Multiparameter Flow Cytometry in the Diagnosis and Management of Acute Leukemia

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• Context.—Timely and accurate diagnosis of hematologic malignancies is crucial to appropriate clinical management. Acute leukemias are a diverse group of malignancies with a range of clinical presentations, prognoses, and preferred treatment protocols. Historical classification systems relied predominantly on morphologic and cytochemical features, but currently, immunophenotypic, cytogenetic, and molecular data are incorporated to define clinically relevant diagnostic categories. Multiparameter flow cytometry provides rapid and detailed determination of antigen expression profiles in acute leukemias which, in conjunction with morphologic assessment, often suggests a definitive diagnosis or a narrow differential. Many recurrent molecular or cytogenetic aberrations are associated with distinct immunophenotypic features, and therefore flow cytometry is an important tool to direct further testing. In addition, detection of specific antigens may have prognostic or therapeutic implications even within a single acute leukemia subtype.

After initial diagnosis, a leukemia’s immunophenotypic fingerprint provides a useful reference to monitor response to therapy, minimal residual disease, and recurrence.

Objective.—To provide an overview of the application of flow cytometric immunophenotyping to the diagnosis and management of acute leukemias, including salient features of those entities described in the 2008 World Health Organization classification.

Data Sources.—Published articles pertaining to flow cytometry, acute leukemia classification, and experiences of a reference flow cytometry laboratory.

Conclusion.—Immunophenotypic evaluation is essential to accurate diagnosis and classification of acute leukemia. Multiparameter flow cytometry provides a rapid and effective means to collect this information, as well as providing prognostic information and a modality for minimal residual disease evaluation.

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The 4th edition of the World Health Organization’s Classification of Tumours of Haematopoietic and Lymphoid Tissues incorporates recent advances in understanding of the molecular, genetic, and clinical aspects of hematolymphoid neoplasms to revise categorization of these processes into the most clinically relevant entities possible, facilitating both the management of patients as well as future investigational studies. In many cases, criteria for prior entities have been revised and new entities introduced based primarily on recurrent molecular and genetic lesions. Despite this fact, morphologic and immunophenotypic characterization remain crucial for accurate diagnosis as well as monitoring response to therapy. Indeed, “For acute leukemia, multiparameter flow cytometry (3 or more colors) is the method of choice for determining the blast lineage as well as for detecting aberrant antigenic profiles that may prove useful for disease monitoring.”

In this context, we will first review some general considerations in the application of flow cytometry to the diagnosis of acute leukemias, followed by brief discussions of the characteristic immunophenotypic features of the different subtypes of acute leukemia currently recognized. A schematic summary of the current World Health Organization classification for acute leukemia is presented in Table 1.

GENERAL CONSIDERATIONS IN THE APPLICATION OF FLOW CYTOMETRY TO LEUKEMIA DIAGNOSIS

Despite the increasing importance of molecular and genetic features in the subclassification of acute leukemias, morphologic and immunophenotypic analysis remain the primary modalities by which these diseases are first evaluated. It is currently impractical to perform a comprehensive, undirected molecular analysis of hematolymphoid malignancies owing to the myriad different genes involved, but an initial rapid interpretation of morphologic and immunophenotypic data can efficiently direct a narrow and specific search for molecular lesions. Thus, flow cytometry serves as a tool to triage cases for recurrent molecular and genetic testing. In addition, molecular assays may require a high level of technical expertise, available only in specialized reference laboratories, necessitating prolonged specimen processing and analysis times, which may be incompatible with timely clinical management of acute leukemia. By contrast, flow cytometric analysis can be completed within a matter of hours on virtually any case and is often sufficient, when combined with conventional morphologic review, to arrive at a narrow differential or even definitive diagnosis.
A prominent component of the specimen. On extremely rare occasions, buffy coat and bone marrow aspirate samples can yield useful results, especially if the neoplastic clone is restricted to myeloblasts or promyeloblasts. However, as few as 50,000 cells may be sufficient for analysis. Aliquots of the processed cells are then added to individual "tubes" for staining with panels of fluorochromes. Panels using as many as 10 colors are coming into clinical use and may offer improved sensitivity in recognizing small populations of myeloblasts.

A wide range of specimens are amenable to flow cytometric evaluation, although in general, the best results are achieved with fluid-based specimens such as peripheral blood and bone marrow aspirates. These specimens are routinely subjected to red blood cell lysis with ammonium chloride as a first step in processing. The remaining leukocyte population is then resuspended in cell culture medium and subjected to a cell count, so that controlled numbers of cells can be submitted for staining with antibodies conjugated to a variety of fluorochromes. Aliquots of the processed cells are then added to individual "tubes" for staining with panels of antibodies. For best results, 500,000 to 1 million cells are stained per tube, although as few as 50,000 cells may yield useful results, especially if the neoplastic clone is a prominent component of the specimen. On extremely paucicellular specimens there will be a practical limit to the number of tubes—and hence antigenic markers—that can be analyzed. Finally, the samples are scanned by the flow cytometry instrument, and the resultant data histograms saved for evaluation. Biopsies from solid organs, including bone marrow, undergo a similar process after the addition of a manual disaggregation step and coarse filtration of large particles. Even with careful processing, biopsy specimens, particularly from fibrotic organs such as skin, generally produce specimens with reduced viability and greater distortion of cellular populations. Even in a nearly ideal specimen, such as a high-quality marrow aspirate, flow cytometry should not be the sole arbiter of blast percentages, as processing may introduce artifacts due to differential susceptibility of cells to degradation during processing and variability in the efficacy of the erythrocyte lysis step. Certain cell types such as megakaryocytes and, to a lesser extent, plasma cells, are selectively vulnerable to loss during processing.

In general, it is preferable to report flow cytometric findings by describing patterns of antigen expression on any aberrant population as well as an estimated percentage of all cell types identified, both aberrant and normal. Simple listings of percentages of antigen positivity for an entire sample represent underutilization of the technology and are less likely to be diagnostic in complex specimens consisting of a small neoplastic population in a reactive background, as in a posttreatment specimen. For data analysis, most laboratories use a "gating" system in which predetermined combinations of cellular characteristics, for example, intrinsic light scatter properties combined with level of CD45 (leukocyte common antigen) expression, are used to define populations of cells that are then characterized with respect to expression of specific antigens. A smaller number of laboratories use a "cluster analysis" process, whereby the raw data from each tube of cells stained with an antibody combination are subjected to manual review by a pathologist, who uses a combination of expected cellular characteristics (similar to gating), combined with visual inspection, to isolate all unique cellular populations. Both methods of analysis can yield similar results if correctly implemented, although cluster analysis is more sensitive when multiple or only small neoplastic populations are present. However, this benefit requires additional effort during analysis, which may not be practical in all settings. Currently, there is a broad range in the number of antibodies per analysis tube used by clinical laboratories, with most using either 3, 4, or 5 "colors" (ie, different fluorochromes). Forward scatter (cell size) and orthogonal/side scatter (cytoplasmic complexity) provide 2 additional analytic parameters. Panels using as many as 10 colors are coming into clinical use and may offer improved sensitivity in recognizing subtly aberrant immunophenotypes, low numbers of neoplastic cells in minimal residual disease analysis, and studies of low-cellularity samples in which only 1 or a few tubes may be analyzed.1,2,4

A large and ever-increasing number of specific, monoclonal antibodies are available for use in flow cytometry. Laboratories will select different panels of markers on the basis of prior experience and personal preference. Nevertheless, there is a subset of markers that are particularly well characterized and broadly useful, forming the core of most immunophenotypic panels. A

### Table 1. World Health Organization (2008) Categories of Acute Leukemia*

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
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<tbody>
<tr>
<td>Acute myeloid leukemia</td>
<td>AML with recurrent cytogenetic abnormalities (see Figure 1)</td>
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<tr>
<td></td>
<td>AML with myelodysplasia-related changes</td>
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<td></td>
<td>Therapy-related myeloid neoplasms</td>
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<td></td>
<td>AML NOS</td>
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<td></td>
<td>Morphologic and immunophenotypic subtypes</td>
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<td></td>
<td>Acute panmyelosis with myelofibrosis</td>
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<td></td>
<td>Myeloid sarcoma</td>
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<tr>
<td></td>
<td>Myeloid leukemia associated with Down syndrome</td>
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<tr>
<td></td>
<td>Blastic plasmacytoid dendritic cell neoplasm</td>
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<tr>
<td>Acute leukemia of ambiguous lineage</td>
<td>Acute undifferentiated leukemia</td>
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<tr>
<td></td>
<td>Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); BCR-ABL1</td>
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<tr>
<td></td>
<td>Mixed phenotype acute leukemia with t(15;17); APL</td>
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<tr>
<td></td>
<td>Mixed phenotype acute leukemia, NOS (subclassify by phenotypes)</td>
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<td>B-lymphoblastic leukemia/lymphoma</td>
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<td>B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities</td>
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<td>B-lymphoblastic leukemia/lymphoma NOS</td>
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<td>T-lymphoblastic leukemia/lymphoma</td>
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Abbreviations: AML, acute myeloid leukemia; NOS, not otherwise specified.

* The current World Health Organization classification includes entities defined by recurrent genetic abnormalities, cytomorphology, and clinical and immunophenotypic features. Although acute myeloid leukemias with recurrent genetic abnormalities are not strictly linked to specific morphologic or immunophenotypic characteristics, in many cases an association exists (refer to Figure 1). The unique subtypes of acute myeloid leukemia listed have a variable association with specific morphologic and immunophenotypic features. For example, the myeloid neoplasms associated with Down syndrome show a marked relative increase in apparent megakaryocytic differentiation.

For example, a new diagnosis of acute myeloid leukemia lacking expression of CD34 and HLA-DR raises the possibility of an acute promyelocytic leukemia and would prompt careful morphologic review, consideration of cytogenetic fluorescence in situ hybridization (FISH) for the t(15;17); PML-RARA, and clinical evaluation of the patient for evidence of disseminated intravascular coagulation. It should be emphasized that, as a general rule, a specific diagnosis should not be based solely on immunophenotypic findings without morphologic and preferably molecular/genetic correlation.
Selection of these markers is listed in Table 2, along with their expected expression patterns in normal bone marrow specimens submitted for flow cytometry.

Only a few antigens are narrowly restricted to a single cell type (lineage specific); most show more complex reactivity. At one extreme, expression of CD3 virtually defines T lymphocytes. By contrast, CD5 is predominantly associated with T cells but is also seen on a small subset of natural killer cells. At the opposite end of the spectrum, markers such as CD38, HLA-DR, and CD45 are broadly expressed across a wide range of cell types, but they remain crucial to analysis owing to complex but reproducible patterns of expression during maturation, as well as their propensity to be overexpressed or underexpressed in neoplastic populations. The specific immunophenotypic signature of the abnormal cells, as established at diagnosis, will be useful for follow-up after treatment and detection of minimal residual disease, as discussed below.

In a typical case, the presence of immature “blast” cells in peripheral blood or bone marrow initiates a request for flow cytometric analysis. The first step would be to confirm the presence of blasts and preliminarily assign a lineage to them. This may be accomplished through a screening panel as shown in Figure 2. Depending on the phenotype of the cells, further testing is performed and a specific diagnosis or differential is rendered. Selected panels of antibodies used in our laboratory are given in the legend of Figure 2. The number of tubes and the specific antibodies will vary among laboratories, depending on preference and experience. Some laboratories will choose to initially perform a complete marker panel, reducing turnaround time, as compared to this staged process.

**ACUTE MYELOID LEUKEMIAS WITH RECURRENT GENETIC ABNORMALITIES**

Many of the acute myeloid leukemias in this category do not have distinct immunophenotypic features, as they...
have been defined by recurrent genetic and molecular changes, which may be associated with a range of morphologic and immunophenotypic features. Nevertheless, some subtypes have a characteristic immunophenotypic fingerprint which, when present, should prompt directed evaluation for the suspected genetic lesion.

**Acute Myeloid Leukemia With t(8;21)(q22;q22); RUNX1-RUNX1T1**

Acute myeloid leukemia with recurrent t(8;21) generally shows granulocytic differentiation with maturation, and no specific immunophenotypic or morphologic evidence of monocytic, erythroid, or megakaryoblastic differentiation. By flow cytometry, common granulocytic/neutrophilic lineage–associated antigens are expressed including CD13, CD15, CD33, and myeloperoxidase. The blasts are often bright positive for CD34 and may express terminal deoxynucleotidyl transferase (TdT). In addition, these leukemias frequently show expression of B lineage–associated markers, especially CD19, with PAX5 and CD79a less commonly seen (representative example in Figure 3, a). In cases of acute myeloid leukemia showing strong expression of CD19, it is important to evaluate for t(8;21), as this recurrent cytogenetic abnormality supersedes a diagnosis of mixed phenotype acute leukemia (discussed below). If a satisfactory conventional cytogenetic analysis is not available, remaining materials from the flow cytometry specimen may be sent directly for interphase FISH. Some cases show expression of CD56, which may be associated with a worse prognosis. Additional cytogenetic abnormalities, most frequently loss of chromosome 9q, may be present.

**Acute Myeloid Leukemia With inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11**

Acute myeloid leukemia with recurrent inv(16) or t(16;16) characteristically shows myelomonocytic differentiation with a variable expansion of eosinophils, which may have unusually large cytoplasmic granules with violet coloration. Flow cytometry may identify distinct aberrant blast and myeloid/monocytic populations. Expression of CD14, CD4, CD11b, or combined CD36/CD64 by flow cytometry suggests monocytic differentiation. Although most easily noted by morphologic evaluation, the eosinophils are usually also evident by flow cytometry as a distinct population of cells with high cytoplasmic complexity (side scatter) similar to mature neutrophils, but which appear slightly smaller (less forward scatter) as well as being uniformly CD16+.

**Acute Promyelocytic Leukemia With t(15;17)(q22;q12); PML-RARA**

Corresponding to the French-American-British morphologic category of acute myeloid leukemia M3, acute promyelocytic leukemia with recurrent t(15;17) consists of a neoplastic granulocyte population at the promyelocyte stage of maturation. Normal myeloblasts express CD34, HLA-DR, and CD79a less commonly seen (representative example in Figure 3, a). In cases of acute myeloid leukemia showing strong expression of CD19, it is important to evaluate for t(15;17), as this recurrent cytogenetic abnormality supersedes a diagnosis of mixed phenotype acute leukemia (discussed below). If a satisfactory conventional cytogenetic analysis is not available, remaining materials from the flow cytometry specimen may be sent directly for interphase FISH. Some cases show expression of CD56, which may be associated with a worse prognosis. Additional cytogenetic abnormalities, most frequently loss of chromosome 9q, may be present.
complexity. The microgranular variant more commonly shows at least partial expression of CD34 and HLA-DR and may be positive for CD2. A new diagnosis of acute myeloid leukemia with CD34+ HLA-DR+ blasts should prompt careful morphologic review and/or consideration of rapid testing for PML-RARA (eg, by FISH) to rule out acute promyelocytic leukemia owing to the increased risk of potentially fatal disseminated intravascular coagulation. However, it should be noted that many CD34+ HLA-DR+ acute myeloid leukemias will not have an associated PML-RARA1, instead representing clinically and molecularly distinct entities, such as the so-called acute myeloid leukemia with cuplike nuclear invagination.

Acute Myeloid Leukemia With t(1;22)(p13;q13); RBM15-MKL1

Acute myeloid leukemia with recurrent t(1;22) generally shows evidence of megakaryocytic differentiation, which can be demonstrated by using antibodies targeting CD41 (glycophorin IIb) and CD61 (glycophorin IIIa). While these markers have good specificity, false-positive expression may be seen owing to adherence of platelets to blasts. Comparison to other cell populations may be helpful; for example, if mature granulocytes in the same specimen show apparent expression of CD41 and CD61, then similar staining on the blast population should be regarded with skepticism. Marrow fibrosis may cause difficulty in aspirating neoplastic cells, resulting in marked underestimation of blast percentage by flow cytometry. The blasts are often negative for CD34, HLA-DR, and CD45.

Additional Categories of Acute Myeloid Leukemia With Recurrent Genetic Abnormalities

The remaining examples of acute myeloid leukemia with recurrent genetic abnormalities show less distinctive immunophenotypic features. Acute myeloid leukemia with recurrent t(9;11)(p22;q23); MLLT3-MLL and acute myeloid leukemia with mutated NPM1 frequently, but not
Figure 3. Examples of flow cytometric findings in acute leukemia. Representative findings are shown for 5 different cases of acute leukemia, organized into horizontal rows. The neoplastic populations are identified in red. Relevant reference populations include maturing granulocytes (green), B lymphocytes (light blue), and T lymphocytes (yellow). In all cases, additional markers (not shown) were also analyzed; uncharacterized events are depicted in gray. a, Acute myeloid leukemia with expression of CD34, CD15, CD33, and myeloperoxidase. In addition, CD19 is present in the absence of strong expression of other B-lineage markers, such as CD20, CD22 (not shown), or CD79a. These features suggest a possible recurrent t(8;21)(q22;q22), which was later confirmed by cytogenetic analysis. b, CD13+, CD33+, CD117+ acute myeloid leukemia with minimal expression of CD34 and HLA-DR. In addition, the blasts show increased orthogonal (side) scatter, suggestive of cytoplasmic complexity/granularity. As predicted, cytogenetic fluorescence in situ hybridization studies performed on the remaining specimen showed the recurrent t(15;17)(q22;q12) of acute promyelocytic leukemia. c, B-lymphoblastic leukemia that is CD34+, CD19+, CD22+, and CD10+. Aberrancies in comparison to normal B-lymphoid precursors include the partial loss of CD45 and gain of the myeloid antigen CD11c. d, T-lymphoblastic leukemia, which is dim to negative for surface CD3 (second plot) but positive for intracellular CD3 and TdT (fifth plot). e, Acute leukemia with expression of multiple myeloid antigens including CD14, CD33, and most significantly myeloperoxidase, as well as multiple B-lymphoid markers including CD19 and CD79a. This neoplasm also expressed myeloid-associated CD36 and CD64 and B-lymphoid associated CD22. No cytogenetic abnormalities were identified; hence, this case is best classified as a mixed phenotype acute leukemia, B/myeloid by 2008 World Health Organization criteria. Abbreviations: IC, intracellular staining; TdT, terminal deoxynucleotidyl transferase.
invariably, are associated with monocytic differentiation; the latter usually has CD34− blasts. A subset of acute myeloid leukemias with NPM1 mutations has been reported to show an unusual immunophenotype with strong expression of CD33 and myeloperoxidase but minimal expression of other myeloid and monocytic antigens.7 This group is apparently distinct from the better-known subset of acute myeloid leukemias with “cuplike” nuclear invaginations, which have both NPM1 and FLT3-ITD mutations, and are also CD34+, HLA-DR−, and myeloperoxidase− but show less restricted myeloid antigen expression patterns.6,8

Acute myeloid leukemia with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 may show granulocytic, myelomonocytic, or more rarely, megakaryocytic differentiation. Acute myeloid leukemia with t(6;9)(p23;q34); DEK-NUP214 and acute myeloid leukemia with mutated CEBPA generally show nonspecific granulocytic or myeloid differentiation, although monocytic features may also be present.

**ACUTE MYELOID LEUKEMIA NOT OTHERWISE SPECIFIED**

A subset of acute myeloid leukemias are not currently assigned to a distinct molecular/genetic subtype, nor do they meet criteria for inclusion in one of the additional distinct World Health Organization acute myeloid leukemia categories described below. These cases are grouped by combined morphologic, cytochemical, and immunophenotypic features into categories reminiscent of the prior French-American-British system. Results of flow cytometric analysis support the morphologic impression, but immunophenotyping is perhaps more important to evaluate for unexpected antigen expression, which might prompt recategorization, for example, expression of lymphoid markers indicative of a mixed phenotype acute leukemia.

By definition, acute myeloid leukemia with minimal differentiation does not show definitive evidence of myeloid differentiation by morphologic or routine cytochemical methods. A comprehensive immunophenotypic evaluation is crucial in these cases to assign lineage to the neoplastic population and rule out a leukemic presentation of a mature lymphoma process. Antigens normally expressed on myeloid blasts are frequently present including CD34, CD117, and HLA-DR; TdT expression is also not uncommon. Pan-myeloid antigens such as CD13 and CD33 are present, but markers normally restricted to maturing myeloid cells such as CD15, CD36, and CD64 are typically absent. Although cytochemical stain results for myeloperoxidase are negative, limited expression may be detected by flow cytometry. B- and T-lineage markers are absent, but CD7 may be seen in the absence of CD3. Acute myeloid leukemia without maturation, acute myeloid leukemia with maturation, and the blasts of acute panmyelosis with myelofibrosis show nonspecific features, with frequent expression of markers associated with myeloid immaturity, such as CD34 and CD117, combined with variable expression of more general markers of myeloid lineage, including CD13, CD15, CD33, and CD64. The rare cases of acute basophilic leukemia reported have frequently shown expression of common myeloid antigens such as CD13 and CD33, as well as CD9, CD11b, and CD123.9

In keeping with the morphologic impression, acute myelomonocytic leukemia and acute monoblastic or monocytic leukemias display immunophenotypic evidence of monocytic differentiation. In the former, there may be distinct populations of immunophenotypically aberrant blasts not showing definitive evidence of monocytic differentiation, as well as a population of cells demonstrating expression of CD14, coexpression of CD36 and CD64, or bright expression of CD33, CD4, CD11b, or CD11c. By contrast, acute monoblastic and monocytic leukemias usually show a single population of aberrant cells with evidence of monocytic differentiation, which are usually CD34+ and may be CD117+. A recent study10 has suggested that bright expression of CD64 shows strong correlation with this latter group of leukemias.

Although morphologic examination will generally suggest the possibility of an acute myeloid leukemia with erythroid or megakaryocytic differentiation, this information may not be available to the flow cytometry laboratory, and in some cases the morphologic features are not typical. Moderate to bright expression of CD36 is common for acute myeloid leukemias with erythroid or megakaryocytic differentiation, and when present, may prompt additional studies to evaluate this possibility. CD41 (glycoprotein IIb) and CD61 (glycoprotein IIIa) are strongly associated with megakaryocytic differentiation, but care must be taken to rule out artifact due to platelets adhering to the neoplastic cells, as described above for acute myeloid leukemia with recurrent t(1;22). In general, if platelet adherence is a problem, there will also be similar findings with 1 or more nonblast populations, such as mature granulocytes or monocytes, and comparison may be helpful. Although CD42 (glycoprotein Ib) is less frequently expressed in acute leukemia, it offers strong evidence of megakaryocytic differentiation. Moderate to bright expression of CD71 (transferrin receptor) and glycoporphin A (CD235a) provides evidence of erythroid differentiation, but it should be noted that dim expression of the former is seen on many activated cell types, including neoplastic myeloblasts.

**UNIQUE CATEGORIES OF ACUTE MYELOID LEUKEMIA**

Three subtypes in this group—therapy-related acute myeloid leukemia, acute myeloid leukemia with myelo-dysplasia-related changes, and myeloid sarcoma—are defined predominantly on the basis of patient history, morphologic and cytogenetic findings, or anatomic location. All 3 frequently have morphologic and immunophenotypic features of a myelomonocytic, monocytic, or monoblastic leukemia. However, a range of immunophenotypes may be seen and do not usually impact diagnostic categorization, as the other features supersede the immunophenotypic findings. However, complete characterization should be performed and reported to support the diagnosis and aid in monitoring of response to therapy.

Myeloid leukemia associated with Down syndrome often shows megakaryocytic features including expression of CD36, CD41, and CD61. The most important differential is generally with transient abnormal myelo-poesis, which has a similar phenotype, although expression of CD11b and CD13 is more common in the leukemia form.11 Clinical and cytogenetic correlation, including the patient’s age, is crucial in this diagnosis.
Blastic plasmacytoid dendritic cell neoplasm most frequently involves the skin, and therefore is less likely to be submitted for flow cytometry as an initial diagnosis. However, staging evaluation does reveal peripheral blood or bone marrow disease in most cases, and in this context, flow cytometry is frequently helpful, especially when involvement of these sites is limited. This neoplasm characteristically expresses CD4, CD45RA, CD56, CD123 (bright), and HLA-DR. There is minimal expression of B-lineage, T-lineage, or myeloid-specific markers, with CD7 and CD33 being most common. Results with CD34 and CD117 are usually negative, but results with TdT may be positive. Careful correlation with clinical, morphologic, immunohistochemical, and cytochemical features is required to avoid erroneous diagnosis of this rare entity.

**Monitoring Minimal Residual Disease in Acute Myeloid Leukemia**

Current chemotherapy protocols are highly effective in producing clinical remission in patients diagnosed with acute myeloid leukemia, but most will eventually have a relapse. Cytogenetic and molecular characteristics stratify a proportion of patients into low- and high-risk groups, but most lack definitive prognostic features, resulting in classification as “standard” risk. For these patients, presence of minimal residual disease after chemotherapy, defined as the persistence or recurrence of neoplasia at a level not recognizable by conventional morphologic review, has been shown to have prognostic significance in both adults and children. These results may be used to tailor postinduction therapy, as the likelihood of relapse in patients with and without minimal residual disease after induction chemotherapy may vary by as much as 5-fold. Furthermore, early reductions in peripheral blood blast counts have been shown to correlate with eventual clinical remission and disease-free survival. Two major modalities have been used to measure minimal residual disease in patients with acute myeloid leukemia: molecular testing and multiparameter flow cytometry. The former is highly sensitive for those patients with recurrent genetic abnormalities detectable by polymerase chain reaction, such as t(15;17), t(8;21), and inv(16). An advantage of flow cytometry is that it provides a direct quantification of viable leukemia cells as a proportion of total leukocytes. In those cases in which both flow cytometry and molecular analyses of minimal residual disease are informative, there has been overall concordance, although the sensitivity of the 2 techniques is highly variable, depending upon the molecular and immunophenotypic features of the diagnostic specimen. However, at the current time, most acute myeloid leukemia cases are not amenable to molecular monitoring of minimal residual disease.

By contrast, multiparameter flow cytometry may identify an abnormal/aberrant “leukemia-associated immunophenotype” in more than 90% of cases, if a broad spectrum of monoclonal antibodies is used. Aberrancies include absence of expected antigens (eg, loss of CD13 or CD33), overexpression of antigens (eg, bright expression of CD33), asynchronous expression of antigens (eg, simultaneous coexpression of immature and mature markers like CD34 and CD11b), lineage infidelity (eg, expression of CD19), and more rarely, aberrant light scatter properties (eg, blasts with increased orthogonal scatter). Aberrant expression of CD2, CD7, CD11b, CD19, and CD56 has been reported as the most helpful. The lower limit of sensitivity is affected by several parameters including sample quality, number of cells analyzed, the degree of immunophenotypic aberrancy, and the technical expertise of the testing laboratory. Ideally, levels of leukemic cells as low as 0.01% may be recognized. Because of the importance of minimal residual disease detection, a thorough immunophenotypic characterization should be performed on all new acute myeloid leukemias even if the flow cytometric results are not required for the initial diagnosis, as subtle immunophenotypic features may be crucial to distinguish persistent disease from reactive changes related to therapy. This immunophenotype should be explicitly documented in the flow cytometry report, as patients frequently receive follow-up treatment in different centers from those where their conditions were originally diagnosed. Rarely, no overt immunophenotypic abnormality will be present at diagnosis, and in these cases, the sensitivity of flow cytometric evaluation for minimal residual disease may be greatly reduced. An additional complicating factor is antigenic shift, although the number of cases in which immunophenotypically aberrant blasts convert to an entirely normal pattern of antigen expression is probably low.

**LYMPHOBLASTIC LEUKEMIA/LYMPHOMA**

As for acute myeloid leukemia, immunophenotypic evaluation of lymphoblastic leukemia/lymphoma (formerly known as acute lymphoblastic leukemia) is essential to confirm the diagnosis and perform further subclassification. With the exception of Auer rods, which when present, unequivocally indicate a myeloid lineage, there is no definitive morphologic feature distinguishing acute myeloid from lymphoblastic leukemia. In addition, there is no reliable morphologic criterion by which B-lineage and T-lineage lymphoblastic leukemias/lymphomas can be distinguished. This latter subdivision is important, as lineage does provide meaningful clinical information, which impacts both prognostic and therapeutic considerations. After lineage has been established, clinical features, such as peripheral leukocyte count and patient age, as well as genetic features including DNA ploidy, stratify patients into different risk groups. Although morphologic variants of lymphoblastic leukemia/lymphoma do exist, in general, cytologic features do not correlate well with prognostic or specifically known molecular/genetic lesions. Some recurrent genetic lesions are associated with particular immunophenotypic features, however.

**B-Lymphoblastic Leukemia/Lymphoma**

Most cases of B-lymphoblastic leukemia/lymphoma show strong expression of multiple B-lineage markers including CD19, CD22, and CD79a. Expression of CD10, often somewhat brighter than for normal immature B-lymphocytes (hematogones), is classic but neither specific nor required for diagnosis. PAX5 and TdT are also commonly seen, while expression of leukocyte common antigen (CD45), CD34, and CD20 may show any pattern of expression from bright to negative (see Figure 3, c for a representative example). Surface immunoglobulin is very rarely present, even in cases with immunoglobulin gene rearrangement. Inappropriate expression of myeloid lineage markers, especially CD13 and CD33, and less frequently, CD15, is common, but myeloperoxidase...
positivity would indicate a mixed phenotype acute leukemia. In some cases, the B lymphoblasts do not show aberrant gain and loss of any single antigen, but instead show only an abnormal or “asynchronous” pattern of marker coexpression. For this reason, careful evaluation of the pattern of marker expression may be crucial, particularly in a regenerating posttreatment bone marrow, as normal B-lymphocyte precursors (hematogones) may be increased in these samples. In particular, normal hematogones are initially CD10+, CD22(dim+), CD34+, and TdT+, losing expression of CD34 and TdT before acquiring expression of CD20, and finally losing expression of CD10 only as they develop into mature B lymphocytes with surface antibody expression. B-lymphoblastic leukemia may show a similar spectrum of antigen expression, but often with a more restricted pattern and derangement of the normal maturational sequence. For example, individual neoplastic lymphoblasts may simultaneously express CD34 and CD20, which would not be observed in normal maturing hematogones.

B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities generally conforms to the pattern of CD marker expression described above, although some subtypes show reproducible but not absolute differences in marker profiles. Perhaps the most distinctive are examples associated with t(v;11q23); MLL rearranged, which are often CD10+ and CD15+, particularly when t(4;11)(q21;q23); AF4-MLL is present. The aberrations t(9;22)(q34;q11.2); BCR-ABL1 and t(12;21)(p12;q22); TEL-AML1 also show expression of myeloid-associated antigens more frequently than most B-lymphoblastic leukemias, especially antigens CD13 and CD33. B-lymphoblastic leukemia with recurrent t(1;19)(q23;p13.3); TCF3-PBX1 is reportedly associated with absence of CD34 as well as expression of CD9. The remaining forms of B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities lack known distinguishing immunophenotypic features.

**T-Lymphoblastic Leukemia/Lymphoma**

Although recurrent genetic abnormalities are known in T-lymphoblastic leukemia/lymphoma, their role in prognosis is less clear than for B-lymphoblastic leukemia/lymphoma and currently distinct categories are not recognized in the World Health Organization classification. Moderate to bright expression of CD3, either on the cell surface or within the cytoplasm, is considered the most lineage-specific marker of T-cell differentiation. In addition, other T lineage–associated markers including CD1a, CD2, CD4, CD5, CD7, and CD8 are variably expressed; it would be rare to see expression of CD3 in isolation. Terminal deoxynucleotidyl transferase, CD34, and CD1a each suggest an immature T-lymphoid process, with TdT the most likely to be present (Figure 3, d). CD10, CD79a, and rarely, PAX5, may be positively expressed and individually do not disprove a diagnosis of T-lymphoblastic leukemia/lymphoma. Myeloid antigens, especially CD13 and CD33, may also be seen. More rarely, CD117 expression is present and has been associated with FLT3-activating mutations. In adults, expression of CD1a and absence of CD13 have been associated with improved survival. Although it is possible in some cases to further correlate the neoplastic phenotype with a stage of normal thymocyte differentiation, this categorization has only inconsistently been correlated with prognosis, and a recent study suggests it is of limited significance in adults.

**Monitoring of Minimal Residual Disease in Lymphoblastic Leukemia/Lymphoma**

Detection of minimal residual disease in lymphoblastic leukemia/lymphoma has similar implications as in acute myeloid leukemia, correlating with a worse prognosis, especially when present at the end of consolidation. In addition, early treatment responses (blast reduction) also predict remission status after induction as well as likelihood of clinical relapse after therapy. Pediatric studies have demonstrated that it is not only the presence of blasts that is important in minimal residual disease, but also their absolute percentage, with levels as low as 0.1% to 0.01% correlating with increased risk of relapse. As significant numbers of T-lineage precursor cells are not normally present in the bone marrow, the identification of T-lineage cells that are CD34+ or TdT+ at this site strongly suggests residual disease. This is in contrast to B-lineage precursors (hematogones). In fact, hematogones are frequently increased in regenerating, posttherapy bone marrow, but they do retain their characteristic immunophenotypic features. Therefore, the presence of immature B-lineage precursors is not necessarily indicative of persistent/recurrent B-lymphoblastic leukemia. Careful evaluation for immunophenotypic aberrancy, including an abnormal pattern of maturation, is crucial and will be diagnostic in most cases. Newer markers, such as CD81, may also prove helpful in this distinction. A significant fraction of recurrent lymphoblastic leukemias/lymphomas will show an immunophenotypic shift, and therefore it is important that minimal residual disease panels not be too narrowly restricted.

Multiple studies have shown correlation between flow cytometric and molecular genetic studies of minimal residual disease in lymphoblastic leukemia/lymphoma, the latter predominantly by real-time quantitative polymerase chain reaction detection of B- and T-cell gene rearrangements or recurrent genetic translocations. However, the utility and sensitivity of these 2 modalities for any specific patient may vary. Although molecular studies with patient-specific oligonucleotide probes are generally considered more sensitive than flow cytometry, the creation and application of these probe sets require molecular expertise. In addition, subsets of patients are not amenable to polymerase chain reaction–based methods owing to the absence of a specific, detectable gene rearrangement or ongoing mutation in the variable regions of the immunoglobulin or T-cell receptor genes. It has been suggested that the most sensitive strategy may be to apply both flow cytometry and molecular studies to assess minimal residual disease.

**ACUTE LEUKEMIAS OF AMBIGUOUS LINEAGE**

The 2008 World Health Organization classification combines the former categories of “bilineal” and “biphenotypic” acute leukemias into the single category of mixed phenotype acute leukemia. In combination with the rare acute undifferentiated leukemias, these neoplasms constitute the acute leukemias of ambiguous lineage. Extensive immunophenotypic analysis is required for evaluation of these neoplasms, as morphologically distinct blast populations may not be evident. In general, a mixed phenotype acute leukemia indicates the presence of either (1) two distinct blast populations meeting criteria for at
least 2 of the following entities: B-lymphoblastic leukemia, T-lymphoblastic leukemia, and acute myeloid leukemia; or (2) a single population showing definitive evidence of differentiation toward 2 of these lineages. In this latter case, the World Health Organization provides specific guidelines, that is, myeloperoxidase staining or expression of 2 or more mononcyclic antigens (CD11c, CD14, CD64, non-specific esterase, lysosome) indicates myeloid lineage; strong cytoplasmic or surface CD3 expression indicates T lineage; and strong CD19 expression associated with weak expression of CD10, CD22, or CD79a or weak CD19 expression plus strong expression of 2 of the same markers indicates B-lineage. Therefore, at a minimum, all acute leukemias should be assayed for myeloperoxidase, a selection of mononcyclic markers, CD19, and cytoplasmic/surface CD3 to detect unsuspected cases of mixed phenotype acute leukemia. Currently, there is some ambiguity in the interpretation of these markers, particularly in cases in which an apparently lymphoblastic leukemia shows limited expression of myeloperoxidase by flow cytometry not corroborated by cytochemical staining. In the absence of the recurrent genetic abnormalities (t(9;22)(q34;q11.2); BCR-ABL1 or t(v;11q23); MLL rearranged, subclassification of mixed phenotype acute leukemias is based upon the lineages present. With the current criteria, the most commonly identified form of mixed phenotype acute leukemia appears to be the combination of B-lymphoid and myeloid differentiation (Figure 3, e).

CONCLUSION

Ongoing developments in the understanding of hematolymphoid malignancies have resulted in revision to prior classification systems, better reflecting clinically relevant entities that affect prognosis and therapy. Increasingly, acute leukemias are defined by discrete, recurrent genetic abnormalities. Despite these advances, morphologic and immunophenotypic analyses remain as the foundation for initial evaluation, providing a rapid assessment and directing specific molecular genetic tests, which are impractical to apply in a universal or “shotgun” approach. In addition, immunophenotypic data obtained via flow cytometry may directly correlate with prognosis. Finally, as molecular medicine evolves, specific antitumor agents, including monoclonal antibodies to tumor antigens, are transitioning from investigational to clinical use.94-96 Flow cytometry provides a direct assessment of the varied surface antigen expression on hematolymphoid neoplasms,42 facilitating a rational and individualized selection of such targeted immunotherapy strategies.

References


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Plan now to submit abstracts and case studies for the College of American Pathologists (CAP) 2011 meeting, which will be held September 11th through the 14th in Grapevine, Texas. Submissions for the CAP ’11 Abstract Program will be accepted from:

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