Multi-color flow cytometric analysis of cell surface and cytoplasmic antigens in the diagnosis of acute leukemia in children

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**Background:** Acute leukemia (AL) is 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. Hence the distinction between lymphoid and myeloid leukemia, most often made by flow cytometry (FCM), is crucially important. This study was undertaken to evaluate the value of multi-color flow cytometry in the immunophenotyping of acute leukemia in children.

**Methods:** Three- or four-color flow cytometry and CD45/SSC gating were used to analyze the surface and cytoplasmic antigen expressions from 222 children with acute leukemia.

**Results:** Cells from the 222 children were analyzed. Based on the diagnostic criteria proposed by EGIL, four categories of the cells could be identified: undifferentiated type, 2 patients (0.9%); acute myeloid leukemia (AML), 78 (35.1%); acute lymphoblastic leukemia (ALL), 124 (55.9%); and mixed lineage AL, 18 (8.1%). Of the 124 patients with ALL, 94 (75.8%) were classified as having B lineage and 30 (24.2%) T lineage ALL. Antigen aberrant expressions were found in 19 (24.4%) of 78 patients with AML, 34 (36.2%) of 94 with B lineage ALL and 9 (30.0%) of 30 with T lineage ALL. The most commonly expressed lymphoid antigen in 78 patients with AML was CD7, 10 patients (12.8%), followed by CD19, 5 (6.4%), and CD2, 4 (5.1%). The most commonly expressed myeloid antigen in 124 patients with ALL was CD13, 23 patients (18.5%), followed by CD15, 14 (11.3%), CD11b, 8 (6.5%) and CD33, 4 (3.2%). CD17 and CD56 were present in 55 (73.3%) and 27 (38.6%) of the 75 patients and 71 patients with AML, respectively, but were generally absent in blast cells of ALL. Cytoplasmic (Cy) CD22, CyCD3 and CyMPO were specifically expressed in B lineage, T lineage and myeloid lineage leukemia, respectively, and the first two could be more sensitively detected than they were on the cell membrane surface.

**Conclusions:** Multi-color flow cytometry is a reliable method in the diagnosis, differential diagnosis and classification of acute leukemia in children.

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**Key words:** flow cytometry; leukemia; acute; diagnosis; childhood

**Introduction**

Acute leukemia (AL) is 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. Hence the distinction between lymphoid and myeloid leukemia is crucially important. The standard of acute leukemia, as reflected in the French-American-British (FAB) system, is based on the criteria derived from the morphologic and cytochemical examination of bone marrow specimens. However, these criteria often fail to discriminate reproducibly between subtypes of leukemia arising from the same hematopoietic lineage, and at times even between subtypes of leukemia of different lineages. Several advances in flow cytometry (FCM) including availability of new monoclonal antibodies, improved gating strategies and multiparameter analytic techniques have all dramatically improved the utility of flow cytometry in the diagnosis and classification of leukemia. Acute leukemia reflects the pattern of antigen acquisition in normal hematopoietic differentiation,
yet invariably demonstrates 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. In this study, a three- or four-color flow cytometry method was used in the routine investigation of bone marrow samples by means of a primary CD45/side scatter (SSC) gating procedure. Blast cells were first identified by the CD45/SSC gating in 222 children with acute leukemia.

Methods

Patients

From 1999 through 2002, a total of 222 consecutively admitted, pre-treated AL patients (138 were male and 84 female) at our hospital and other referral hospitals were enrolled into this study. The median age of the patients was 8 years with a range of 6 months to 14 years. The FAB criteria were adopted for the diagnosis and subclassification of all patients. In this series, 135 patients were diagnosed as having acute lymphoblastic leukemia (ALL), 78 acute myeloid leukemia (AML) including 8 cases of M0/M1, 33 M2, 20 M3, 3 M4, 12 M5, 1 M6, 1 M7, and 9 morphologically unclassified AL.

Sample preparations

Fresh heparinized blood or bone marrow samples were used for immunophenotypic analysis before chemotherapy. Mononuclear cells (MNC) were separated after the samples were layered onto the Ficoll-Hypaque medium with a density of 1.077, centrifuged at 1500 rpm for 10 minutes, and washed. The MNC suspensions at a concentration of 10^7 cells/ml were prepared in phosphate buffer saline (PBS, 0.01 mol, pH 7.4).

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD2, CD3, CD5, CD7, CD10, CD15, CD22, CD34, CD45, CD61, CD64, CD71, Lambda F (ab')2, TCR (a/b), mouse IgG1; phycoerythrin (PE)-conjugated anti-CD4, CD11b, CD13, CD14, CD19, CD20, CD33, CD38, CD56, CD117, myeloperoxidase (MPO), P-glycoprotein (P-GP), TCR (gamma/delta), mouse IgG1; peridinin chlorophyll protein (PerCP) -conjugated anti-CD8, HLA-DR, mouse IgG1; and APC-conjugated anti-CD38, CD3, mouse IgG1 were purchased from Becton Dickinson Company, USA. PE-conjugated anti-CD41a was purchased from PharMingen Company, USA.

Flow cytometry analysis

The direct immunofluorescent method was applied basically according to what was described in the literature. A panel of 37 antibodies was used to phenotype the acute leukemia. Specimens were pre-incubated with human AB serum for 30 minutes at 37°C to block the non-specific binding of Fc receptors followed by incubation with specific monoclonal antibodies (MoAbs) at 4°C in the dark for 20 minutes. After the incubation, the cells were washed twice with PBS. For the cytoplasmic staining of MPO, CD22 and CD3, directly conjugated antibodies PE-MPO, FITC-CD22 and APC-CD3 were added into the same tube after fixing the cells in 1% paraformaldehyde and permeablized in permeablizing solution. Isotype-matched control MoAbs were used to determine the negative limit. The samples were analyzed with multi-parameter 4-color flow cytometry (FACSCalibur with CELL Quest software, Becton Dickinson, USA) on list mode by collecting 10,000 cells/tube. Leukemic cells were selected on the basis of CD45 expression vs side scatter gating (Fig.), then the expressions of antigens on these populations of blast cells were evaluated. An antigen was considered positive if 20% or more of the cells were stained with individual cognate antibody except for MPO, which was considered positive if ≥ 3% of the leukemic cells were stained.

![Analysis of normal (left) and leukemic (right) bone marrow by CD45/SSC gating](image_url)

**Diagnostic criteria of immunophenotyping**

AL was classified according to the scoring system proposed by the European Group for the Immunological Characterization of Leukemia. This is based on the number and its degree of lymphoid/myeloid specificity of the antigens expressed by the leukemic cells (Table 1). Biphenotypic acute leukemia is defined when scores for the myeloid and one of the lymphoid lineages are over two points; the criteria for the definition of biphentotypic AL proposed here are those adopted in the WHO classification of haemopoietic malignancies.
Table 1. Scoring system for the diagnosis of acute leukemia 

<table>
<thead>
<tr>
<th>Score</th>
<th>B-lymphoid</th>
<th>T-lymphoid</th>
<th>Myeloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>CD79a</td>
<td>CD8</td>
<td>MPO</td>
</tr>
<tr>
<td></td>
<td>cytCD22</td>
<td>anti-TCRα/b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cytlyM</td>
<td>anti-TCRδ/d</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CD19</td>
<td>CD2</td>
<td>CD17</td>
</tr>
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<td>CD20</td>
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<td>CD13</td>
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<td></td>
<td>CD10</td>
<td>CD6</td>
<td>CD33</td>
</tr>
<tr>
<td>0.5</td>
<td>TdT</td>
<td>TdT</td>
<td>CD14</td>
</tr>
<tr>
<td></td>
<td>CD24</td>
<td>CD7</td>
<td>CD15</td>
</tr>
</tbody>
</table>

* Biphenotypic acute leukemia is defined when scores for the myeloid and one of the lymphoid lineages are >2 points.

Statistical analysis
The chi-square test or Fisher’s exact test of probabilities was performed with SPSS 10 software.

Results
Use of CD45/SSC gating in bone marrow (Fig.)

Immunophenotypic features
The cells from the 222 children with AL were analyzed. Based on the diagnostic criteria proposed by EGIL, four categories of the cells could be identified: the undifferentiated type, 2 patients (0.9%); acute myeloid leukemia (AML), 78 (35.1%); acute lymphoblastic leukemia (ALL), 124 (55.9%); and mixed lineage AL, 18 (8.1%). Of the 124 patients with ALL, 94 (75.8%) were classified as having B lineage and 30 (24.2%) T lineage ALL.

Of 2 patients with undifferentiated AL, 1 expressed CD34, and the other expressed CD34 and HLA-DR. Of 78 patients with AML, 19 (24.4%) expressed lymphoid-associated antigens. The most frequently expressed lymphoid-associated marker was CD7 in 10 patients (12.8%), which was followed by CD19, 5 (6.4%), CD2, 4 (5.1%) and CD20, 1 (1.3%). In 94 B lineage ALL patients, progenitor-B type, 7 patients, accounted for 7.4%, common ALL, 75 (79.8%), pre-B ALL, 11 (11.7%), and B-ALL, 1 (1.1%). Of the 94 B lineage ALL patients, 34 (36.2%) expressed myeloid-associated antigens. In 19 patients (20.2%), CD13 was the most frequently expressed myeloid-associated marker.

The next most frequently expressed myeloid-associated marker was CD15, which was present in 12 patients (12.8%), followed by CD11b, 5 (5.3%), and CD3, 4 (4.3%). In 30 T lineage ALL patients, 9 (30.0%) expressed myeloid-associated antigens. CD13 was present in 4 patients (13.3%), and it was followed by CD11b, 3 (10.0%), and CD15, 2 (6.7%). The FAB morphologic type and immunophenotypic features of 18 patients with mixed lineage AL are summarized in Table 2.

Table 2. Cases in which immunotyping made a major contribution to final diagnosis

<table>
<thead>
<tr>
<th>Final diagnosis</th>
<th>FAB</th>
<th>L1</th>
<th>L2</th>
<th>ALL</th>
<th>M1</th>
<th>M5</th>
<th>Mix</th>
<th>Total</th>
<th>MPO</th>
<th>CyCD22</th>
<th>CyCD3</th>
<th>CD10</th>
<th>CD19</th>
<th>CD7</th>
<th>CD2</th>
<th>CD8</th>
<th>CD3</th>
<th>CD13</th>
<th>CD15</th>
<th>CD34</th>
<th>HLA-DR</th>
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<tbody>
<tr>
<td>B/M</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>15</td>
<td>2</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>13</td>
<td>0</td>
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<td>11</td>
<td>13</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>T/M</td>
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<td>-</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T/B</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Total</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>18</td>
<td>2</td>
<td>12</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
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<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
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</table>

Table 3. Distribution of childhood AL cases according to immunophenotypes

<table>
<thead>
<tr>
<th>AL subtype</th>
<th>Total cases</th>
<th>Proportion (%)</th>
<th>Cases detected</th>
<th>Positive cases (percentage)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD34 CyCD3</td>
<td>CyCD22</td>
<td>CD34 HLA-DR</td>
<td>CD10 CD11 CD15 CD56 MPO CyCD22 CyCD3</td>
</tr>
<tr>
<td>AUL</td>
<td>2</td>
<td>0.9</td>
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<td>78</td>
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<td>33</td>
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<td>M3</td>
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<td>M4</td>
<td>3</td>
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<td>M5</td>
<td>12</td>
<td>5.4</td>
<td>12</td>
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</tr>
<tr>
<td>M6</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M7</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ALL</td>
<td>124</td>
<td>55.9</td>
<td>124</td>
<td>98</td>
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<tr>
<td>B lineage</td>
<td>94</td>
<td>46.3</td>
<td>94</td>
<td>74</td>
</tr>
<tr>
<td>T lineage</td>
<td>30</td>
<td>13.5</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Mix AL</td>
<td>18</td>
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<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>222</td>
<td>100.0</td>
<td>222</td>
<td>192</td>
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</table>

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Expressions of early hematopoietic cell surface and cytoplasmic antigens in AL

The expressions of early hematopoietic antigens and cytoplasmic antigens on all kinds of AL cells are summarized in Table 3.

In the 222 patients with AL, the positive rate of CD34 in T-ALL was significantly lower than those in AML and in B lineage ALL (χ^2 = 6.933, P < 0.01, χ^2 = 20.19, P < 0.01, respectively). The positive rates of HLA-DR in AML and in B lineage ALL were significantly higher than those in T-ALL (χ^2 = 33.970, P < 0.01 and χ^2 = 108.000, P < 0.01, respectively).

The positive rates of CD56 and CD117 in AML were significantly higher than those in ALL (χ^2 = 38.154, P < 0.01 and χ^2 = 97.774, P < 0.01, respectively).

In the patients with AML-M3, the positive rates of CD34 and HLA-DR were low (10% and 5%, respectively), but the positive rate (50%) of CD117 was significantly higher than those of CD34 and HLA-DR (P = 0.021 and P = 0.012, respectively).

Cytoplasmic antigens CD22, CD3 and MPO were the earliest identifiable, specific B, T, and myeloid markers. In the 58 (61.7%) of 94 patients with B lineage ALL, CD22 expression was found. CyCD22 was detected in 62 of the B lineage ALL patients, and it was expressed in 51 patients (82.3%). Of 30 patients with T-ALL, 17 showed CD3 expression, but all patients with T cell showed CyCD3 expression. MPO was expressed in all patients with AML except those with M0/M1.

Discussion

Recently, immunophenotyping by flow cytometric analysis has been used in the diagnosis and classification of acute leukemia. Flow cytometric analysis of acute leukemia is interpretive, combining the patterns and intensity of antigen expression to reach a definitive diagnosis. Gating is critical to isolate the abnormal cells because leukemic phenotype should be determined as a pure population as possible. Standard forward and side scatter gating is not optimal for separating bone marrow cells because of the overlap between monocytes, blasts, myelocytes, lymphocytes and erythrocytes. As bone marrow cells mature, they express increasing CD45. Thus, when CD45 is combined with SSC, which separates lineages based on cytoplasmic complexity, bone marrow sample is readily separated into its cellular constituents. In this study, CD45/SSC gating clearly separates the five cell populations of blast cells, erythrocytes cells, lymphocytes, monocytes and granulocytes. We found that flow cytometry (CD45/SSC gating) is reliable in detecting the cell sub-populations of the samples in normal bone marrow analysis as well as in acute leukemia. Moreover, recently we found that this gating procedure is helpful in detecting minimal residual disease (MRD), which often presents with low percentages of blast cells. Kern et al reported that CD45/SSC gating improves the sensitivity of FCM-based MRD monitoring in AML by 1 log.

Following the EGIL criteria, we analyzed the 222 consecutive patients and found that 91.0% of them could be diagnosed as having myeloid or lymphoid lineage AL (AML, 78 patients; B-cell lineage ALL, 94; T-cell lineage ALL, 30), 8.1% mixed AL, and less than 1% undifferentiated. The percentage of mixed ALL was consistent with that reported previously. According to morphological criteria, 9 patients with AL were unable to be classified. Depending on the reactive patterns of both cytoplasmic and surface antigen expression or co-expression, these patients were distributed as follows: acute undifferentiated leukemia (AUL) in 2 patients, B/myeloid biphenotypic type in 5, and T/myeloid biphenotypic type in 2. Eleven patients diagnosed as having ALL according to the FAB criteria were analyzed by FCM and classified into B/myeloid biphenotypic type (9), T/myeloid biphenotypic type (1), and T/B biphenotypic type (1).

In this study, antigen aberrant expressions were found in patients with AML (24.7%), those with B lineage ALL (36.2%), and those with T lineage ALL (30.0%). The fixing/permeabilizing technique employed in this study was highly sensitive in FCM detection of both cytoplasmic and nuclear antigens. Hence if it is used with immunophenotyping, the improvement of diagnosis and immunological classification may ensure appropriate selection of effective therapies for the patients. The results of this study also confirmed the findings that CyCD22, CyCD3 and CyMPO are the earliest identifiable, specific B, T, and myeloid markers, which are expressed virtually in all patients with B and T cell ALL or those with subtypes of AML. Thus, we think that cytoplasmic antigen detection is of utmost importance in establishing correct lineage affiliation in patients lacking expressions of B, T, and myeloid surface antigens or in demonstrating equivocal or ambiguous phenotypic features. It is also helpful in identifying biphenotypic AL. Although aberrant antigen expressions would be difficult to classify AL patients into relevant subtypes accurately, using the expression of leukemia or myeloid-associated phenotypes, MRD can be quantified by immunophenotyping with flow cytometry. Of 78 patients with AML in this study, 19 (24.4%) expressed lymphoid-associated antigens, the most frequently expressed lymphoid-associated marker was CD7, which was followed by CD19, 5 (6.4%)
and CD2, 4 (5.1%). Previous studies indicated that CD7 positivity at diagnosis is associated with the low remission rate and the expression of CD19 in the context that M2 is associated with the presence of t(8;21), a favorable prognostic marker, and that the presence of CD2 is correlated with an important subtype M4EO that is associated with abnormalities of chromosome 16 and a better prognosis. [11-13]

In this study, although CD56 was a NK cell marker, its expression was significantly higher in AML patients than in those with ALL. CD117 was present in 73% of patients with AML but was generally absent in patients with ALL except for 2 T-ALL patients who accounted for only 2% of 98 patients with ALL. Whereas CD117 was a myeloid lineage associated marker, its myeloid specificity was higher markedly than that of CD13 and CD33. [14] Hence, we suggested that CD56 and CD117 be included in a basic panel of antibodies for the immunophenotypic diagnosis of AML. In previous studies, CD117 was expressed in a minority (<4%) of bone marrow cells. [15,16] In this study, in the patients with AML-M3, the positive rates of CD34 and HLA-DR were low (10% and 5%, respectively), but the positive rate of CD117 was high (50%), which was significantly higher than those of CD34 and HLA-DR. Thus, we think that analysis of CD117 may be helpful in distinguishing blast cell populations from normal cell populations, especially in the diagnosis of M2b and M3 patients in whom CD34 and HLA-DR are usually negative. Over-expression of CD117 and CD56 in AML may be valuable to detect minimal residual disease. [17,18] Our data [19] showed that CD56 positivity at diagnosis with a low remission rate which was also confirmed by Tiftik et al. [20] We suggest the evaluation of CD56 in all patients with acute leukemia at the time of diagnosis in view of poor clinical outcome.

The FCM analysis of AML subtypes insufficiently identifies the monocytic components of FAB-M4 and FAB-M5 cells since CD14 is insensitive for confirming the monocytic cells. Evaluation of cytochemical analysis with alpha-naphthyl acetate esterase (ANAE) staining in connection with immunophenotyping may help to identify M4 and M5 more accurately. [20,21]

In conclusion, immunophenotyping with multi-parameter flow cytometry is a powerful approach for the diagnosis and classification of acute leukemia in children. It has been crucial for clinicians in therapeutic decision-making for children with AL.

**Contributors:** All authors contributed to the design and interpretation of the study and to further drafts.

**References**


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**Competing interest:** None declared.

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