Routine Immunophenotyping in Acute Leukemia: Role in Lineage Assignment and Reassignment

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Diagnostic evaluation of acute leukemia at Roswell Park Cancer Institute has routinely included immunophenotyping by multiparameter flow cytometry. In a retrospective analysis of 646 cases, morphology and cytochemistry established lineage in 612, but not in 34 (5%), of which 26, 5, and 3 were myeloid, undifferentiated, and lymphoid, respectively, based on immunophenotyping. In addition, immunophenotyping changed the lineage assigned based on morphology and cytochemistry in 11 cases (2%); 8 changed from lymphoid to myeloid, and 3 from myeloid to lymphoid. The data support routine inclusion of at least limited immunophenotyping in the diagnostic evaluation of acute leukemia. © 2006 International Society for Analytical Cytology

Key terms: acute leukemia; immunophenotyping; multiparameter flow cytometry

Assignment of lineage is critical in the diagnostic evaluation of acute leukemia, as treatment for acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) differs. Myeloid and lymphoid lineage may be distinguished based on cellular morphology, cytochemical staining, and expression of lineage-specific antigens (1). In particular, myeloid antigen expression in acute leukemia that is morphologically and cytochemically undifferentiated serves to define the French–American–British (FAB) M0 subtype of AML in the FAB classification (2) or minimally differentiated AML in the World Health Organization (WHO) classification (1). Nevertheless, optimal use of immunophenotyping in the diagnostic evaluation of acute leukemia remains to be defined. Current National Comprehensive Cancer Network (NCCN) guidelines state that immunophenotyping and cytochemistry are complementary techniques, and that their prioritization is at the discretion of the pathologist (3).

At Roswell Park Cancer Institute (RPCI), diagnostic evaluation of acute leukemia has routinely included immunophenotyping by multiparameter flow cytometry, in addition to morphology and cytochemical staining. We retrospectively analyzed the frequency with which immunophenotyping was needed for lineage assignment because of lack of morphologic and cytochemical differentiation, and the frequency with which it changed the lineage assigned based on morphology and cytochemistry.

MATERIALS AND METHODS

Patients

Six hundred and forty six cases of acute leukemia diagnosed consecutively in adults at RPCI between 1990 and 2002 were reviewed.

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Morphology and Cytochemistry

Bone marrow aspirate smears were stained with Wright-Giemsa for morphological analysis. Cytochemical stains included myeloperoxidase (MPO), Sudan Black B (SBB), chloroacetate esterase (CAE), alpha naphthyl butyrase esterase (ANBE), and periodic acid-Schiff (PAS).

Immunophenotyping

Bone marrow samples were immunophenotyped by multiparameter flow cytometry in the Laboratory of Flow Cytometry at RPCI, as previously described (4,5). Briefly, cell suspensions were stained with multiple panels of three monoclonal antibodies (until March 1998) labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and either peridinin chlorophyll protein (PerCP) or phycoerythrin-CY5 tandem complex (PECY5), or four monoclonal antibodies (starting in March 1998) labeled with FITC, PE, PerCP or PECY5 and allophycocyanin (APC). Cell suspensions were also stained with panels of identically conjugated isotype controls for the antibodies in each panel. Listmode data were acquired on either a FACScan or a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using WinList multiparameter analysis software (Verity Software House, Topsham, ME). Antigen-positivity was defined by reactivity above isotype controls on ≥10% of leukemia cells, as identified by multiparameter flow cytometry (4,5). Intracellular antigen expression was not studied.

Immunological classification of acute leukemias utilized the criteria of the European Group for the Immunological Characterization of Leukemias (EGIL) (6). As noted, however, cytoplasmic antigen expression was not studied in this retrospective series, and classification was therefore based on surface expression.

**Fig. 1. Outcome of immunophenotyping.**

- Patients with Acute Leukemia between 1990 and 2002 N=646
  - Morphology and Cytochemistry
    - Lineage assigned N=612
      - AML N=525
        - Immunophenotyping
          - AML N=522
          - ALL N=3
      - ALL N=87
        - Immunophenotyping
          - Stem cell leukemia N=5
          - ALL N=3
          - AML N=26
  - Lineage not assigned N=34 (5%)
    - Immunophenotyping
      - AML N=3
      - ALL N=79
      - Reversal of Lineage N=11 (2%)
Cytogenetics

Pretreatment bone marrow samples were processed for chromosome analysis by standard techniques, with 24–48 h unstimulated cultures. Air-dried chromosome preparations on glass slides were G-banded. At least 20 metaphases were analyzed in each case. Karyotypes were designated according to the International System for Human Cytogenetic Nomenclature (7).

Study Design

Morphological characteristics and cytochemical staining, on the one hand, and immunophenotype, on the other hand, were reviewed independently (by MB and CCS, respectively) and independent assessments were made. In cases with discrepant findings, all findings were subsequently reviewed together, along with clinical input, to establish a final diagnosis, and in more recent years, a single pathology report encompassing all findings has been prepared for all cases. However, the analyses reported here compare the initial findings of independent review of morphology and cytochemical staining and of immunophenotype.

RESULTS

Six hundred and forty six cases of acute leukemia were diagnosed at RPCI between 1990 and 2002 (Fig. 1). Morphology and cytochemistry established lineage in 612 patients (95%); lineage was myeloid in 517 and lymphoid in 86, and bilineal (myeloid and lymphoid) in 9. Morphology and cytochemistry did not establish lineage in 34 cases (5%).

Among the 34 cases in which lineage could not be assigned based on morphology and cytochemistry, immunophenotyping established myeloid lineage in 26 (AML

<table>
<thead>
<tr>
<th>No.</th>
<th>Morphology</th>
<th>Cytochemical staining</th>
<th>Antigens expressed</th>
<th>Antigens not expressed</th>
<th>Karyotype</th>
</tr>
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<tr>
<td>1</td>
<td>ALL, L2</td>
<td>PAS</td>
<td>HLADr, CD13, CD14, CD15, CD32, CD34, CD38, CD4d</td>
<td>CD33, CD15, CD14, CD11b, CD11c, CD64, CD19, CD20, CD22, kappa, lambda, CD10, CD56, CD2, CD3, CD4, CD5, CD7, CD8</td>
<td>47, XY, +13</td>
</tr>
<tr>
<td>2</td>
<td>ALL, L2</td>
<td>PAS</td>
<td>HLADr, CD13, CD32, CD34, CD38, CD4d</td>
<td>CD33, CD15, CD14, CD11b, CD11c, CD64, CD19, CD20, CD22, kappa, lambda, CD10, CD56, CD2, CD3, CD5, CD7, CD8</td>
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<td>46, XX, i(12)(q10), der(18)t(13;18) (q11;p11)</td>
</tr>
<tr>
<td>4</td>
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<td>HLADr, CD33, CD15, CD11c, CD32, CD34, CD38, CD4d</td>
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<td>47, XY, +12</td>
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<td>5</td>
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<td>PAS</td>
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<td>CD15, CD14, CD32, CD64, CD19, CD20, CD22, kappa, lambda, CD10, CD56, CD2, CD3, CD4, CD5, CD7, CD8</td>
<td>45, X, −Y, −2, add(3)(q22), add(5)(q21), +mar</td>
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<tr>
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<td>PAS</td>
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<td>CD33, CD19, CD20, kappa, lambda, CD10, CD2, CD3, CD5, CD8</td>
<td>46, XY</td>
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<tr>
<td>7</td>
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<td>HLADr, CD13, CD34, CD38</td>
<td>CD33, CD14, CD15, CD11b, CD11c, CD32, CD64, CD19, CD20, CD22, kappa, lambda, CD10, CD56, CD2, CD3, CD4, CD5, CD7, CD8</td>
<td>46, XY</td>
</tr>
<tr>
<td>8</td>
<td>ALL</td>
<td>PAS</td>
<td>HLADr, CD13, CD34, CD56, CD4d, CD7</td>
<td>CD33, CD14, CD11c, CD32, CD64, CD19, CD20, CD22, kappa, lambda, CD10, CD2, CD3, CD5, CD8</td>
<td>46, XY</td>
</tr>
</tbody>
</table>

Assays for MPO, SBB, CAE, and ANBE were negative or <3% in all cases.

*PAS, Periodic acid-Schiff, granular, dot, block or ring reactivity.
FAB M0), lymphoid lineage in 3, and an undifferentiated phenotype (1) in 5.

Among the 87 cases with ALL based on morphology and cytochemistry, immunophenotyping demonstrated lymphoid immunophenotypes in 79, but demonstrated myeloid immunophenotypes in 8. The 8 cases with lymphoid morphology/cytochemistry but myeloid immunophenotypes are summarized in Table 1. Blasts in all cases had lymphoid morphology. All cases showed granular, dot or block PAS staining, present in 6–70% blasts, and four cases had partial or complete ring-type positivity. Staining with MPO, SBB, and CAE was absent or present in less than 5% of blasts in all cases. Immunophenotypes were myeloid in all cases, with expression of CD13 and/or CD33 in all, expression of other myeloid markers, such as CD11b, CD11c, CD14, CD15, CD32, or CD64, and dim expression of CD4 (CD4d) in a variable number of cases, and absence of lymphoid markers, with the exception of CD56 in one case. Case 2 in Table 1 is illustrated in Figure 2. Cytogenetic data are also shown in Table 1. Among the eight patients with lymphoid morphology and cytochemistry but myeloid immunophenotypes, one had trisomy 13, one tetrasomy 13, one trisomy 1q and 13q, and one trisomy 12.

Among the 525 cases with AML based on morphology and cytochemistry, 522 patients had myeloid immunophenotypes, but 3 had lymphoid immunophenotypes (Table 2). SBB reactivity was seen in two cases, along with ANBE in one, while MPO staining was seen in the third. The immunophenotype was B-lineage (1,6) in all three cases, as evidenced by the HLA-DR, CD34, CD19, CD22 phenotype in all three, with surface immunoglobulin in one. CD13 was coexpressed in one case, and CD15 in another. Case 1 in Table 2 is illustrated in Figure 3. As shown in Table 2, cytogenetic findings included t(2;11)(p21;q23) in one and hypodiploidy in the other two.

**DISCUSSION**

Lineage assignment is critical for optimal therapy for acute leukemia, as treatment regimens for AML and ALL differ markedly. In this retrospective analysis of routine immunophenotyping in the diagnostic evaluation of 646 cases of acute leukemia, immunophenotyping was necessary for lineage assignment in 34 cases (5%) that were morphologically and cytochemically undifferentiated, and also corrected the lineage that was assigned based on morphology and cytochemistry in an additional 11 cases (2%).

In 8 of the 686 cases in our series, blasts had lymphoid morphology and stained with PAS and did not stain with other cytochemical stains, but nevertheless had a myeloid immunophenotype. Snower et al. found that PAS staining by itself was 52% sensitive and 81% specific for lymphoid lineage, but was 100% specific when other cytochemical stains, including MPO, SBB, and ANBE, were negative (8). The cases in our series would represent exceptions.

Three out of 686 cases in our series had myeloid morphology and stained with SBB and/or MPO, but had a B-lineage immunophenotype. SBB or MPO positivity in 3% or more blasts in acute leukemia marrow is considered to be diagnostic of myeloid lineage (9). Nevertheless, SBB positivity has been found in patients with B-lineage ALL with frequencies of 1.3 and 2.7% (10,11). Additionally, Arber et al. recently reported immunoreactivity with
a polyclonal MPO antibody (pMPO), but not MPO cytochemical staining, in 19 of 82 patients (23%) with ALL, all of whom had precursor B-cell phenotypes, with frequent coexpression of CD13 or CD15 and frequent (42%) Philadelphia chromosome-positivity (12).

Four of the eight patients in our series with lymphoid morphology and cytochemical staining but myeloid immunophenotypes had trisomy or tetrasomy of chromosomes 13q/13 and/or 12q/12. Trisomy 13/13q has previously been associated with morphologically undifferentiated leukemia and mixed lineage leukemia (13–15). Trisomy 12 is characteristic of chronic lymphocytic leukemia, with an incidence of up to 20% (16), but has also been reported in both AML and ALL (17,18). Like trisomy 13/13q, trisomy 12/12q has also been associated with undifferentiated acute leukemia (15). Reciprocal chromosome translocations with 11q23 breakpoints, involving the MLL gene, are found in both AML and ALL (18). In particular, t(2;11)(p21;q23), seen in one of our three cases with myeloid morphology and cytochemistry but lymphoid immunophenotype, is a rare 11q23 translocation reported in both AML and ALL (18).

The original FAB classification scheme relied on morphologic and cytochemical characteristics to classify leukemias (2,9,19). The EGIL proposed an immunological classification of leukemias with development of a scoring system to differentiate between various leukemia subtypes (6), which was recently validated (20). However immunophenotyping is still sometimes considered to be a complementary technique, rather than an essential part of diagnostic evaluation (3). In our retrospective analysis of routine immunophenotyping in 686 cases of acute leukemia, immunophenotyping was essential for lineage assignment in 5% of cases and for lineage correction in 2% of cases. These data provide an argument for routine inclusion of at least limited immunophenotyping in the diagnostic evaluation of acute leukemia.

Table 2
Characteristics of Cases in Which the Diagnosis Changed from AML to ALL Based on Immunophenotyping

<table>
<thead>
<tr>
<th>No.</th>
<th>Morphology</th>
<th>Cytochemical staining</th>
<th>Antigens expressed</th>
<th>Antigens not expressed</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML, M0</td>
<td>SB</td>
<td>HLADr, CD19, CD22, CD34, CD38, CD15</td>
<td>CD33, CD13, CD14, CD11b, CD11c, CD32, CD64, CD20, CD10, CD56, CD2, CD3, CD4, CD5, CD7, CD8</td>
<td>38–41, inc</td>
</tr>
<tr>
<td>2</td>
<td>AML, M4</td>
<td>MPO</td>
<td>HLADr, CD19, CD22, kappa, CD34, CD13, CD32</td>
<td>CD38, CD33, CD15, CD14, CD11b, CD11c, CD64, CD20, CD10, CD56, CD2, CD3, CD4, CD5, CD7, CD8</td>
<td>46, XX, t(2;11) (p21;q23)</td>
</tr>
<tr>
<td>3</td>
<td>AML, M1</td>
<td>SBB, ANBE</td>
<td>HLADr, CD19, CD22, CD34, CD38, CD32</td>
<td>CD33, CD13, CD15, CD14, CD11b, CD11c, CD64, CD20, CD10, CD56, CD2, CD3, CD4, CD5, CD7, CD8</td>
<td>36, inc[8 cells]</td>
</tr>
</tbody>
</table>

SBB, Sudan black B; MPO, myeloperoxidase; ANBE, alpha naphthyl butyrase esterase.
LITERATURE CITED


