Acute Leukemia Immunohistochemistry

A Systematic Diagnostic Approach

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● *Context.*—The diagnosis and classification of leukemia is becoming increasingly complex. Current classification schemes incorporate morphologic features, immunophenotype, molecular genetics, and clinical data to specifically categorize leukemias into various subtypes. Although sophisticated methodologies are frequently used to detect characteristic features conferring diagnostic, prognostic, or therapeutic implications, a thorough microscopic examination remains essential to the pathologic evaluation. Detailed blast immunophenotyping can be performed with lineage- and maturation-specific markers. Although no one marker is pathognomonic for one malignancy, a well-chosen panel of antibodies can efficiently aid the diagnosis and classification of acute leukemias.

Objective.—To review important developments from recent and historical literature. General immunohistochemical staining patterns of the most commonly encountered lymphoid and myeloid leukemias are emphasized. The goal is to discuss the immunostaining of acute leukemias when flow cytometry and genetic studies are not available.

Data Sources.—A comprehensive review was performed of the relevant literature indexed in PubMed (National Library of Medicine) and referenced medical texts. Additional references were identified in the reviewed manuscripts.

Conclusions.—Immunophenotyping of blasts using an immunohistochemical approach to lymphoid and myeloid malignancies is presented. Initial and subsequent additional antibody panels are suggested to confirm or exclude each possibility in the differential diagnosis and a general strategy for diagnostic evaluation is discussed. Although the use of immunohistochemistry alone is limited and evaluation by flow cytometry and genetic studies is highly recommended, unavoidable situations requiring analysis of formalin-fixed tissue specimens arise. When performed in an optimized laboratory and combined with a careful morphologic examination, the immunohistochemical approach represents a useful laboratory tool for classifying various leukemias.

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H istorically, acute leukemia (AL) classifications used blast morphology and cytochemical stains to categorize the diseases broadly into acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).[1–4] The current World Health Organization classification of tumors of hematopoietic and lymphoid tissues segregate ALs based on lineage as demonstrated by antigen expression into lymphoid or myeloid malignancies.[5] Within each lineage, distinct subtypes are defined based on clinical and morphologic features in conjunction with immunophenotyping by immunohistochemistry (IHC) and/or flow cytometry (FC) and an emphasis toward classification by molecular genetics.[6] With infrequent exception, AL can be characterized sufficiently to assign a lineage and effort to do so is warranted because of the marked differences in treatment and prognosis resulting from these 2 disparate diagnoses.[7,8] Only occasionally a definitive lineage is

not able to be reliably assigned because of the promiscuous nature of antigen expression, and despite use of the available diagnostic ancillary tools, qualifies for the classification of AL of ambiguous lineage.

Pathologists often classify ALs without difficulty because of the accessibility of the neoplastic cells within the peripheral blood and/or bone marrow aspirate allowing flow cytometric analysis and genetic studies. Flow cytometry represents a powerful methodology because of the ability to rapidly sort neoplastic populations and simultaneously perform multiple antigen analyses[9,10]; however, it has several important limitations (Table 1). Additionally, an appropriate specimen with adequate cellularity is not always readily available. For example, a "dry tap" because of bone marrow fibrosis, amyloid deposition, extreme hypercellularity, or technical problems may lack diagnostic cells and processing delays may lead to poor viability. Flow cytometric studies may not be routinely requested if leukemia is not an initial diagnostic consideration. This is a common scenario in extramedullary or extranodal site biopsies. Similarly, fresh cells may not be consistently submitted for consultation cases, and the technology may not be immediately accessible in community settings.

The application of IHC to diagnostic bone marrow specimens is a relatively new practice.[11–13] The earliest IHC studies were limited by inconsistent methods, low-affinity antibodies, and uncertain interpretations. However, many

Table 1. Comparison of Immunophenotyping Techniques	
Immunohistochemistry	**Flow Cytometry**
• Can use fixed/archived tissue	• Need fresh cells or tissue
• Architectural and cytologic correlation	• Limited morphologic correlation
• Can assess nonviable "ghost" cells	• Cannot assess nonviable cells
• Longer turnaround time (hours to days)	• Shorter turnaround time (minutes to hours)
• Subjective result interpretation	• Less subjective result interpretation
• Semiquantitative results	• Quantitative results
• High background staining for immunoglobulin light chains	• Good to detect immunoglobulin light-chain restriction
• Usually limited to a single antibody per slide	• Multiple antibodies/fluorochromes per test
• Fewer antibodies available	• Greater antibody selection
• Easily transported to reference laboratory for special studies	• Loss of viability issues with transport

recent advances have significantly improved paraffin-section IHC. These include antigen retrieval techniques, automated staining devices, and commercial antibody production.[14–16] Immunohistochemistry now represents a universally accessible immunophenotyping technique that can be rapidly and accurately applied to leukemia diagnosis. It is particularly useful for analyzing malignant cells that are too fragile to remain intact during specimen processing or the hydrodynamic focusing steps of flow cytometric analaysis.[17] Plasmacytoid, megakaryocytic, and nonviable "ghost cells" have a high propensity to shear in the flow chamber. Immunohistochemistry can also readily detect nuclear and cytoplasmic antigens such as terminal deoxynucleotidyl transferase (TdT) and myeloperoxidase (MPO), respectively, which cannot be identified by FC without prior membrane permeabilization. In addition, IHC reveals architectural features and estimates tumor cellularity in paraffin sections.[18,19] The comparative features of FC and IHC are listed in Table 1.

In the event an AL diagnosis is suspected without access to diagnostic fresh tissue, assessment by IHC becomes imperative. Requisite familiarity with certain aspects of immunophenotypic interpretation of hematopoietic neoplasms are (1) knowledge of the antigen expression at normal stages of cell maturation in lymphoid and myeloid cells (ie, CD20 is not usually expressed by B lymphoblasts and CD3 is not expressed on the surface and is only found within the cytoplasm of T lymphoblasts); (2) knowledge of the accepted lineage-defining (lineage-specific) and merely lineage-associated markers (ie, CD3 and CD22 are lineage-specific markers for T and B blasts, respectively, whereas CD2, CD7, and CD56 are lineage-associated markers that can be seen in multiple types of blastic neoplasms); (3) knowledge of marker variability by method (ie, CD10 is more sensitive by IHC, whereas CD15 is more sensitive by FC); and (4) knowledge of marker availability. Some markers commonly used in FC are not routinely available by IHC or are currently not reliably tested in paraffin, such as CD19, CD13, and CD33.

Immunologic markers have proven value for leukemia diagnosis and classification, especially when the blasts are morphologically undifferentiated or cytochemical stains are indeterminate.[10,20] The goals of IHC should be to confirm the leukemic condition, identify its hematopoietic lineage, and establish the diagnosis. An unnecessarily excessive number of markers should not be immediately ordered in an attempt to thoroughly address the original differential diagnosis or maintain a rapid turnaround time. This can become quite costly, and it can be counterproductive when unexpected results occur. Rather, a well-planned initial panel containing a few antibodies followed

by a focused second panel is more appropriate. Careful morphologic evaluation will help guide rational antibody selection.

Because chronic leukemias, chronic myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelomonocytic leukemia are diagnosed using a combination of clinical, molecular, and morphologic features with minimal reliance on immunophenotyping, chronic myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelomonocytic leukemia are not discussed in this manuscript. Plasmacytoma, multiple myeloma, and plasma cell leukemia usually do not pose a significant diagnostic dilemma, and a limited IHC panel may confirm the morphologic diagnosis. This usually includes CD138 to quantify the plasma cell infiltrate, CD56 and CD117 to document the aberrant phenotype, and cytoplasmic κ/λ to demonstrate the light-chain restriction.[21]

This article mainly reviews the application of IHC in the diagnostic assessment of ALs in the absence of FC immunophenotyping. The initial section addresses the expected immunophenotype in precursor B cells, precursor T cells, myeloid cells, and hematodermic neoplasm cells. The subsequent section suggests practical staining panels and discusses a systematic approach to the diagnosis.

PRECURSOR B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOBLASTIC LYMPHOMA

The disease state composed of immature B cells is termed either precursor B-cell acute lymphoblastic leukemia (B-ALL) (if mainly marrow-based with >25% replacement of bone marrow by lymphoblasts) or precursor B-cell lymphoblastic lymphoma (B-LBL) (if mainly tissue based with ≤25% bone marrow involvement by lymphoblasts). This is an arbitrary distinction. The malignant cells are identical by morphology, immunophenotype, and genetics in both entities. Blasts in B-ALL/B-LBL are small to medium sized with a high nuclear-cytoplasmic ratio, fine nuclear chromatin and small or indistinct nucleoli (traditionally called L1 and L2 type blasts), usually scant blue-gray cytoplasm, and only occasionally some small vacuoles (Table 2). These blasts are distinguished from the neoplastic cells in Burkitt lymphoma, which can also have a leukemic phase. Burkitt lymphoma cells are medium sized with relatively coarse nuclear chromatin, deep blue cytoplasm, and many cytoplasmic vacuoles (traditionally termed L3 type blasts).

An important differential in the diagnosis of B-ALL/B-LBL is from a B-cell non-Hodgkin lymphoma (B-NHL) especially Burkitt lymphoma and diffuse large B-cell lymphoma. This distinction relies on the presence (or absence) of various B-cell markers expressed during B-cell matu-

|		Table 2. Assessment of Acute Leukemia by Morphology		
Suggested Blast Lineage	Nuclear Features	Chromatin Pattern	Cytoplasmic Features	Cytoplasmic Granules
Lymphoid	Round, smooth	Fine/dispersed	Scant, blue-gray	None to rare
Myeloid	Regular, reniform	Coarse/vesicular	Variable	Variable, Auer rods
Promyelocytic	Reniform/bilobed	Variable	Variable	Many, Auer rods
Monocytic	Bilobed/indented	Fine to coarse	Blue-gray	Occasional, fine
Erythroid	Round to oval	Coarse to dense	Vacuoles	None to rare
Megakaryocytic	Large, multilobed	Coarse to dense	Pseudopods	None to rare

ration stages (Figure 1). Use of an antibody panel will allow the classification in cases morphologically ambiguous (Table 3). CD45 (leukocyte common antigen [LCA]) is weakly expressed in B-cell ALL; indeed B lymphoblasts may be completely negative for LCA. Staining for TdT (a specialized DNA polymerase) and CD34 can be performed to confirm the immature stage of these cells. About 95% of B-ALL/B-LBL cases are TdT positive, and although the expression of CD34 is seen in 85% to 90% of cases, TdT is a superior marker (more sensitive and easier to read) than CD34 when evaluating by IHC. CD20, widely used as a screening marker for B-cell lineage, is usually negative in B-ALL/B-LBL (if positive is often weak). If CD20 is negative, other B-cell markers such as CD79a, CD22, or PAX-5 (the PAX5 gene encodes the B-cell lineage–specific activator protein expressed in pre-B cells and mature B cells and is not seen in plasma cells) can be used as CD19 is not reliably available by IHC.[22] However, PAX-5 alone is not lineage specific as a subset of AMLs can express PAX-5 (see later).[23] The expression of CD10 favors a B lineage as this marker is not expressed by AML; however, primitive T-cell acute lymphoblastic leukemia (T-ALL) can sometimes express CD10. Expression of BCL-6 protein favors a B-cell NHL (germinal center cell origin) because BCL-6 is negative in B-ALL/B-LBL.[24,25] As always, the use of IHC results in combination and in context is very important (ie, PAX-5 positive and CD10+ with concurrent TdT positivity favors B-ALL/B-LBL over AML). Rarely, B-ALL can have a mature phenotype with expression of surface light chains. To distinguish these cases from B-NHL, the clinical history, blastlike morphology, and weak expression of CD45RB and/or weak CD20 are useful in supporting B-ALL over B-NHL.

PRECURSOR T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOBLASTIC LYMPHOMA

Like B-ALL/B-LBL, the distinction between precursor T-cell acute lymphoblastic leukemia (T-ALL) and precursor T-cell lymphoblastic lymphoma (T-LBL) is arbitrary and carries the same diagnostic criteria as previously mentioned. Also important in the diagnosis of immature T-cell neoplasms is the phenotype of T cells during the maturation process in the thymus (Figure 2). Generally, T-cell maturation can be divided into cortical and medullary stages. T-ALLs express the cortical thymocyte phenotype, and T-NHLs express the medullary thymocyte phenotype. Like B-cell neoplasms, CD45 (LCA) is usually weak in T-ALL and strong in T-NHL.[26] Terminal deoxynucleotidyl transferase is positive in T-ALL and negative in T-NHL. Other T-cell markers such as CD2, CD5, and CD7 can be variably expressed and are not reliable in distinguishing T-ALL and T-NHL. Coexpression of CD4 and CD8 (double positive) and lack of expression of CD4 and CD8 (double negative) would favor T-ALL. However, a double-negative phenotype is often seen in γ/δ T-NHL and double positivity can be seen in 20% of T-cell prolymphocytic leukemia and rarely in adult T-cell leukemia/lymphoma. A helpful feature is that these mature processes are always

Figure 1. *Immunophenotype during B-cell differentiation. ALL indicates acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; NHL, non-Hodgkin lymphoma; TdT, terminal deoxynucleotidyl transferase; BCL-6, B-cell lymphoma 6; and PAX-5, paired box gene 5. Modified with permission from IARC Press.[5(p121)]*

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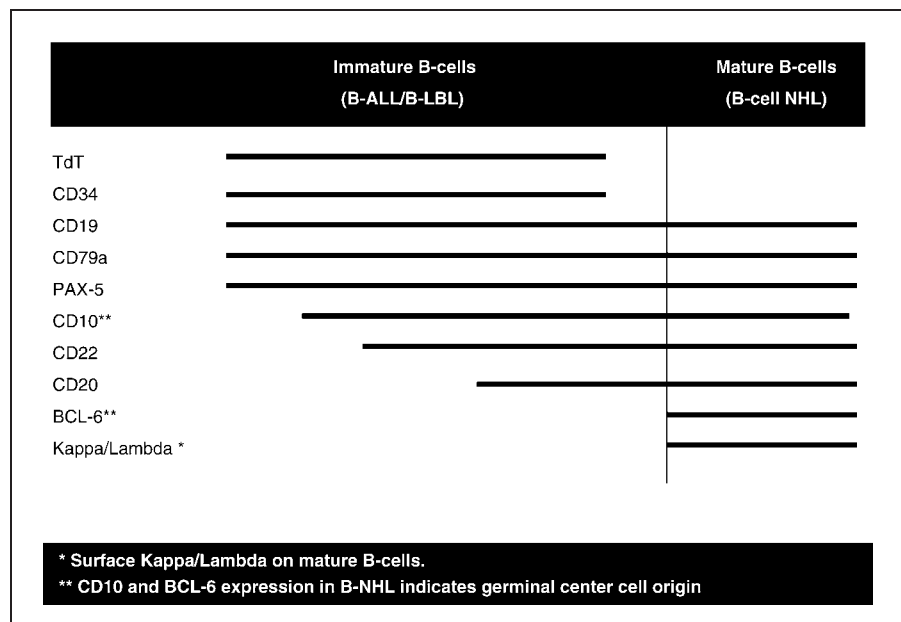
Table 3.

Table 3. Immunohistochemistry Panel Used to Distinguish Between B-Cell Acute Lymphoblastic Leukemia (B-ALL)/ B-Cell Lymphoblastic Lymphoma (B-LBL) and B-Cell Non-Hodgkin Lymphoma (B-NHL)*

	B-ALL/B-LBL	B-Cell NHL
CD45RB (LCA)	Weak positive or negative	Strong positive
CD20	Negative or weak positive	Strong positive
CD22	Negative or positive	Positive
CD79a	Positive	Positive
PAX-5	Positive	Positive
TdT	Positive (95%)	Negative
CD34	Positive (85%)	Negative
CD10	Positive or negative†	Positive or negative†
BCL-6	Negative	Positive or negative‡
Surface light chains	Negative§	Positive

* LCA indicates leukocyte common antigen; PAX-5, paired box gene 5; TdT, terminal deoxynucleotidyl transferase; and BCL-6, B-cell lymphoma 6.
† Early precursor B-ALL can be CD10⁻. CD10 expression is seen in B-NHL of germinal center cell origin (follicular lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma).
‡ BCL-6 is positive in B-NHL of germinal center cell origin (follicular lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma).
§ Rare cases of B-ALL may have surface light-chain expression.

TdT negative. Expression of CD1a also helps to favor T-ALL. A diagnosis of T-ALL should be reconsidered if TdT and cytoplasmic CD3 are negative as other T-cell antigens (CD2, CD5, and CD7) are not lineage specific and can be associated with AML. Although the double-positive or double-negative expression of CD4/CD8 with TdT, CD34, and/or CD1a at peripheral sites (such as blood, bone marrow, lymph node, pleural effusion) favors T-ALL, it is imperative to remember this phenotype is not necessarily diagnostic of lymphoblastic lymphoma if the biopsy contains thymic lymphoid tissue (which normally expresses an immature phenotype). Therefore, the differential diagnosis between T-ALL and lymphocyte-rich thymoma or thymic hyperplasia is challenging on a small biopsy or fine-needle aspirate of the mediastinum as phenotypically the distinction between cortical thymocytes and T-ALL is difficult. However, multicolor FC may help in differentiating thymoma from T-ALL.[27] Rarely, T-ALL can have a mature phenotype (with surface CD3 and CD4 or CD8 class assignment) and lack TdT expression. To distinguish these cases from T-NHL, the clinical history, blastlike morphology, and weak expression of CD45 are useful in supporting T-ALL over T-NHL.

ACUTE MYELOID LEUKEMIA

The current World Health Organization classification subclassifies AML into various categories based on genetic findings, the presence of dysplasia, and/or a history of previous therapy. The World Health Organization categories of AML are as follows:

A. AML with recurrent genetic abnormalities
B. AML with multilineage dysplasia
C. AML and myelodysplastic syndrome, therapy related
D. AML (not otherwise categorized)
E. Acute leukemia of ambiguous lineage

The first 3 categories require genetic studies and/or clinical history to classify. Immunohistochemistry becomes useful in group D (AML not otherwise categorized), which is subdivided based on the traditional French-American-British classification. Acute myeloid leukemia may not be definitively classified with IHC but differentiation toward myeloid, monocytic, erythroid, or megakaryocytic lineages can be demonstrated with appropriate staining panels. Certain staining characteristics may guide genetics testing such as fluorescence in situ hybridization studies on the paraffin-embedded tissue according to the type of blasts present. With these issues in mind, for the purposes of discussion within this manuscript, it is possible to subclassify AML into one of the following groups with IHC:

1. AML–minimally differentiated (AML-MD)
2. AML–myeloid lineage (includes AML without mat-
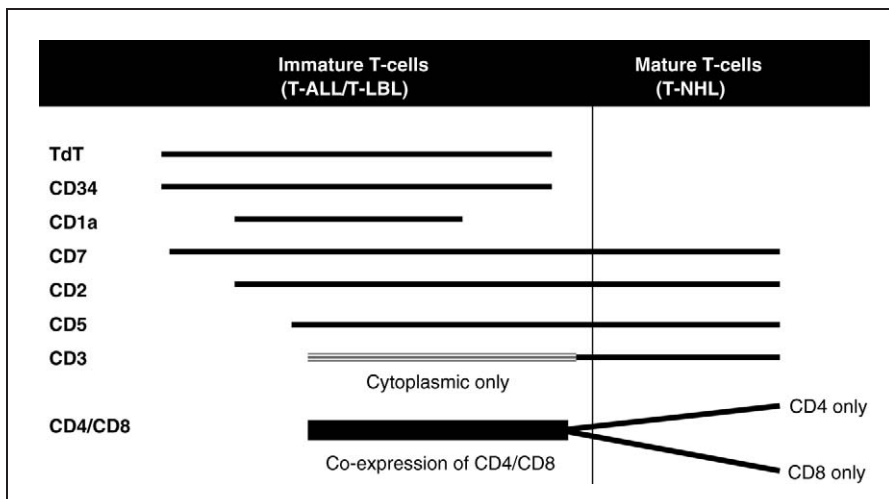
Figure 2. *Immunophenotype during T-cell differentiation. ALL indicates acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; NHL, non-Hodgkin lymphoma; and TdT, terminal deoxynucleotidyl transferase. Modified with permission from IARC Press.[5(p192)]*

uration, with maturation and acute promyelocytic leuke-mia)

3. AML–myelomonocytic (AML-MM)
4. AML–monocytic lineage
5. AML–erythroid lineage (AML-E)
6. AML–megakaryocytic lineage (AML-Meg)

The morphologic features of blasts in AML are widely variable according to the type of leukemia and are described in Table 2. The commonly available antibodies for AML include CD117, MPO, CD68, lysozyme, CD163, MAC 387, HAM56, CD31, CD41, CD61, factor VIII (FVIII), hemoglobin A1, glycophorin A, CD15, and HLA-DR. By IHC, CD34 is not a sensitive marker as it is expressed in only 50% of cases.[28–31] CD117 (also known as c-Kit is a membrane receptor for stem cell factor and is expressed on most hematopoietic progenitor cells including multi-potent hematopoietic stem cells as well as committed myeloid, erythroid, and lymphoid precursor cells) is much more sensitive than CD34; however, mast cells will be positive. Also, CD15 is not as sensitive by IHC as by FC and marks mature granulocytes and therefore may not be reliably assessed with this method. The more sensitive CD13 and CD33 antibodies (positive in >95% of all AMLs) are not commonly available by IHC in most clinical laboratories and have not been shown to give reliable results.

Staining patterns for blasts in ALs can guide a diagnosis into these general groups, which carry different prognostic and sometimes diagnostic implications. A discussion of each category follows.

AML–Minimally Differentiated

Blasts are usually positive for CD117 and HLA-DR. All other markers listed previously are usually negative including MPO. Expression of CD68 is variable. Acute myeloid leukemia as a group can have TdT expression in 20% of cases, and about 90% of AML-MD are TdT positive. The AML-MD cases also commonly express T-cell associated markers such as CD2, CD5, or CD7 without many myeloid-associated antigens except CD13 and CD33, which are not reliably available by IHC. These characteristics make this type of AML difficult to diagnose by IHC alone; however, if CD7, CD2, and/or CD5 are seen in a case of AL, it is essential to do a wide battery of lymphoid and myeloid markers including CD3 and TdT. Expression of CD117 and TdT without CD79a, PAX-5, CD79a, MPO, and CD3 would suggest (not confirm) the diagnosis of AML-MD.

AML–Myeloid Lineage

A myeloid lineage can be established based on the expression of CD117 and MPO. Even when CD117 is negative, these cases are almost always MPO positive and also positive for CD68 and lysozyme. PAX-5 (and CD19) staining correlates highly with AML having t(8;21) abnormality.[23]

AML–Myelomonocytic

As both myeloid and monocytic differentiation is present, blasts are positive for MPO as well as for CD68 and lysozyme. Expression of CD4 can be seen.

AML–Monocytic Lineage

Blasts are less often positive for CD117 and CD34. Myeloperoxidase is negative. CD68, lysozyme, CD15, and HLA-DR are positive. Expression of CD4 in AML suggests monocytic differentiation.

AML–Erythroid Lineage

It is extremely rare for this subtype of AML to present at an extramedullary site. Erythroblasts are variably CD117+ and negative for all myeloid markers (including HLA-DR). However, myeloblasts present will show myeloid associated markers. The erythroid lineage can be established based on hemoglobin A1 expression (or glycophorin A). In the bone marrow core biopsy, the distinction by IHC alone from myelodysplastic syndrome is difficult.

AML–Megakaryocytic Lineage

It is also extremely rare for this subtype of AML to present at an extramedullary site but is very common to receive a dry tap on bone marrow aspiration. Blasts are variably CD117+ and may express myeloid markers but are negative for LCA, CD34, and HLA-DR. The lineage can be established based on CD41 (platelet glycoprotein IIB), CD61 (platelet glycoprotein IIIa), and/or FVIII expression. It is important to note that primitive megakaryoblasts can be negative for FVIII, and CD41 expression can sometimes be observed in other subtypes of AML. CD31 (platelet endothelial cell adhesion molecule) was initially thought to be lineage specific for megakaryocytes but has been seen in other subtypes of AML and in vascular lesions such as angiosarcomas (which are also positive for FVIII and CD34).

Certain cases are not easily classified neatly into one of the previously mentioned groups because of the expression of multiple lineage-specific antigens and therefore require a separate category of AL termed ALs of ambiguous lineage.

AL OF AMBIGUOUS LINEAGE OR AL WITH LINEAGE HETEROGENEITY

Blasts that fail to express the morphologic, cytochemical, and immunophenotypic features of either lymphoid or myeloid differentiation would qualify as an acute undifferentiated leukemia. Nonhematopoietic entities should be sufficiently ruled out before this diagnosis is rendered. If a single population of blasts has morphologic and immunophenotypic evidence showing coexpression of lineage-specific antigens for both lymphoid and myeloid cell types (or B and T lymphoid), a diagnosis of acute biphenotypic leukemia is warranted. Acute bilineal leukemia describes the entity containing a dual population of blasts, each with a separate phenotypic lineage.[32–34] These are rare forms of leukemias and occur in both pediatric and adult patients.

Extensive data on the therapeutic response of these rare leukemias are not yet available, but the current consensus is to treat them with AML-induction chemotherapy followed by bone marrow transplantion[35–37] making the distinction of bilineal or biphenotypic leukemia from ALL with aberrant expression of myeloid markers (20%–40% of lymphoblasts express CD13, CD15, or CD33)[38] an important diagnostic consideration. Acute myeloid leukemia with lymphoid markers (such as expression of CD2, CD4, CD5, CD7, CD19, or PAX-5 in myeloblasts) should be distinguished because some entities have a significantly better prognosis (ie, AML with t(8;21), which commonly coexpresses CD19 and/or PAX-5) and may also require alternative treatment strategies (ie, AML with t(15;17),

Table 4. Gene Rearrangements in Leukemias and Lymphomas*		
	Clonal *IgH*, %	Clonal *TCR*, %
B-ALL	100	20–60
B-NHL	100	10–15
T-ALL	20	>90
T-NHL	<5	>90
AML	<5	<5

* *IgH* indicates immunoglobulin heavy chain gene rearrangement; *TCR,* T-cell receptor gene rearrangement; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; and AML, acute myeloid leukemia.

which commonly coexpresses CD2).[39,40] Therefore, molecular genetic studies may be beneficial.

Diagnosing this form of AL by IHC alone is difficult as the sensitivity and specificity of antibodies varies when compared with monoclonal antibodies used by FC and antigen expression is not as easily assigned to separate blast populations. Also, certain markers such as CD19, CD13, and CD33 are not routinely available by IHC. The European Group for the Immunologic Classification of Leukemia has established a tiered scoring system for the expression of granulocytic, B-cell–specific, and T-cell–specific antigens defining this entity[41,42] However, the assignment should not be made based merely on this scoring system, and the contributions of ancillary testing such as cytogenetics and molecular genetics are important in these situations. The polymerase chain reaction (PCR) for clonal immunoglobulin gene rearrangement (immunoglobulin H [*IgH*]) and clonal T-cell receptor rearrangement (*TCR*) are routinely available for formalin-fixed tissue in paraffin; however, because these may have lineage crossover (ie, B-ALL and T-ALL may have both *IgH* and *TCR* clones), a negative result is more useful than a positive one. In addition, a small percentage of AMLs can be positive for a *TCR* or *IgH* clone.[43] Polymerase chain reaction results should be interpreted with these factors in mind (Table 4).

Because this diagnosis is difficult, especially when limited to only the tools available by IHC, requesting a repeat sample for FC and genetic studies is prudent before making a definitive diagnosis of AL with lineage heterogeneity.

CD4⁺/CD56⁺ HEMATODERMIC NEOPLASM

A morphologic mimic of AL and a commonly overlooked entity in the differential diagnosis of ALs is CD4⁺/CD56⁺ hematodermic neoplasm, formerly known as blastic natural killer (NK)–cell neoplasm. This is an uncommon but very aggressive hematopoietic neoplasm that usually presents in the skin as multiple cutaneous nodules with subsequent involvement of the bone marrow and peripheral blood.[44] The precise lineage of this neoplasm is unresolved and an origin from the plasmacytoid dendritic cell has been proposed.

Skin is the most common biopsy site, and therefore the sample is usually received in formalin. The lesion is characterized by medium-sized blasts resembling lymphoblasts or myeloblasts, and the initial impression is of skin involvement by AL (leukemia cutis). With this in mind, a typical initial IHC panel includes CD45 (LCA), CD3, CD20, PAX-5 (or CD79a), TdT, CD117, CD34, MPO, and CD68. On this panel the neoplastic cells are weakly positive for LCA, variably and focally positive for CD68, and

negative for all the remaining markers except for a few cases (<20%) positive for CD34 and/or TdT. This pattern can be confusing because of the absence of lineage-specific markers. An additional panel of IHC markers will allow a diagnosis to be rendered (CD2, CD7, CD5, CD4, CD8, CD43, CD56, CD1a, and CD123). Of these CD4, CD43, CD56, and CD123 (plasmacytoid dendritic cell marker) are consistently positive, whereas the expression of CD2 and CD7 is variable. CD5 and CD1a are negative. It is interesting to note that CD4, CD43, CD56, and CD123 are also positive when AML[45] involves the skin (granulocytic sarcoma). Classically, CD4⁺/CD56⁺ hematodermic neoplasm is distinguished from AML by clinical presentation, CD123 positivity, and MPO, CD117, CD13, and CD33 negativity[46,47]; thus the distinction between the two must be resolved by a more extensive panel including CD13 or CD33 expression (which are only readily available by FC and are present in AMLs). Focal CD33 positivity has been described in some cases of CD4⁺/CD56⁺ hematodermic neoplasm,[48] and hence the understanding of this entity is not complete and is evolving.

CD4⁺/CD56⁺ hematodermic neoplasm is negative for T-cell intracellular antigen 1 and Epstein-Barr virus and should not be confused with other NK-cell neoplasms such as aggressive NK-cell leukemia or extranodal NK-cell lymphoma, which are CD56⁺, T-cell intracellular antigen 1 positive, Epstein-Barr virus positive, and CD4 negative.[49]

USING IMMUNOHISTOCHEMICAL MARKERS

A working knowledge of the many markers used in hematopoietic neoplasms is imperative in the diagnosis of AL.[50,51] The following discussion addresses each antibody and the staining pattern expected in the different ALs and the most common entities from which they should be distinguished (Table 5). Although no one marker is pathognomonic for one malignancy, a well-chosen panel of antibodies can efficiently aid the diagnosis and classification of ALs (Table 6; Figures 3 and 4).

CD45 (LCA)—Recognizing a Hematopoietic Origin

An undifferentiated high-grade neoplasm warrants an initial panel that includes pancytokeratin, S100, and CD45 (LCA). The expression of CD45 (LCA) is strong in NHLs and weak in ALs, thus weak expression in tumor cells would favor an immature process and the differential diagnosis would include ALL (T or B), AML, and blastic NK-cell neoplasm. It is important to note that some cases of B-ALL/B-LBL and all cases of AML with erythroid and megakaryocytic lineages are CD45 (LCA) negative. Therefore, a negative LCA does not exclude these neoplasms. Although LCA is commonly used to exclude hematopoietic origin in undifferentiated neoplasms, many mature hematopoietic neoplasms can be LCA negative, such as classical Hodgkin, anaplastic large cell lymphomas, and neoplasms with plasmacytic differentiation.

Markers of Immaturity—CD34, TdT, CD117

A weak LCA should prompt antibody selection to confirm an immature process (CD34, TdT, and CD117). CD34 and CD117 are membrane glycoproteins expressed by hematopoietic progenitor cells.[28,29] The expression is strongest in undifferentiated blasts and progressively declines with maturation.[30] CD34 is the more commonly used blast marker; however, it is less sensitive by IHC than by FC. CD117 demonstrates a similar expression pattern and is

Antigens	T-ALL, %	B-ALL, %	AML-MD, %	AML-Myeloid	AML-MM	AML-Monocytic	AML-E	AML-Meg
TdT	99	95	†	†	†	†	†	†
CD34	75	85–90	†	†	†	†	†	†
HLA-DR	10–20	95	Pos	Pos/neg†	Pos	Pos	Neg	Neg
CD1a	20–30	Neg	Neg	Neg	Neg	Neg	Neg	Neg
CD2	90–95	Neg	<5‡	‡	‡	‡	‡	‡
CD3	99	Neg	Neg	Neg	Neg	Neg	Neg	Neg
CD4/CD8	§	Neg			‖	‖		
CD5	95	Neg	<5‡	‡	‡	‡	‡	‡
CD7	>95	Neg	5–10‡	‡	‡	‡	‡	‡
CD10	10–20	85	Neg	Neg	Neg	Neg	Neg	Neg
CD15	20	20–40	Neg	Pos/neg	Pos/neg	Pos/neg	Pos/neg	Pos/neg
CD20	Neg	40–50 (weak)	Neg	Neg	Neg	Neg	Neg	Neg
PAX-5	Neg	>98	Neg	Pos¶	Neg	Neg¶	Neg	Neg
CD22	Neg	90	Neg	Neg	Neg	Neg	Neg	Neg
CD79a	Rare pos	80	Neg	Neg	Neg	Neg	Neg	Neg
CD43	>95	80	†	†	†	†	†	†
MPO	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg
CD56	<5	Neg	†	†	†	†	†	†
CD117	<1	Neg	†	†	†	†	†	†
CD41	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
CD61	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
FVIII	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
HgbA	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg

* The arbitrary classification of AMLs in this article is based on the diagnostic workup by immunohistochemistry. For actual classification please refer to *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.*[5] AML-MD indicates acute myeloid leukemia–minimally differentiated; AML-MM, AML–myelomonocytic; AML-E, AML–erythroid; AML-Meg, AML–megakaryocytic; TdT, terminal deoxynucleotidyl transferase; Pos, positive; Neg, negative; PAX-5, paired box gene 5; MPO, myeloperoxidase; FVIII, factor VIII; and HgbA, hemoglobin A.

† TdT is positive in 20% of AML cases and most TdT-positive AML cases are AML-MD (90%). Overall CD34 is seen in 50% of AMLs. HLA-DR and CD34 are often negative in acute promyelocytic leukemia. CD43 can be seen in more than 95% of AML cases. CD56 expression can be seen in 20% of AMLs. CD117 is often positive in AML (80% or more); however, expression can be lost with differentiation.

‡ CD2, CD5, and/or CD7 expression can be seen in 5% to 10% AMLs (CD2 frequent in acute promyelocytic leukemia).

§ T-ALLs are either double CD4+/CD8+ or double CD4−/CD8−.

‖ CD4 expression suggests monocytic differentiation.

¶ PAX-5 expression correlates highly with AML showing t(8;21) abnormality.

Table 6. Lineage-Specific Antigens in Acute Leukemia by Immunohistochemistry	
Lineage	Markers*
Hematopoietic	CD45 (LCA)†
Immature antigens	TdT, CD34, CD117
T cells	CD3‡, TCRβ F1§
B cells	CD22, CD79a, cytoplasmic IgM, PAX-5‖
Myeloid	CD117, MPO
Megakaryoblast	CD41, CD61, FVIII
Erythroid	Hemoglobin A, glycophorin A

* LCA indicates leukocyte common antigen; TdT, terminal deoxynucleotidyl transferase; TCR, T-cell receptor; IgM, immunoglobulin M; PAX-5, paired box gene 5; MPO, myeloperoxidase; and FVIII, factor VIII.

† CD45RB is negative in acute myeloid leukemia (AML)–erythroid, AML–megakaryocytic, and a few cases of B-cell acute lymphoblastic leukemia.

‡ Cytoplasmic CD3 is seen in natural killer cells.

§ TCRβ F1 is an immunostain specific for T cells (α/β type).
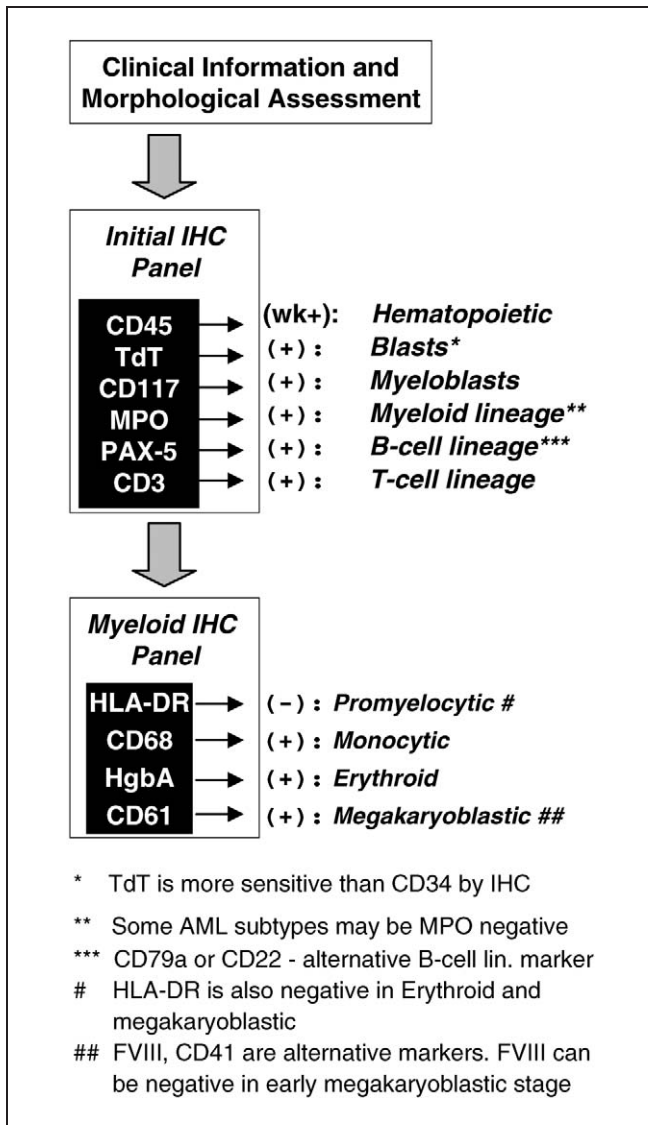
‖ PAX-5 is also expressed in AML with t(8;21).

more sensitive than CD34 in AML.[52] Thus, CD34 alone should not be relied on as a blast marker. CD117 strongly favors a myeloid blast lineage because it is not seen in B-ALL/B-LBL and is reported only very rarely in T-ALLs[53] (<2%). CD117 is also expressed by mast cells, so it can confirm a diagnosis of mast cell leukemia, systemic mastocytosis, and other related disorders.[54,55] Terminal deoxynucleotidyl transferase is the most sensitive by IHC as

99% of T-ALL, 95% of B-ALL, and 20% of AML (90% of AML-MD) are TdT positive. Although TdT is not helpful in differentiating various ALs, it is a useful sensitive marker for ALs, which if positive, excludes high-grade NHLs, which are a common consideration.[56,57]

Recognizing the Lineage of Blasts (Lineage-Specific Markers)

CD3 is a lineage-specific protein considered to be a pan–T-cell marker.[58,59] CD3 is a multidomain component of the T-cell receptor complex that is normally located in the cytoplasm of the immature blast and on the membrane of mature thymic lymphocytes. CD3 is also expressed in the cytoplasm of NK cells; thus, the subcellular localization of CD3 can suggest the lineage and maturation of T-cell and NK-cell neoplasms (NK cells contain the cytoplasmic ε chain component of CD3). Surface CD3 expression is seen in T lymphocytes but not in T lymphoblasts and NK cells; however, cytoplasmic CD3 by IHC is seen in T lymphocytes, T lymphoblasts, and NK cells.[60]

Almost all cases of T-ALL are cytoplasmic CD3+ and TdT positive; in fact, a diagnosis of T-ALL would require careful consideration in the absence of cytoplasmic CD3 staining. CD3 by IHC is negative in B-ALL, AML, and blastic NK-cell neoplasm. CD2, CD5, and CD7 are T-cell–associated antigens that are frequently found on other neoplasms, and T-ALL can be variably positive for these antigens. These T-cell–associated antigens are negative in

Clinical Information and Morphological Assessment

↓

Initial IHC Panel

CD45	→	(wk+):	*Hematopoietic*
TdT	→	(+):	*Blasts**
CD117	→	(+):	*Myeloblasts*
MPO	→	(+):	*Myeloid lineage***
PAX-5	→	(+):	*B-cell lineage****
CD3	→	(+):	*T-cell lineage*

↓

Myeloid IHC Panel

HLA-DR	→	(−):	*Promyelocytic* #
CD68	→	(+):	*Monocytic*
HgbA	→	(+):	*Erythroid*
CD61	→	(+):	*Megakaryoblastic* ##

* TdT is more sensitive than CD34 by IHC

** Some AML subtypes may be MPO negative

*** CD79a or CD22 - alternative B-cell lin. marker

\# HLA-DR is also negative in Erythroid and megakaryoblastic

\#\# FVIII, CD41 are alternative markers. FVIII can be negative in early megakaryoblastic stage

Figure 3. *Antibody panel selection and lineage differentiation. In B-cell acute lymphoblastic leukemia (B-ALL), paired box gene 5 (PAX-5) is more sensitive than CD79, CD22, and CD20. By immunohistochemistry (IHC), terminal deoxynucleotidyl transferase (TdT) is a more sensitive stain than CD34 in acute lymphoblastic leukemias. Two other B-cell markers, B-cell Oct-binding protein 1 (BOB.1) and octamer-binding transcription factor 2 (Oct2), are also expressed by B-ALL in 90% and 20% of cases, respectively.[21] MPO indicates myeloperoxidase; HgbA, hemoglobin A; AML, acute myeloid leukemia; and FVIII, factor VIII.*

B-ALL, but 1 or 2 of the antigens are seen in 5% to 10% of AMLs. CD2 expression is commonly seen in acute promyelocytic leukemia, especially the microgranular variant.[39,40] IHC studies for T-cell receptor protein (β F1) have rather limited value in T-ALL diagnosis.

CD79a forms part of the B-cell receptor complex and translocates from the cytoplasm to the surface membrane as the B cell matures from a blast to the resting B-cell stage.[61,62] Although frequently treated as such, CD20 is not a pan–B-cell marker and is often negative in B-ALL. A negative stain should be evaluated carefully as B-ALL can sometime be weak to focally positive. When CD20 is negative, assignment to a B-cell lineage can be evaluated by CD79a, PAX-5, or CD22.[63,64] All 3 markers are available by

IHC. CD22 is lineage specific for B cells[59] but is not expressed in the early stage of B lymphoblasts. As B cells mature, first cytoplasmic then surface expression becomes detectable. PAX-5 is almost always expressed in B lymphoblasts. PAX-5, however, is seen in some AMLs with t(8;21) abnormality.[23,64] CD79a is expressed in 80% cases of B-ALL[21] and has been described only rarely in T-ALL.[65] Therefore, expression of these markers should be evaluated in conjunction with other stains. The cytoplasmic IgM stain can be used to demonstrate the B lineage of blasts. Immunohistochemistry studies for immunoglobulin light chain have rather limited value in B-ALL diagnosis. Rare cases of B-ALL can have surface light-chain expression.

For practical purposes, CD117 is a specific marker for AML (<2% of T-ALLs have been reported as positive).[53] Rarely AML-MD can be CD117 negative, and because they are also usually negative for MPO, a diagnosis by IHC can be problematic. Expression of markers such as HLA-DR, CD56, or CD15 (not always positive), along with the absence of lymphoid markers, supports a myeloid lineage. The results of PCR for TCR should be interpreted in context as IgH and TCR clones have been described in cases of AML.[43]

Myeloperoxidase is an enzyme present in myeloid primary granules. It is predominantly negative in AML-MD (by definition, less than 3% blasts are positive) and strongly positive in most blasts of AML-myeloid.[31,66,67] It is negative in monocytic, erythroid, and megakaryoblastic leukemia cells.

Frequency of Various Markers in Acute Leukemia

Lymphoid blasts may demonstrate considerable immunophenotypic heterogeneity.[68] Approximately 40% and 20% of B-ALL and T-ALL cases, respectively, aberrantly express markers of the opposite lymphoid lineage and uncommonly aberrantly express myeloid markers such as MPO or activation markers such as CD56.[69,70] After narrowing the differential diagnosis with results from the initial IHC panel, additional blast or lineage markers may be used to verify the suspected classification or exclude other options. However, each interpretation should be considered in the overall morphologic and IHC context when using these less specific antibodies.

T-Cell Antigens—CD1a, CD2, CD3, CD4, CD5, CD7, CD8.—*B-Cell Acute Lymphoblastic Leukemia.*—B-ALL is usually negative for all T-cell markers.

T-Cell Acute Lymphoblastic Leukemia.—About 20% to 30% of cases are CD1a positive. Almost all cases of T-ALL are cytoplasmic CD3 and TdT positive. CD2, CD5, and CD7 are variably present. T-ALL can be double positive for CD4 and CD8 or lack both CD4 and CD8. The diagnosis of T-ALL should be made with caution if cytoplasmic CD3 is negative as CD2 or CD5 or CD7 can be seen in AMLs.

Acute Myeloid Leukemia.—CD1a, CD3, and CD8 are not seen in AMLs. About 5% to 10% of AMLs can express CD2, CD5, or CD7 (aberrant expression of 1 or 2 T-cell–associated antigens but not 3).[71–73] CD2 is commonly seen in acute promyelocytic leukemia, especially the microgranular variant.[39,40] The expression of CD4 in AMLs suggests monocytic differentiation.

CD4+/CD56+ Hematodermic Neoplasm.—Neoplastic cells are negative for CD1a, CD3, CD5, and CD8. The expression of CD2 and CD7 is variable, whereas CD4 is positive in all cases.

B-Cell Antigens—CD20, CD22, CD79a, PAX-5, Cyto-

Figure 4. *Algorithm demonstrating use of various antibodies and assigning lineage to acute leukemias. TdT indicates terminal deoxynucleotidyl transferase; PAX-5, paired box gene 5; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MPO, myeloperoxidase; and AML-MD, acute myeloid leukemia–minimally differentiated.*
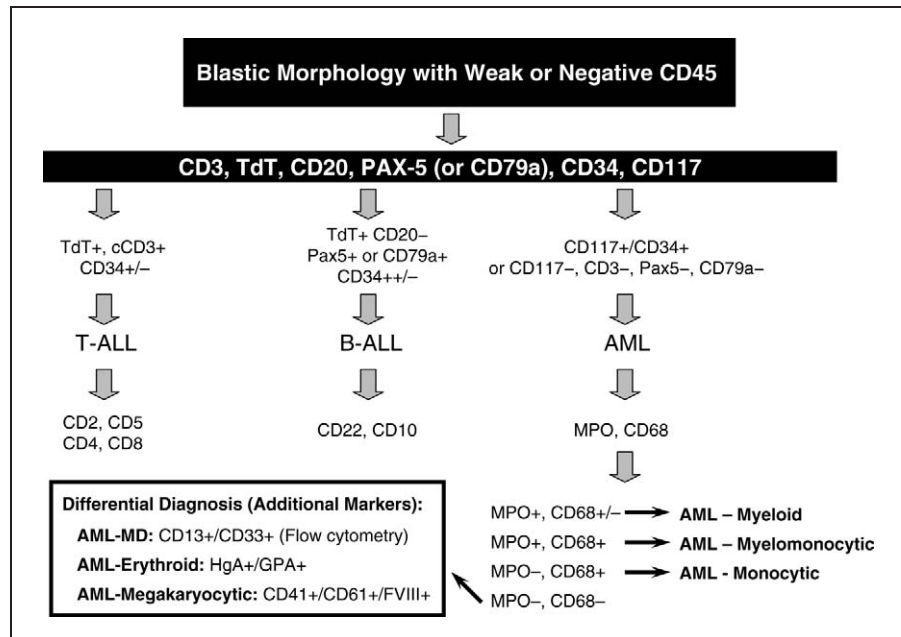
Table 7. Immunoreactivity of Histiocytic Markers by Paraffin Immunohistochemistry*

	CD163, %	CD68 (KP-1), %	CD68 (PG-M1), %
Nonneoplastic monocytes/macrophages	++†	+ in all sites	+ in all sites
Langerhans cells	Negative	Variably +	Variably +
Mast cells	Negative	+	Variably +
CMML	100	100	100
AML (nonmonocytic lineage)	Negative	80	Negative
AML with monocytic lineage	<5	75	60
Granulocytic sarcoma	<5	85	15
Histiocytic sarcoma	100	100	100

* + indicates positive; CMML, chronic myelomonocytic leukemia; and AML, acute myeloid leukemia. Modified with permission from the American Society for Clinical Pathology.[79]

† Positive in interfollicular macrophages and sinus histiocytes of lymph nodes. Tingible body macrophages are negative. Bone marrow macrophages are positive. Macrophages in the red pulp of spleen are positive, and macrophages of white pulp are negative. Epithelioid histiocytes are negative.

plasmic IgM, BCL-6.—*B-Cell Acute Lymphoblastic Leukemia.*—CD20 is usually negative and if positive is weak and focal. CD79a is positive in 80% of cases, whereas PAX-5 is almost always positive.[22] CD22 is lineage specific for B-ALL but is not always positive as the antigen is acquired late during the B-cell blast stage. BCL-6 is negative in B lymphoblasts. Cytoplasmic IgM is positive in pre-B stage of blasts.

T-Cell Acute Lymphoblastic Leukemia.—Expression of CD20, CD22, and PAX-5 is not seen in T-ALL. However, rare cases can be CD79a and BCL-6 positive.[74]

Acute Myeloid Leukemia.—CD20, CD22, CD79a, and BCL-6 are not expressed in AMLs. However, the expression of PAX-5 correlates highly with AML showing the t(8;21) abnormality.[23]

CD4+/CD56+ Hematodermic Neoplasm.—B-cell markers are not expressed in this neoplasm.

Myelomonocytic Antigens—MPO, CD68, CD15, CD31, Lysozyme, CD163, MAC 387, HAM56, Hemoglobin A, FVIII, CD41, CD61.—Myeloblasts are well recognized for demonstrating marked immunophenotypic heterogeneity.[31,75] Thus, multiple lineage-specific antibodies may be necessary to confirm the AML classification.[76–78] CD13 and CD33 are the most sensitive myeloid markers, but al-

though commonly used in FC analysis, they are not widely available for IHC. Myeloperoxidase is specific myeloid marker but is negative in AML-MD (<3% of blasts are positive) and blasts of monocytic, erythroid, and megakaryocytic lineages. Hemoglobin A and glycophorin A are positive in 90% to 100% of erythroid lineage cells, and FVIII is positive in 90% of megakaryocytic cells, but rare cases demonstrating inadequate lineage maturation (early megakaryoblasts) may be negative.[31,66] CD41 and CD61 expression favor megakaryoblastic lineage; however, CD41 expression can be sometimes observed in other subtypes of AML. As discussed previously, the CD31 initially thought to be lineage specific for megakaryocytes can be seen in other subtypes of AML.

CD163 and CD68 (KP-1 and PG-M1) are markers of monocytic and /or histiocyte differentiation (Table 7), and these stains are available by IHC on formalin-fixed samples.[79,80] Using IHC antibody, AMLs (monocytic or non-monocytic) and granulocytic sarcomas are usually CD163 negative (<5% are positive). However, CD163 has been shown in AML with monocytic differentiation by FC.[81] The CD68 antigen (KP-1 and PG-M1) is localized to lysosomes, phagosomes, and neutrophil primary granules and is not regarded as lineage specific as any cells with

these organelles will express CD68. The monoclonal KP-1 antibody is more sensitive but less specific as 80% of AMLs (monocytic and nonmonocytic) and 85% of granulocytic sarcomas will be positive. In contrast, the PG-M1 antibody is less sensitive (24%) but more specific (65%) for AML with monocytic differentiation. All cases of histiocytic sarcomas and chronic myelomonocytic leukemia are positive for CD163, KP-1, and PG-M1. CD163 is negative in mastocytosis, and KP-1 and PG-M1 are positive in 100% and 30%, respectively.[79]

MAC 387 and HAM56 are other monoclonal antibodies that are available in paraffin-embedded tissue samples. About 30% to 60% of all AMLs express these markers with MAC 387 also expressed in 40% to 80% of myeloid sarcomas.[79] However, the specificity of these antibodies is low as other types of sarcomas and adenocarcinomas are positive as well.

B-Cell Acute Lymphoblastic Leukemia.—Myeloperoxidase, CD68, and lysozyme are not expressed in B-ALL. CD15 expression can be seen in B lymphoblasts.

T-Cell Acute Lymphoblastic Leukemia.—Myeloperoxidase, CD68, and lysozyme are not expressed in T-ALL. CD15 expression can be seen in T lymphoblasts in approximately 20% of cases.

Acute Myeloid Leukemia.—Myeloperoxidase expression is seen in AML with myeloid component, whereas expression of CD4 suggests monocytic differentiation. CD15, CD68, and lysozyme are seen in any subtype of AML. AML-MD will typically express blast markers strongly (CD117+, CD34+, TdT positive), is myeloperoxidase negative (<3% of blasts are positive), will variably express CD68, and is usually negative for CD15. Expression of CD163 and CD68 in AML is discussed previously.

CD4+/CD56+ Hematodermic Neoplasm.—Neoplastic cells are negative for MPO. CD15 and CD68 expression is variably seen in these cases.

Other Antigens—HLA-DR, CD10, CD56.—*B-Cell Acute Lymphoblastic Leukemia.*—Almost all cases are HLA-DR positive. About 85% cases are CD10+. Those with the 11q23 translocation (mixed lineage leukemia) are commonly CD10− and CD15+. CD56 is rarely seen.

T-Cell Acute Lymphoblastic Leukemia.—Ten percent to 20% of cases of T-ALL are HLA-DR positive. CD56+ cases are uncommon. Expression of CD10 is seen in 10% to 20% cases.

Acute Myeloid Leukemia.—HLA-DR is often positive. Acute promyelocytic leukemia and AML with erythroid and megakaryocytic lineage are often HLA-DR (and CD34) negative. CD10 is negative and CD56 is positive in 20% of cases.

CD4+/CD56+ Hematodermic Neoplasm.—HLA-DR and CD10 are variably positive. All cases are CD56 positive.

CD43, BCL-2, and CD99 Stains in ALs.—CD43 is a T-cell marker but is not lineage specific.[72] Its expression can be seen in T-ALL (>95%), B-ALL (80%), AML (>95%), and blastic NK-cell neoplasm (>95%). Because the sensitivity of CD43 is so high in these blastic hematopoietic neoplasms, the hematopoietic origin should be questioned in the event it is negative.

Morphologically, Ewing sarcoma is in the differential diagnosis of ALL. It is important to note that CD99 (MIC-2) has been reported in AML, T-ALL, and B-ALL.[82] CD99+ and LCA-negative phenotype does not mean small blue cell tumor such as Ewing sarcoma, as LCA may be negative in B-ALL. Terminal deoxynucleotidyl transferase and/or PAX-5 would be useful discriminating markers. Expression of both CD99 and TdT would favor ALL as TdT positivity is not seen in Ewing sarcoma.[83,84]

The BCL-2 stain is not useful in ALs or in differentiating ALs from NHLs as the protein can be overexpressed in ALL as well as in NHL. Similarly, nonspecific staining of blasts with nonhematopoietic antigens such as vimentin, HMB-45, thyroglobulin, and actin should not be misinterpreted as evidence of metastatic carcinoma.[85] Neuroendocrine tumors are typically positive for CD56, but like all epithelial neoplasms, they are consistently CD45 negative and positive for other epithelial markers.[86–88]

IHC MARKERS HAVING CLINICAL SIGNIFICANCE

Several IHC markers conferring prognostic or therapeutic significance have been recently characterized, and specific studies identifying their presence or absence are frequently requested by clinicians. The use of chimeric anti-CD20 human/murine monoclonal antibodies such as Rituximab (Genentech Inc, San Francisco, Calif), Ibritumomab (Biogen Idec, Cambridge, Mass), and Tositumomab (GlaxoSmithKline, Brentford, Middlesex, United Kingdom) have received tremendous attention because of their success in treating B lymphoid neoplasms including B-ALL and Burkitt leukemia/lymphoma.[89–91] The availability of these agents now define the presence of CD20 as a good prognostic marker.[92] Similarly, Gemtuzumab (Wyeth, Madison, NJ) has shown efficacy in CD33+ AML cases.[93,94] Clinical trials are currently examining Denileukin (anti-CD25) (Seragen, Inc, Hopkinton, Mass) and Alemtuzumab (anti-CD52) (Berlex Oncology, Seattle, Wash) in T-cell leukemia/lymphoma and chronic lymphocytic leukemia.[95–97]

GENETICS ON PARAFFIN-EMBEDDED TISSUE SAMPLES

Because many of the known genetic abnormalities are diagnostically important, the ability to test common translocations using paraffin-embedded tissue would be ideal.

Fluorescence in situ hybridization studies for AL can be performed on paraffin-embedded tissue samples.[98,99] Although not a standard approach, studies can be performed in selected cases. Fluorescence in situ hybridization probes for t(15;17), t(8;21), 11q23, inv(16), t(9;22), and other breakpoints are commercially available. Stringent quality control procedures and validation of every probe is required. When the sections are cut, the preservation of the whole cell nucleus is difficult to achieve. Therefore, a negative result should be interpreted with caution; however, a positive signal most likely reflects a true positive result. Fluorescent in situ hybridization studies are commonly performed on touch imprint slides or smears, which are preferred over formalin-fixed tissue.

DNA can be extracted from paraffin-embedded tissue samples to perform molecular genetic studies by PCR for *IgH, TCR, BCL1,* and *BCL2.*[98,100] Commercial kits are available for this purpose. However, PCR testing in AL (for fusion proteins such as BCR/ABL and PML/RARα) would require extraction of RNA for chimeric protein and amplified by reverse transcriptase–PCR. Fresh samples are preferred for reverse transcriptase–PCR testing, and results using paraffin-embedded tissue samples might not be reliable because of degradation of RNA.

Table 8. Assessment of Acute Leukemia by Cytochemistry*					
Leukemia Classification	SBB/MPO	CAE	ANA	ANB	PAS
B-ALL	−	−	−	−	Block
T-ALL	−	−	−	−	Block
AML–minimally differentiated	−	−	−	−	−
AML–myeloid	+	+	−	−	−
AML–myelomonocytic	+/w+	w+/−	+	+	−
AML–monocytic	−	−	+	+	−
AML–erythroid	−	−	−	−	Diffuse
AML–megakaryoblastic	−	−	+	−	Punctate

* SBB indicates Sudan black B; MPO, myeloperoxidase; CAE, chloroacetate esterase; ANA, α-naphthyl acetate esterase; ANB, α-naphthyl butyrate esterase; PAS, periodic acid–Schiff; B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; −, negative; AML, acute myeloid leukemia; +, positive; +/w+, variably positive to weak positive; and w+/−, variably weak positive to negative.

Table 9.   Diagnostic Pearls in Acute Leukemias*
• Aberrant expression of a single myeloid antigen is found in up to 40% of B-cell ALL.
• If CD7, CD2, and/or CD5 are seen in a case of acute leukemia, it is essential to do a wide battery of lymphoid and myeloid markers including cytoplasmic CD3 and TdT. The diagnosis of T-ALL is unlikely in the absence of CD3 and TdT.
• PAX-5 expression in AML correlates highly with the t(8;21) abnormality, but concurrent PAX-5, CD10, and TdT expression is more likely B-ALL.
• CD4 in AML indicates monocytic differentiation.
• Expression of CD34 is seen in only 50% of AMLs.
• CD56 expression is seen in 20%, CD7 in up to 10%, and CD2 and CD5 in 5% of AML.
• Expression of 2 or more lymphoid markers and absence of myeloid markers suggests ALL, whereas expression of 2 or more myeloid markers and absence of lymphoid markers suggests AML.
• Negative MPO, CD68, lysozyme, CD41, and CD61 and absence of CD79a, PAX-5, and CD3 with a positive TdT suggests AML with minimal differentiation. The demonstration of CD13 and/or CD33 by FC is needed to confirm the diagnosis.
• Expression of myeloid markers with double negativity (90%) for HLA-DR and CD34 suggests APL. Expression of CD2 is seen in APL.
• Double-positive or double-negative CD4/CD8 phenotype at peripheral sites (bone marrow, soft tissue, pleural fluid, etc) suggests T-ALL/ T-LBL. A positive TdT and/or CD1a will confirm the diagnosis.
• Among B-ALL, PAX-5 is more sensitive than CD79a and CD22.
• BCL-6 expression is not seen in B-ALL but has been described in T-ALL.
• CD10 is not seen in AML and expression would favor lymphoid origin (B or less commonly T-ALL). CD4+/CD56+ hematodermic neoplasm can have CD10 expression.

* ALL indicates acute lymphoblastic leukemia; TdT, terminal deoxynucleotidyl transferase; PAX-5, paired box gene 5; AML, acute myeloid leukemia; MPO, myeloperoxidase; FC, flow cytometry; APL, acute promyelocytic leukemia; and LBL, lymphoblastic lymphoma.

CYTOCHEMICAL STAINS

The original French-American-British classification was based on cytochemical evaluation of blasts (Table 8).[3–5] Although it is sometimes considered antiquated in today's modern laboratories, cytochemistry remains a useful ancillary tool.[101–103] These stains can be performed on fresh or archived touch imprint slides, peripheral blood, and bone marrow aspirate smears, so they can provide an additional source of diagnostic material when the original biopsy is inadequate, not available, or cannot be obtained. However, because of a multitude of factors, cytochemistry is losing favor to IHC. Thus, as fewer laboratories are offering these stains and fewer pathologists are ordering them, proficiency is declining. Also, the target enzymes are light and temperature sensitive, so improper slide or reagent storage can lead to false-negative results.[103] Careful comparison of the patient slide to an appropriate control is recommended.

The most valuable stains include MPO, Sudan black B, specific esterase (chloroacetate esterase), nonspecific esterase (α-naphthyl acetate esterase and α-naphthyl butyrate esterase), periodic acid–Schiff, and oil red O (Table 8). Myeloperoxidase, Sudan black B, and chloroacetate esterase stain the primary granules of myeloid cells and can help distinguish among the less differentiated or less mature AML subtypes. By definition, fewer than 3% of blasts seen in AML-MD will demonstrate positive granules, whereas the majority of blasts seen in AML-myeloid will be intensely positive.[82,84] Myeloperoxidase is most specific for granulocytes, but chloroacetate esterase is more sensitive.[104,105] Faint dusty staining in monocytes, especially with Sudan black B, should not be misinterpreted as a positive result.[106] α-Naphthyl butyrate esterase only stains the histiocytic granules of acute myelomonocytic leukemia and acute monoblastic/monocytic leukemia cells, but α-naphthyl acetate esterase additionally stains acute megakaryoblastic leukemia cells[107]; sodium fluoride will inhibit the α-naphthyl acetate esterase monocyte reaction, enabling distinction among these 3 entities.[108] About 20% of monocytic leukemias can be negative for nonspecific esterase. α-Naphthyl butyrate esterase and α-naphthyl acetate esterase can also stain lymphoblasts. Periodic acid–Schiff stains erythroid, megakaryocytic, and lymphoid blasts in a diffuse, punctate, and block cytoplasmic pattern, respectively.[109] Therefore, a positive periodic acid–Schiff stain with negative MPO, Sudan black B, and esterases would favor lymphoblasts, erythroid, megakaryocytic, or AML with minimal differentiation. Immunohistochemistry stains would be needed to further characterize these blasts. Oil red O brightly stains the lipid-containing vacuoles of Burkitt lymphoma/leukemia.[110]

Table 10.   Diagnostic Pitfalls and Limitations in Acute Leukemias*

- Greater than 95% of AMLs have CD33 and/or CD13 expression; however, these markers are not commonly available by IHC.
- When blasts are undifferentiated, differential diagnosis includes B-ALL, T-ALL, and AML-MD, and positive staining for TdT is not diagnostically helpful in this situation as 99% of T-ALL, 95% of B-ALL, and 90% of AML-MD are TdT positive. TdT is, however, beneficial in those cases in which there is difficulty in distinguishing between an AL and NHL.
- The differential diagnosis between T-ALL/T-LBL and lymphocyte-rich thymoma or thymic hyperplasia on a fine-needle aspirate or small core biopsy of the mediastinum is problematic as nonneoplastic cortical thymocytes express CD34 and TdT and are double-positive or double-negative for CD4 and CD8.
- The differential diagnosis of a small round blue cell tumor includes Ewing sarcoma and ALL (B and T), and positive CD99 (MIC-2) staining can be seen in both as well as in AMLs. Both Ewing sarcoma and B-ALL can be LCA negative, but the Ewing sarcoma is TdT negative.
- Rarely, T-ALL can have a mature phenotype (with surface CD3 and CD4 or CD8 class assignment) and lack TdT expression. To distinguish these cases from T-NHL, the clinical history, blastlike morphology, and weak expression of CD45 are useful in supporting T-ALL over T-NHL.
- Rarely, B-ALL can have a mature phenotype with expression of surface light chains. To distinguish these cases from B-NHL, the clinical history, blastlike morphology, and weak expression of CD45 and/or weak CD20 are useful in supporting B-ALL over B-NHL.
- CD43 is a T-cell marker that is often expressed in T-ALL, B-ALL, AML, and CD4$^+$/CD56$^+$ hematodermic neoplasm. It is not lineage specific in ALs. Therefore, the expression even with concurrent TdT positivity is not confirmatory for a lymphoid process.
- A CD4$^+$/CD56$^+$ phenotype does not mean CD4$^+$/CD56$^+$ hematodermic neoplasm as AML can have this phenotype. Even CD123 expression can be seen in both neoplasms.
- *IgH* or *TCR* clonality by PCR cannot be used to assign lineage in ALs because these may cross over between lineages (ie, B-ALL and T-ALL may have both *IgH* and *TCR* clones) and a small percentage of AMLs can be positive for a *TCR* or *IgH* clone.

* AML indicates acute myeloid leukemia; IHC, immunohistochemistry; ALL, acute lymphoblastic leukemia; AML-MD, acute myeloid leukemia–minimally differentiated; TdT, terminal deoxynucleotidyl transferase; AL, acute leukemia; NHL, non-Hodgkin lymphoma; LBL, lymphoblastic lymphoma; LCA, leukocyte common antigen; *IgH,* immunoglobulin heavy chain gene rearrangement; *TCR,* T-cell receptor rearrangement; and PCR, polymerase chain reaction.

CONCLUSION

Tables 9 and 10 list the diagnostic pearls and pitfalls and limitations in ALs. Morphologic features in conjunction with an appropriate IHC panel are sufficient for lineage assignment in most AL cases. The sensitivity and specificity of various antibodies and immunophenotypic heterogeneity are important considerations in these diagnostic situations. Leukemic cells are primitive cells and heterogenous phenotypes are commonplace. A cost-effective practical approach is warranted; however, it should be balanced with ensuring the most clinically relevant accurate diagnosis. In certain situations, an incorrect diagnosis may result in inappropriate therapy with subsequent adverse impact, which possibly could be avoided with a few additional relevant stains in difficult cases.

This article has provided a systematic diagnostic approach by IHC in the assessment of AL cases in the event a sample for FC and genetic studies is not available. This should not be considered a standard diagnostic approach in ALs as the classification, therapy, and prognosis of various leukemias optimally require flow cytometric immunophenotyping and most importantly genetic studies. The role of the pathologist is to identify those cases in which genetic studies are imperative to facilitate additional sampling of the neoplasm for these definitive studies.

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