8 ⁺	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (53.8%)

AML subtypes because its expression was seen in only 1 of 26 APL cases. The precision of this distinction is further enhanced if expression pattern of CD34 is also taken into account. HLADR and CD34 double negativity in APL was observed in 80.8% of cases, this incidence was near to that reported by Wang et al. [16]. On the other hand, none of APL cases expressed both antigens simultaneously, thus the expression of both markers in AML can effectively exclude a diagnosis of APL. There was a strong association between HLA-DR positivity and AML-M4/M5 subtypes and Callea et al. reported similar results [17].

MPO was expressed in all APL cases and expressed with some variation in non-APL AML with the highest positivity seen in AML-M1/M2 (96.3%). Expression of CD117 was seen in 72.4% of non-APL AML cases and in 80.8% of APL cases. Similar findings were reported by previous studies [18–20]. So, MPO and CD117 are not reliable markers for differentiation between APL and non-APL AML.

The expression pattern of CD33 contributes to some extent in the distinction between APL and AML-M1/2 because none of our 26 APL cases lacked this antigen in contrast to approximately 11.1% of AML-M1/2 cases and

these results were similar to that stated by other researcher [19].

Lymphoid antigens expression in our AML cases varied from 1.8%–23% with the highest positivity seen in expression of CD7 (23%). An association was found between lymphocyte antigens positivity and expression of CD34 in our AML group as this aberrant expression was observed with highest percentage (93.8%) in AML-M0. Some published studies reported that lymphoid antigen positivity in AML between 16 and 22% and CD7 appeared to be the most commonly expressed marker [21–23].

CytoplasmicCD3 and CD5 expression were seen in 100 and 84.6% of our T-ALL cases respectively but in none of AML-M0 tested cases so they are the best markers to distinguish AML-M0 from T-ALL and this is in agreement with Kaleem et al. [24]. So, the lack of both cCD3 and CD5 dictates the diagnosis of AML-M0 more often than expression of MPO.

Immunophenotypic criteria of B-ALL

All our B-ALL cases express CD19 while TdT was expressed in 97.4% and this found to be similar to Bachir et al. [25]. In B-ALL, myeloid aberrant phenotype has been

reported in 10–47% of cases [23, 26, 27]. In our study, expression of CD13 and CD33 in B-ALL were 7.9 and 10.5%, respectively.

Immunophenotypic criteria of T-ALL

In our T-ALL cases, CD7 was the most detected antigen (92.3%) but was not totally specific, as it was demonstrated to cross react with AML cases. CD5 also came out as a highly sensitive marker (positive in 84.6% of the cases). The high sensitivity of these markers is in accordance with the previous reports and is thus the most commonly used antigens for T-ALL diagnosis [28]. Positivity of CD3 was 38.5% and like some previous studies was not confirmed as the most sensitive marker, which is limited by the fact that it is uncommonly expressed on the surface of T-ALL and is almost always present abundantly in the cytoplasm of these cells [13]. All T-ALL cases in this study were negative for HLADR and this incidence is much higher than reported elsewhere [13, 25] which may be explained by low number of cases in our study. Regarding aberrant B-cell marker expression on T-ALL, it was reported that CD10 and CD19 are expressed in various percentages on such cases (19–43% and 0–2.3%, respectively) [13, 19]. About twenty three percent of our T-ALL cases showed CD10 expression while none expressed CD19 or CD20. CD13 was not expressed at any of our T-ALL cases while CD33 observed in 7.7%. Aberrant expression of these markers varied from 0–11.5 to 4.8–6.3% of T-ALL in previous studies [13, 19].

In summary, Beside the routine role of the flowcytometric immunophenotyping in identification and enumeration of blasts in the clinical specimen, it could be uniquely useful in the diagnosis of AML-M0 and in differentiation of APL from non APL-AML. Immunophenotypic criteria of our AL patients were comparable to the internationally published data. On the other hand, our AML FAB subtypes showed some geographical variation from most of the previous reports in the form of predominance of M4/5 and more frequent APL.

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