Applications of Flow Cytometry and Immunohistochemistry to Diagnostic Hematopathology

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• ***Objective.*—Diagnostic hematopathology depends on the applications of flow cytometric immunophenotyping and immunohistochemical immunophenotyping combined with the cytomorphology and histologic features of each case. Select cases may require additional ancillary cytogenetic and molecular studies for diagnosis. The purpose of this review is to focus on the applications of flow cytometric and immunohistochemical immunophenotyping of paraffin-embedded tissue to diagnostic hematopathology. Advantages and disadvantages of these techniques are examined.**

***Data Sources.*—The literature is extensively reviewed (PubMed 1985–2003) with an emphasis on the most recent applications and those that are most useful clinically, both diagnostically and prognostically.**

***Study Selection.*—Studies were selected based on statistically significant results in large studies with reported adequate clinical follow-up.**

***Data Extraction.*—The methodology was reviewed in the selected studies to ensure reliable comparison of reported data.**

***Data Synthesis.*—Flow cytometric immunophenotyping offers the sensitive detection of antigens for which antibodies may not be available for paraffin immunohistochemical immunophenotyping. However, paraffin immunohistochemical immunophenotyping offers preservation of architecture and evaluation of expression of some proteins, which may not be available by flow cytometric immunophenotyping. These techniques should be used as complimentary tools in diagnostic hematopathology.**

***Conclusions.*—There are extensive applications of flow cytometric and immunohistochemical immunophenotyping to diagnostic hematopathology. As cytogenetic and molecular findings evolve in diagnostic hematopathology, there may be additional applications of flow cytometric and immunohistochemical immunophenotyping to this field of pathology.**

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Diagnostic hematopathology relies heavily on combining cytomorphology and histology with ancillary techniques that apply immunophenotyping and molecular/cytogenetic analysis. This review will focus on the applications of flow cytometric and paraffin immunohistochemical immunophenotyping to diagnostic hematopathology.

FLOW CYTOMETRY

General Overview

Flow cytometric immunophenotyping (FCI) is a useful tool in diagnostic hematopathology. Types of specimens suitable for FCI include peripheral blood, bone marrow (BM) aspirates, and core biopsies,[1] fine-needle aspirates (FNAs),[2] fresh tissue biopsies, and all types of body fluids.[3]

Advantages of FCI include the following:

1. Distinct cell populations are defined by their size (forward light scatter) and granularity (side light scatter).
2. Dead cells may be gated out of the analysis.
3. Weakly expressed surface antigens may be detected.
4. Multicolor (2-, 3-, 4-) analysis may be performed, allowing for an accurate definition of the surface antigen profile of specific cells.
5. Two simultaneous hematologic malignancies may be detected within the same tissue site.
6. Tissue biopsy may be obviated by the relatively noninvasive diagnostic evaluation of body fluids and FNA specimens.

Disadvantages of FCI include the following:

1. Sclerotic BM may yield too few cells for adequate analysis.
2. A markedly hypercellular or ''packed'' BM may yield too few cells for analysis.
3. Sclerotic tissue may be difficult to suspend for individual cellular analysis.
4. There is loss of architectural relationships.
5. A small population of monoclonal B cells may not be detected in a T-cell–rich or lymphohistiocytic-rich B-cell lymphoma.
6. T-cell lymphomas that do not have an aberrant immunophenotype may not be detected.
7. An aberrant T-cell immunophenotype (ie, absence or down-regulation of pan–T-cell antigens, particularly CD7) does not necessarily indicate malignancy and may be observed in infectious mononucleosis,[4] reactive dermatoses, and inflammatory disorders.[5–7]

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8. Partial tissue involvement by lymphoma with sampling differences or poor tumor preservation may result in falsely ''negative'' FCI results.[8]

9. Inability to detect/diagnose Hodgkin lymphoma (HL) due primarily to the low number of neoplastic cells normally present in this disease.

Due to these disadvantages, FCI data should always be correlated with light microscopy if no FCI abnormalities are detected. Immunohistochemistry (IHC) may need to be performed in selected cases. In addition, as mentioned, an aberrant T-cell immunophenotype does not necessarily indicate malignancy and requires correlation with light microscopy as well as clinical data and additional ancillary studies (ie, molecular/cytogenetic analysis), in some situations.

In addition, when a monoclonal or aberrant B cell population or an aberrant T-cell population, characterized by a loss of a T-cell antigen, is identified, HL may be excluded only after correlation with the histology, in order to exclude the possibility of a composite lymphoma. In cases in which FCI data is diagnostic, microscopic observations may provide additional information, not only due to sampling, but also due to patterns of involvement and the cytological features of the malignant cells.

The following list represents applications of FCI to diagnostic hematopathology: diagnosis and subclassification of non-Hodgkin lymphoma (NHL) in FNA specimens; distinguishing between follicular hyperplasia (FH) and follicular lymphoma (FL); detection of the lack of surface immunoglobulin (sIg) and light-chain expression by a significant number of B cells indicating malignancy; subtyping B-cell lymphomas/leukemias composed predominantly of small cells; identifying prognostic markers in chronic lymphocytic leukemia (CLL); differentiating lymphoplasmacytic lymphoma or other types of B-cell lymphomas from plasma cell dyscrasias (PCDs); identifying prognostic markers in PCDs; differentiating various types of large B-cell lymphomas (LBCLs) from anaplastic PCDs and from anaplastic $CD30^+$ large cell lymphoma (LCL); immunophenotyping B-cell lymphomas/leukemias; distinguishing between hematogones and neoplastic lymphoblasts; differentiating NHL from HL and T-cell from B-cell NHL; identifying composite lymphomas; distinguishing between T-cell lymphoblastic lymphoma (T-LL) and thymoma; and immunophenotyping T-cell lymphomas/leukemias, natural killer (NK) cell lymphoproliferative disorders, posttransplant lymphoproliferative disorders (PTLDs), granulocytic/monocytic sarcomas (including leukemia cutis), acute myelogenous leukemias (AMLs), and myelodysplastic syndromes (MDSs). In addition, FCI may be useful in excluding a diagnosis of NHL in cases of non-hematopoietic malignancies. These applications will now be discussed in more detail.

Lymphoid/Plasma Cell Disorders

Fine-needle aspiration combined with FCI has been reported to be successful in evaluating sites for lymphomatous involvement in 75% to 90% of cases. False ''negatives'' may result from the following: tumoral necrosis or sclerosis, partial tissue involvement, T-cell NHL without an aberrant immunophenotype, or a T-cell–rich large B-cell lymphoma (TCRBCL) or lymphohistiocytic-rich large B-cell lymphoma (LHRBCL) without detectable monoclonal B cells. Accurate subclassification of NHL by FNA and FCI has been reported to be attainable in 71% to 77% of positive cases. Furthermore, evaluation of cell size by combining FCI and cytomorphology has recently been studied; large cell lymphoma/transformation may be diagnosed reliably if greater than 40% large cells are present.[9] The following situations require biopsy, based on FNA and FCI results: NHL of follicle center cell origin with a mixed cellular composition, indeterminate results, the presence of necrosis and polymorphonuclear cells in evaluation of recurrent NHL, fewer than 10% neoplastic cells detected by FCI, a predominance of small cells detected by cytomorphology or by FCI with clinical signs of transformation, and evaluating for an initial diagnosis of or recurrence of HL, due to the possibility of a composite lymphoma or a subsequent NHL.

In FNA specimens as well as in excised tissue specimens, FCI may be useful in differentiating florid FH from FL. This differentiation is most often accomplished by the detection of a monoclonal B-cell population by FCI. However, there may be occasional cases of florid FH in which the clonality of the germinal center B cells is indeterminate by FCI. These cases may be distinguished by HLA-DO, a flow cytometric marker that is markedly down-regulated in $CD10^+$ germinal center B cells of florid FH, in comparison to $CD10^-$ polytypic B cells and to $CD10^+$ neoplastic cells of FL.[10] In addition, multicolor flow cytometric analysis of expression of Bcl-2, CD10, and CD20 may be useful in distinguishing FH from FL. In a recent study by Cook et al,[11] the presence of $CD10^+$ cells with high Bcl-2 expression predicted the presence of FL rather than FH with a positive predictive value of 100%. The analysis was performed on lymph node and BM specimens.

As detection of monoclonal B cells by FCI may be useful in establishing a diagnosis of B-cell NHL, it should also be noted that the lack of sIg light-chain expression by FCI helps identify peripheral B-cell lymphoma. In a recent study by Li et al,[12] cases with greater than 25% B cells lacking sIg light-chain expression all represented lymphoma. By FCI, the identified sIg light-chain–negative population was distinctly separate from the normal polytypic B cells; in 90% of cases, the identified population was larger by forward angle light scatter than the reactive T cells and polytypic B cells. In their review of reactive cases, no reactive case revealed greater than 17% sIg-negative B cells.

Flow cytometric immunophenotyping is particularly useful in subtyping B-cell lymphomas/leukemias composed predominantly of small cells (Table 1). Cases of small lymphocytic lymphoma (SLL)/CLL with irregular nuclei and various morphologic variants of SLL may occur.[13] Thus, immunophenotyping is essential in diagnosing and differentiating these cases from other subtypes of B-cell lymphoma. Aberrant CD5 expression is characteristic of SLL/CLL and mantle cell lymphoma/leukemia (MCL). SLL/CLL may be reliably differentiated from MCL if CD23 is negative. However, dimly positive CD23 expression may be seen in SLL/CLL and MCL; thus, molecular analysis for cyclin D1 may be necessary in selected cases.[14] Another marker that may be useful in the differentiation of SLL/CLL from MCL is CD79b. This marker is characteristically absent or dimly expressed in SLL/CLL; CD79b expression in MCL is significantly higher.[15] However, one should recognize that higher intensity CD79b expression may occur in 20% of CLL cases. Of interest, this expression correlates with trisomy 12 and

Table 1. Small B-Cell Lymphomas/Leukemias Surface Antigens*

NHL	sIg	CD5	CD10	CD23	CD11c	CD103	CD25
CLL/SLL†	Dim	+	−	++/+	−	−	−
MCL†	+	+	−/+	−/+	−	−	−
Follicular	+	−	+	+/−	−	−	−
LPL	+	−	−	−	−	−	−
MZL	+	−	−	−	+/−wk	−	−
SMZL	+	−	−	−	+/−wk	+/−wk	−
Hairy cell	+	−	−/+	−	++	+	+

* NHL, non-Hodgkin lymphoma; sIg, surface immunoglobulin; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma/leukemia; MZL, marginal zone lymphoma; and SMZL, splenic marginal zone lymphoma.

† CLL/SLL and MCL may further be distinguished by expression of CD76b (absent/dim expression in CLL/SLL, higher expression in MCL).

atypical immunophenotypic features; in turn, trisomy 12 in CLL correlates with a worse prognosis.[16] In addition, there may be cases of CD5-negative MCL.[17] These cases may be identified by evaluation of Bcl-1 expression by immunohistochemical or molecular genetic techniques. Atypical CLL, characterized by at least 10% lymphocytes with clefted and folded nuclei in the peripheral blood, demonstrates significantly higher expression of CD23 than the expression seen in typical CLL. These patients generally have higher white blood cell counts and probability of disease progression.[18] In addition, one should be aware that there are rare cases of $CD5^+$ extranodal marginal zone B-cell lymphoma (mucosa-associated lymphoid tissue [MALT] type).[19] There has been a reported increased propensity for BM involvement and relapse associated with these rare cases.[20]

CD5-negative small B-cell leukemias are unlikely to represent CLL and are classified more appropriately as NHL in the leukemic phase.[21] CD10 expression is encountered in approximately 80% of FL and is negative in up to 20% of FL. In addition, occasional cases of FL may not reveal a monoclonal B-cell population due to an extremely high content of reactive T cells[22] or to the presence of the floral variant of FL.[23,24] This variant may contain an increased number of reactive T cells or benign $CD5^+$ mantle cells that may obscure monoclonality. Of interest, there have also been reports of $CD5^+$ monoclonal B cells in this variant, which are important to recognize and distinguish from nodular variants of SLL and MCL.[25] CD10 positivity may also rarely be seen in MCL and hairy cell leukemia (HCL). $CD10^+$ HCL cases seem to be morphologically and clinically similar to $CD10^-$ HCL cases.[26,27] The recognition of $CD10^+$ HCL cases is important and emphasizes the consideration that the FCI data must always be correlated with the morphologic features of each case. In addition, there is a variant of HCL with prolymphocytic morphology (termed *hairy cell variant* [HCL-V]), which typically presents with an elevated white blood cell count. The immunophenotype differs somewhat from classical HCL in that there is variable expression of CD103 and lack of CD25. It is important to recognize this prolymphocytic variant of HCL because the response to treatment with agents effective in typical HCL is usually poor; median survival is significantly shorter in this variant. Lymphoplasmacytic lymphoma may be differentiated from other types of B-cell lymphomas by applying the characteristic FCI findings outlined in Table 1 and by identifying the presence of monoclonal plasma cells by IHC. Likewise, lymphoplasmacytic lymphoma may be differentiated from a PCD by the finding of a prominent population of monoclonal B cells by FCI in lymphoplasmacytic lymphoma.

In addition to subtyping B-cell lymphoma/leukemia composed predominantly of small cells, FCI offers the ability to detect prognostic markers in CLL/SLL. Expression of aberrant markers by FCI, particularly CD2, CD7, CD10, CD13, CD33, and CD34, has been demonstrated to be associated with significantly shortened overall survival and increased aggressiveness.[28] Expression of CD38 by greater than 20% to 30% of the neoplastic cells in CLL has been associated with an unfavorable prognosis.[29,30] Likewise, the expression of ZAP-70 as detected by FCI has been shown to correlate with immunoglobulin heavy-chain variable region (IgV_H) mutational status, more rapid disease progression, and poorer survival (Table 2).[31,32]

Flow cytometric immunophenotyping has also been useful in immunophenotyping LBCLs and differentiating them from anaplastic $CD30^+$ LCL and from anaplastic PCD. As previously mentioned, expression of CD10 is seen in approximately 80% of lymphomas of follicle center cell origin and is characteristically strongly expressed in Burkitt lymphoma (BL). The blastic and pleomorphic variants of MCL have the same immunophenotype as MCL; these variants are important to recognize because these patients have a significantly worse prognosis. Anaplastic $CD30^+$ LCL, as defined by the World Health Organization (WHO), is of T-cell origin and strongly expresses CD30 by

Table 2. Immunophenotypic Prognostic Markers in Lymphoid/Plasma Cell Disorders*

Disease	Markers Expressed	Impact on Prognosis	References
CLL/SLL	CD2, CD7 CD10 CD13, CD33 CD34 CD38 ZAP-70 MUM1	All associated with shortened OS and increased disease progression	28–32 67
Plasma cell dyscrasia	CD11c, CD13, CD14, CD15, CD10	Multiple myelomonocytic marker and CD10 expression independently associated with shortened OS and more aggressive disease	46, 47
Large B-cell lymphoma	Bcl-2 $CD10^+$, Bcl-6^+, $MUM1^-$ (germinal center phenotype)	Poorer OS, advanced stage Increased OS and event-free survival	81, 83, 84

* CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; and OS, overall survival.

Table 3. Flow Cytometric Immunophenotypic Comparisons: Diffuse Large B-Cell Lymphomas (LBCLs) Versus Anaplastic Large-Cell Lymphoma (ALCL) Versus Anaplastic Plasmacytoma*

Marker	Diffuse LBCLs (FCCL, MCL, BL)	ALCL	Anaplastic Plasmacytoma
CD20, CD19	+	−	−
Other B-cell markers	CD10$^+$ (FCCL and BL)	−	−
T-cell markers	CD5$^+$ (MCL)	−/+	−
CD45 (LCA)	+	+/−	−
CD30	−/+	+	−/+
CD138	−	−	+
Surface Ig	+	−	−
Cytoplasmic Ig	−	−	+

* FCCL, follicle center cell lymphoma; MCL, mantle cell lymphoma; BL, Burkitt lymphoma; Ig, immunoglobulin.

Table 4. Immunophenotypes of B-Lineage Lymphoblastic Leukemias*

Early Pre-B	Pre-B	Mature B (Burkitt)
CD19$^+$, CD20$^-$	CD19$^+$, CD20$^+$	CD19$^+$, CD20$^-$
CD24$^+$	CD24$^+$	CD24$^+$
CD10$^{+/-}$	CD10$^{+/-}$	Intense CD10
sIg/light chain$^-$	sIg/light chain$^-$	sIg/light chain$^+$
TdT$^+$	TdT$^+$	TdT$^-$

* sIg indicates surface immunoglobulin; TdT, terminal deoxynucleotidyl transferase.

Table 5. Normal Maturational Sequence of Bone Marrow Hematogones*

Hematogones				Mature B Cells
TdT				
CD34				
CD10 (brt)	CD10	CD10	CD10	
CD19	CD19	CD19	CD19	CD19
CD22 (dm)	CD22 (dm)	CD22 (dm)	CD22 (dm)	CD22
CD38 (brt)	CD38 (brt)	CD38 (brt)	CD38 (brt)	CD38 (brt/−)
		CD20 (dm)	CD20	CD20
		sIg	sIg	sIg

* brt indicates bright; dm, dim; and sIg, surface immunoglobulin.

Table 6. Immunophenotypes of T-Cell Acute Lymphoblastic Leukemia (T-ALL)*

Ag	Prethymocytes	Pre–T-ALL (I) Prothymocytes	Pre–T-ALL (II) Common Thymocytes	T-ALL (III) Mature Thymocytes	
				T-H	T-S
CD1	−	−	+	−	−
CD2	+/−	+/−	+/−	+	+
cCD3	+/−	+	+/−	−	−
sCD3	−	−	−/+	+	+
CD5	−/+	+	+	+	+
CD7	+	+	+	+	+
CD4	−	−	+	+	−
CD8	−	−	+	−	+
HLA-DR	+/−	−	−	−	−
CD34	+/−	−	−	−	−
TdT	+	+	+	+	+

* T-H indicates T-helper; T-S, T-suppressor; cCD3, cytoplasmic CD3; and sCD3, surface CD3.

FCI. Anaplastic PCD characteristically does not express CD45, B-cell antigens, or sIg by FCI, but does express CD138 and variable CD56 (Table 3).

Prolymphocytic leukemia (PLL) and B-lineage acute lymphoblastic leukemia (ALL) may be immunophenotyped by FCI. B-cell PLL may be divided into CD5$^+$ PLL (arising in CLL) and CD5$^-$ PLL (de novo PLL). CD5$^+$ PLL has a longer median survival than CD5$^-$ PLL; thus, it is important to distinguish between these 2 types of B-PLL. Likewise, FCI is extremely helpful in immunophenotyping and subtyping B-lineage ALL (Table 4). FCI allows for the detection of aberrant myeloid antigen expression in B-lineage ALL, which in adults is associated with a significantly lower complete remission rate and shorter survival. Likewise, FCI allows for the detection of CD15$^+$ early precursor B-ALL, which may occur in infants younger than 1 year of age and in adults and is associated with t(4;11). This group of ALL is associated with a rearrangement of *MLL* and a poor prognosis. In addition, FCI is able to distinguish BM hematogones from leukemic B lymphoblasts, which is often crucial in a post-therapy ALL BM. Hematogones always exhibit a typical complex spectrum of antigen expression that defines the normal antigenic evolution of B-cell precursors and lacks aberrant expression (Table 5). In contrast, lymphoblasts in precursor B-ALL show maturation arrest and exhibit varying numbers of immunophenotypic aberrancies (expression of CD13, CD33, etc).[33]

The immunophenotypes of T-cell ALL are outlined in Table 6 and are best evaluated by FCI.[34] T-cell lymphoblastic lymphoma most often has an immunophenotype that corresponds to the common thymocyte stage of ALL; the immunophenotype of thymoma is identical to this stage. In addition, it should be recognized that T-cell lymphoblastic lymphoma/leukemia and thymoma may also aberrantly express CD10.[35] However, FCI allows for the distinction between thymoma and T-LL. Flow cytometric immunophenotyping features characteristic of thymoma include a smear pattern of CD4/CD8 co-expression (Figure 1, A), a smear pattern of CD3 and terminal deoxynucleotidyl transferase (TdT) expression (Figure 1, B), and lack of T-cell antigen deletion (with the exception of partial CD3). In contrast, T-LL shows much more variability in expression patterns and is characterized by a tight pattern of CD4/CD8 expression (Figure 1, C), significant T-cell antigen deletion, and absence of the CD3 or TdT smear pattern (Figure 1, D).[36] In addition, distinguishing between thymoma and T-LL must always also rely on correlation of the FCI data with the morphology.

Mature T-cell lymphomas may have variable immunophenotypes by FCI. There may be variable loss of a pan–T-cell antigen (ie, CD2, CD3, CD5, CD7). Most cases are CD4$^+$; some are CD8$^+$, CD4$^-$CD8$^-$, or CD4$^+$CD8$^+$. T-cell CLL/PLL expresses pan–T-cell antigens (CD2, CD3, CD5, CD7) with CD4$^+$CD8$^-$ > CD4$^+$CD8$^+$ > CD4$^-$CD8$^-$. T-CLL is distinguished from the small cell variant of T-PLL by electron microscopy. The characteristic immunophenotype of mycosis fungoides (MF) is CD4$^+$CD8$^-$, with CD7 commonly lost and variable expression of CD2. In addition, although not absolutely specific, a low CD8/CD3 ratio in the epidermal component of a lymphocytic infiltrate supports the diagnosis of MF.[37] CD25 is negative in MF. Adult T-cell leukemia/lymphoma (ATLL) has a similar immunophenotype to MF; however, CD25 is characteristically

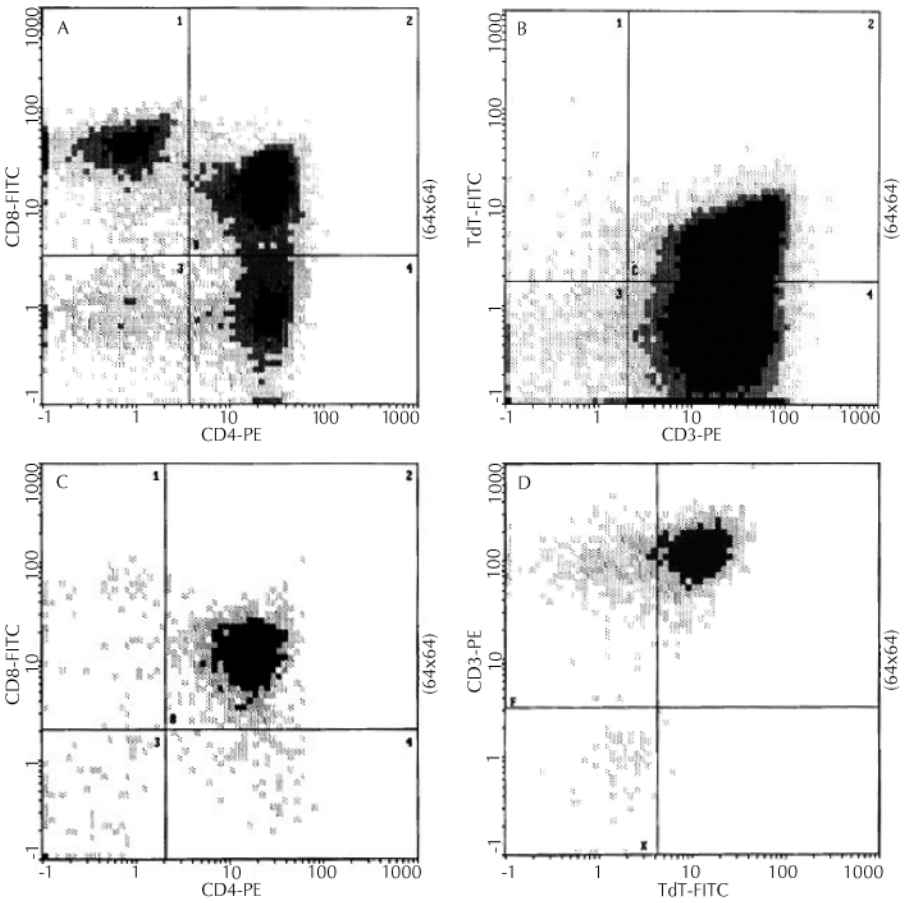

Figure 1. *A, Smear pattern of CD4 (x-axis)/CD8 (y-axis) characteristic of thymoma. B, Smear pattern of terminal deoxynucleotidyl transferase (TdT) (y-axis)/CD3 (x-axis) characteristic of thymoma. C, Tight pattern of CD4 (x-axis)/CD8 (y-axis) characteristic of T-cell lymphoblastic lymphoma. D, Tight pattern of TdT (x-axis)/CD3 (y-axis) characteristic of T-cell lymphoblastic lymphoma.*

Table 7. Immunophenotypes of Natural Killer (NK) and T-Cell Large Granular Lymphocytic (T-LGL) Processes*

Disorder	Immunophenotype
Chronic NK cell expansion	$CD2^+$, $CD3^-$, $CD4^-$, $CD8^-$, $CD16^+$, $CD56^+$, CD57 wk
NK-LGL leukemia	$CD2^+$, $CD3^-$, $CD4^-$, $CD8^+$, $CD16^+$, $CD56^+$, CD57 v
T-LGL leukemia	$CD2^+$, $CD3^+$, $CD4^-$, $CD8^+$, $CD16^{+/-}$, $CD56^{-/+}$, $CD57^+$

* wk indicates weak; v, variable.

expressed in ATLL. The neoplastic T cells of angioimmunoblastic T-cell lymphoma have been shown to characteristically express CD10; recent reports have suggested that the demonstration of a $CD10^+$ T-cell population may aid in the diagnosis of this specific type of T-cell lymphoma.[38–40] However, as has been discussed previously, $CD10^+$ T cells exist in lymphoblastic lymphoma/leukemia and thymoma and it has more recently been shown that there is a normal small subset of $CD10^+$ peripheral T cells that may exist in reactive lymphoid proliferations as well as B-cell lymphoma.[41] Anaplastic $CD30^+$ LCLs of T-cell origin characteristically express CD30 by FCI. Extranodal NK/T-cell lymphomas of nasal type may be defined by FCI. They typically have the following immunophenotype: $CD2^+$, $CD56^+$, surface $CD3^-$, cytoplasmic $CD3^+$, $CD16^-$, and $CD57^-$. Enteropathy-type T-cell lymphoma is $CD3^+$, $CD5^-$, $CD7^+$, $CD8^{-/+}$, $CD4^-$, and $CD103^+$. The neoplastic cells of hepatosplenic T-cell lymphoma are $CD3^+$, variably $CD56^+$, and usually $CD4^-$, $CD8^-$, and $CD5^-$. These entities may further be distinguished based on clinical features, morphology, IHC, and cytogenetic/molecular findings. Flow cytometric analysis of a broad array of antibodies directed against the variable (V) region of the T-cell receptor (TCR)β(V_β) chain may allow for a rapid confirmation of a T-cell malignancy. The recent report by Beck et al[42] describes a sensitivity of 89% for direct detection of pathogenic V_β restriction and a specificity of 88% by this technique. The immunohistochemical findings will be discussed later in this review.

Flow cytometric imunophenotyping is useful in subtyping and classifying lymphoproliferative disorders of large granular lymphocytes. Large granular lymphocytes derive from 2 major cell lines: NK cells and T cells. Clonal expansions of large granular lymphocytes may be categorized based on a combination of clinical features, cytomorphology, FCI, and clonality analysis into the following: chronic NK cell expansion (chronic NK cell lymphocytosis), NK large granular lymphocytic (LGL) leukemia, and T-LGL leukemia. Large granular lymphocytic leukemia is defined as a chronic clonal proliferation of large granular lymphocytes (>2,000/μL) for at least 6 months' duration. T-cell LGL leukemia is usually indolent and associated with neutropenia, recurrent infection, rheumatoid arthritis, and splenomegaly. Natural killer NGL leu-

Table 8. Flow Cytometric Immunophenotyping in Posttransplant Lymphoproliferative Disorder*

Category	I	II		III	
	Plasmacytic Hyperplasia	PBCH	PBCL	IL	MM
FCI data	Polyclonal	Polyclonal	2+ 1− 1+	1+ 1+	1+ 1+
Genotypic data	Polyclonal	Polyclonal	2− 1+ 1+	1+ 1−	1+ 1−

* PBCH, polymorphic B-cell hyperplasia; PBCL, polymorphic B-cell lymphoma; IL, immunoblastic lymphoma; MM, multiple myeloma.

Table 9. Immunophenotype of Acute Myelogenous Leukemia M3 Versus M5

AML, M3	AML, M5
$CD13^{++}$	$CD13^{++}$
$CD33^{++}$	$CD33^{++}$
$HLA\text{-}DR^{-/+}$ (rare)	$HLA\text{-}DR^{++}$
$CD34^{-/+}$ (rare)	$CD34^{-/+}$ (M5a)
$CD14^{-}$	$CD14^{+/-}$
CD64 dim^{+}	$CD64^{++}$
$c\text{-}Kit^{+}$	$c\text{-}Kit^{-}$
CD^{-}; $CD56^{-/+}$ (rare)	$CD4^{+}$; $CD56^{+}$

kemia has a more aggressive clinical presentation associated with a younger disease presentation. The immunophenotypes of the NK cell processes and T-cell LGL process are outlined in Table 7. Clonality is defined by the detection of a single band for the joined termini of the Epstein-Barr viral (EBV) genome in those of NK cell origin and by the detection of a T-cell gene rearrangement in those of T-cell origin. In addition, in chronic NK cell expansion (chronic NK cell lymphocytosis), clonality may be defined by the detection of a restricted phenotype because there are 4 different subsets of normal NK cells by the use of monoclonal antibodies, EB6 and GL183 (ie, $EB6^{+}/GL183^{+}$, $EB6^{+}/GL183^{-}$, $EB6^{-}/GL183^{-}$, and $EB6^{-}/GL183^{+}$).[43,44]

Flow cytometric immunophenotyping may also be useful in identifying a clonal process in PTLDs, even those with negative results by genotypic studies (Table 8). Flow cytometric immunophenotyping and genotypic studies should routinely be performed in PTLDs to detect a clonal process, as the detection of clonality is important to categorize the process and for treatment management.[45]

Although plasma cells do not typically express CD45, B-cell markers, sIg, or light chains, PCDs may aberrantly express B-cell markers (ie, CD19 or CD20) and reveal dim monoclonal sIg or light-chain expression. More importantly, aberrant expression of multiple myelomonocytic markers (ie, CD11c, CD13, CD14, CD15) and CD10 (CALLA) have independently been associated with more aggressive disease and shortened survival (Table 2).[46,47]

Myeloid Disorders

Distinction of malignant lymphoma from a granulocytic or monocytic sarcoma is greatly aided by the use of FCI, as the malignant cells will variably express myelomonocytic markers in these disorders. Likewise, FCI is useful in differentiating AML from ALL to determine appropriate therapy; detecting bilineage and biphenotypic acute leukemias, which generally have a poor prognosis; and defining a blast immunophenotype that may be most useful in evaluating relapse/residual disease. CD117 is an extremely useful marker by FCI in the evaluation of acute leukemias, because this marker has been reported as expressed only in AML.[48] An aberrant AML immunophenotype ($CD7^{+}$, $CD19^{+}$, etc) is particularly useful in detecting residual or relapsing disease and in distinguishing leukemic from recovery blasts. In regards to immunophenotyping AML, FCI defines AML M0, which requires expression of myelomonocytic markers (ie, CD13, CD33) by FCI. Flow cytometric immunophenotyping allows for the detection of $CD19^{+}$ AML, characteristically associated with t(8;21); the myeloblasts in this type of leukemia also typically express CD34 as well as CD56.[49] Aberrant expression of CD19 may also be observed in AML of monocytic lineage.[50] However, the pattern of CD19 expression is distinctly unique in AML with a substantial monocytic/monoblastic component. In 50% of these AML cases, CD19 expression was evident only with the B4 (lytic) antibody and was not observed with B4 89B or SJ25-C1, whereas in the t(8;21)-associated AML M2 cases, CD19 was detected with all three antibodies.[48] Flow cytometric immunophenotyping most often differentiates hypogranular acute promyelocytic leukemia (APL) from acute monocytic leukemia (Table 9).[51] However, rare cases of APL may express HLA-DR and/or CD34. In addition, although CD14 is a monocyte-specific marker, CD14 is often absent or frequently diminished in expression in AML with monocytic differentiation (AMML and AMOL). In addition, other markers characteristically expressed by monocytic cells (CD11b, CD13, CD15, CD33, CD64) are absent or at least partially diminished in AMML and AMOL. Thus, correlation with nonspecific esterase staining, particularly alpha naphthyl acetate esterase (ANAE), is crucial in diagnosing AMLs with monocytic differentiation. Detection of CD34 and CD117 expression by FCI have been shown to be indicative of malignancy in monocytic disorders and thus may be observed in AMML and AMOL (based on unpublished data of C. H. Dunphy, S. M. Orton, and J. L. Mantell, University of North Carolina, Chapel Hill, 2003). FCI also defines acute megakaryocytic leukemia expression of CD41, CD42b, and CD61, which may resemble ALL in pediatric patients.

Flow cytometric immunophenotyping is also useful in immunophenotyping MDS, because it allows for the detection of an accurate percentage of myeloblasts; microblasts are characteristic of MDS and often difficult to morphologically differentiate from lymphocytes. Also of interest, the use of 4-color flow cytometry has allowed for the identification of abnormal myeloid populations in more than 90% of non–chronic myeloid leukemia myeloproliferative disorders (MPDs) and MDSs with a clonal cytogenetic abnormality, supporting the use of FCI in the diagnosis of these disorders.[52] The most useful combinations of these myeloid markers in the study by Kussick and Wood[52] included the following: HLA-DR and CD33, and CD11b and CD16 or CD13 and CD16. Flow cytometric immunophenotyping may also allow for the detection of an accurate percentage of monocytic cells, by analyzing CD14 and CD64, in establishing a diagnosis of chronic myelomonocytic leukemia (CMML). In addition, the morphologically mature monocytes of CMML may reveal abnormalities by FCI (partial loss of CD13, CD14, and CD15 and expression of CD56) that are not observed in normal

monocytes. These abnormalities may indicate clues to a correct classification of CMML in these cases.[53]

Nonhematopoietic Malignancies

Although, in general, nonhematopoietic malignancies are characterized by lack of expression of CD45 (LCA), it may be useful to recognize that primitive neuroectodermal tumor/Ewing sarcoma (PNET/ES) and small cell carcinomas may also be associated with expression of CD56, as these represent small blue round-cell tumors that may be submitted for a lymphoma workup.

PARAFFIN IHC

General Overview

As mentioned previously, FCI data should always be correlated with light microscopy, and IHC may need to be performed in selected cases. Advantages of IHC include the preservation of architectural relationships and the ability to detect a relatively low number of neoplastic cells, such as in HL or a TCRBCL. In addition, some antibodies may be better evaluated in paraffin tissue (eg, CD15 in Reed-Sternberg [RS] cells of HL, and the presence of Bcl-2, Bcl-6, cyclin D1, ALK-1, and cytoplasmic κ and λ). Finally, fresh tissue may simply not be available.

However, one must be aware that it is extremely difficult or may be impossible to detect surface light-chain expression and weakly expressed antigens in paraffin tissue. Variability in tumor preservation and fixation may result in suboptimal results in paraffin IHC. In addition, some markers useful by FCI are simply not routinely available for paraffin IHC (CD13, CD14, CD19, CD33, etc).

The following discussions will focus on the unique applications of paraffin IHC, which enhance the applications of FCI in diagnostic hematopathology.

1. Differentiation of various forms of B-cell hyperplasia from B-cell lymphoma when fresh tissue for FCI is not available or morphology is yet of concern.
2. Subtyping B-cell lymphomas composed predominantly of small cells (ie, when fresh tissue is unavailable for FCI or IHC is necessary to support or define the subtype).
3. Immunophenotyping B-cell lymphomas that may require paraffin IHC due to inconclusive FCI, antibodies uniquely available for paraffin IHC, or infarcted lymphoid tissue. Lymphoid antigens are frequently preserved in cases of lymph node infarction, and paraffin IHC of infarcted lymph nodes may provide clinically useful information.[54,55]
4. Differentiating lymphoplasmacytic lymphoma or other types of B-cell lymphomas associated with monoclonal gammopathy from PCD.
5. Defining a PCD (extramedullary plasmacytoma or multiple myeloma) and predicting clinical behavior (discussed below under ''Primary BM Disorders'').
6. Differentiating various types of LBCLs, including plasmablastic lymphoma from anaplastic plasmacytoma and from anaplastic $CD3^+$ LCL.
7. Differentiating T-NHL from B-NHL when fresh tissue is not available for FCI.
8. Immunophenotyping of T-cell NHL and T/NK cell lymphoproliferative disorders.
9. Detection of a composite lymphoma composed of HL and an NHL.
10. Defining HL.
11. Differentiating classical HL (CHL) from lymphocyte-predominant HL (LPHL).
12. Differentiating HL from various forms of diffuse large B-cell lymphoma (TCRBCL, LHRBCL, primary mediastinal B-cell lymphoma, and so forth).
13. Differentiating HL from T-cell NHL and from anaplastic $CD30^+$ LCL.
14. Differentiating HL from diffuse LPHL/TCRBCL.
15. Immunophenotyping T-cell lymphoma when fresh tissue is not available for FCI or FCI fails to reveal an aberrant T-cell immunophenotype.
16. Differentiating mature (peripheral) large cell NHL from lymphoblastic lymphoma and granulocytic/monocytic sarcoma when fresh tissue is not available for FCI.
17. Immunophenotyping PTLDs.
18. Diagnosing, immunophenotyping, and follow-up of primary BM disorders (eg, acute leukemias, myelodysplasias) when the BM is not aspirable for FCI or the aspirate is markedly hemodiluted.
19. Differentiating acute leukemia from nonhematopoietic malignancies.
20. Diagnosing, determining prognosis, and evaluating follow-up of primary BM disorders that are not generally defined by FCI (eg, multiple myeloma, systemic mast cell disease).
21. Evaluating BM for lymphomatous involvement.

Lymphoid Disorders

Follicular hyperplasia may generally be distinguished from FL with the use of Bcl-2 protein expression. Bcl-2 is nonreactive in reactive germinal centers and is typically strongly reactive in the malignant nodules of FL. However, it should be noted that there are rare cases of FL that are composed of $Bcl\text{-}2^-$ malignant nodules. Another monoclonal antibody available for paraffin IHC, MT2, shows a similar pattern of reactivity to Bcl-2. In a study by Browne et al,[56] 77% of formalin-fixed FLs were $MT2^+$; none of the follicular hyperplasias were $MT2^+$. $Bcl\text{-}2^+$ reactive germinal centers have not been described.[57]

A discussion of Bcl-2 protein expression is warranted at this time. Small lymphocytes show cytoplasmic staining for Bcl-2 in peripheral blood, interfollicular areas, and mantle zones of lymph nodes and the thymic medulla.[58] In addition, Bcl-2 has also been shown to be consistently expressed by reactive marginal zone B cells of the spleen, abdominal lymph nodes, and ileal lymphoid tissue; thus, Bcl-2 expression should not be used as a criterion for discriminating between benign and malignant marginal zone B-cell proliferations involving these sites.[59]

Although expression of the Bcl-2 protein is associated with the t(14;18) chromosome translocation and is expressed on a significantly higher percentage of FLs associated with this translocation, expression of the Bcl-2 oncogene protein is not specific for the t(14;18) chromosomal translocation.[57,60,61] Bcl-2 protein expression may be detected in a substantial number of B cells as well as in T-cell lymphoproliferative disorders not associated with the t(14;18).[62,63] A study by Wheaton et al[62] revealed Bcl-2 expression in 100% of SLLs, 80% of FLs, 38% of diffuse LCLs, 33% of high-grade B-cell Burkitt-like lymphomas, 0% of BLs, and 0% of B-cell lymphoblastic lymphomas. Thus, the significant difference in Bcl-2 expression between Burkitt-like high-grade B-cell lymphoma and BL was suggested as an additional use of Bcl-2. Bcl-2 positivity may rarely occur in BL and has been described when

there is a coexistent t(14;18) and Burkitt translocation. In addition, although marginal zone hyperplasias in the spleen, abdominal lymph nodes, and ileal lymphoid tissues may reveal Bcl-2 expression, Bcl-2 may represent a useful marker for distinguishing reactive monocytoid B-cell hyperplasia from marginal zone lymphoma in other sites. Although T-cell lymphoproliferative disorders had a significantly lower Bcl-2 expression than B-cell disorders, peripheral T-cell lymphoma (including anaplastic $CD30^+$ LCL and angioimmunoblastic-type), and lymphoblastic lymphomas may reveal expression of the Bcl-2 protein.

Progressive transformation of germinal centers (PTGC) represents a reactive condition that may occur simultaneously with or be subsequently followed by nodular LPHL. In addition, PTGC may be difficult to distinguish morphologically from nodular LPHL. Typical nodular LPHL is distinguished by the finding of the characteristic ''popcorn-shaped'' lymphocytic/histiocytic (L&H) cells that have a B-cell immunophenotype ($CD45^+$, $CD20^+$, $CD79a^+$, $Bcl\text{-}6^+$, $BOB.1^+$, $Oct.2^+$, J $chain^{+/-}$, $immunoglobulin^{+/-}$, epithelial membrane antigen $(EMA)^{+/-}$, $CD15^-$, $CD30^-$) set in a non-neoplastic nodular background rich in small reactive B cells. The L&H cells are ringed by T cells that are frequently $CD57^+$. The nodules contain a prominent follicular dendritic cell meshwork that encompasses the L&H cells.[64]

Before embarking on a discussion of subtyping/immunophenotyping of B-cell lymphomas by paraffin IHC, it would be worthwhile to discuss a relatively new marker: anti–Pax-5. Pax-5 encodes for BSAP (Pax-5), a B-cell–specific transcription factor, the expression of which is detectable as early as the pre–B-cell stage and subsequently in all further stages of B-cell development until the plasma cell stage, where it is down-regulated. In a large study by Torlakovic et al,[65] Pax-5 protein (BSAP) was detected in all cases of precursor and mature B-cell NHL/leukemias. Only 4% of diffuse LBCLs did not reveal Pax-5 expression; these lymphomas were exclusively of terminal B-cell origin. In addition, in 97% of CHL, RS cells expressed Pax-5. Pax-5 was not detected in any of the multiple myelomas, solitary plasmacytomas, or T-cell lymphomas/leukemias. Anti–Pax-5 was found to exceed the specificity and sensitivity of L-26 (anti-CD20) because of its earlier expression in B-cell differentiation and its ability to detect all committed B cells, including CHL.[65] Thus, anti–Pax-5 represents a new marker that may be very useful in the differential diagnosis of malignant lymphoma.

Although subtyping of B-cell lymphomas composed predominantly of small cells is most often accomplished effectively by FCI, fresh tissue may not always be available for FCI. In addition, the availability of CD5 for paraffin IHC has increased the ability to subtype this group of B-cell lymphomas by this methodology.[66] One should be aware that CD20 may be so dimly expressed in CLL/SLL that paraffin IHC may yield a negative result with CD20. Thus, paraffin IHC may yield the following immunophenotype: $CD20^-$, $CD3^-$, $CD5^+$, and $CD23^+$, keeping in mind that CD19 is not available for paraffin IHC, and sIg and light-chain expression are not generally effectively evaluated by this method. In addition, there is a paraffin IHC marker, MUM1/IRF4, which, when expressed in CLL/SLL, is associated with shorter overall survival times.[67] This marker may be evaluated in combination with the other prognostic markers already outlined above in the ''Flow Cytometry'' section. Because this marker has not been previously mentioned, discussion regarding *MUM1/IRF4* is warranted at this time.

MUM1/IRF4 is a myeloma-associated oncogene transcriptionally activated as a result of t(6;14)(p23,q32) chromosomal translocation and by virtue of its juxtaposition to the immunoglobulin heavy-chain gene (IgH) locus. MUM1 protein expression appears as a nuclear staining pattern and is strictly regulated during lymphoid differentiation. Tsuboi et al[68] have described the presence of $MUM1^+$ cells in reactive lymph nodes, consisting of plasma cells and a small fraction (approximately 8%) of B cells harboring CD20 and CD38, located in the light zone of the germinal center. MUM1 expression in peripheral blood B/T lymphocytes may be up-regulated by mitogenic stimuli, suggesting that MUM1 expression occurs in the activated state of B/T cells. In B-cell NHL, MUM1 expression was observed in 73.2% (30/41) of diffuse large B-cell lymphoma (DLBCL), 20% (1/5) of marginal zone lymphoma (MZL), and 43% (3/7) of SLL cases, whereas it was not seen in any cases of MCL or FL. Also, MUM1 was aberrantly expressed at high intensity in various types of T-cell lymphomas including adult T-cell leukemia/lymphoma and anaplastic LCL and in the majority of CHL but not in the neoplastic cells of LPHL.[68,69] These results suggest that MUMs are involved in the late stages of B-cell differentiation and in T-cell activation and is deregulated in diffuse LBCL.[70] MUM1 may provide a marker for the identification of transition from $Bcl\text{-}6^+$ (germinal center B cells) to $CD138^+$ (plasma cells).

Now we return to the application of paraffin IHC to subtyping B-cell lymphomas composed predominantly of small cells. Paraffin IHC may be necessary in select cases to support or define the particular subtype. For example, there may be cases in which the morphology and FCI do not clearly distinguish between SLL/CLL and MCL. Such cases may require analysis of cyclin D1 by paraffin IHC. Cyclin D1 is a cell cycle protein that is overexpressed in MCL as a result of t(11;14)(q13;q32). Although fluorescent in situ hybridization analysis for t(11;14) is much more sensitive than cyclin D1 by paraffin IHC (97% vs 69%), oftentimes the only material available for analysis is paraffin-embedded tissue. Real-time polymerase chain reaction (RT-PCR) analysis and routine cytogenetics do not offer increased sensitivity to paraffin IHC in detecting cyclin D1.[71] Although cyclin D1 analysis by paraffin IHC is not highly sensitive, strong and diffuse reactivity of cyclin D1 in most monoclonal B cells has been shown to be highly specific for MCL. Hairy cell leukemia may also be cyclin $D1^+$, but the staining has been described as usually weak and in a subpopulation of the tumor cells.[72] Plasma cell dyscrasias may also show strong cyclin $D1^+$ in tumor cells[73]; however, this finding does not usually cause consternation because MCL is typically easily distinguished morphologically and by other immunophenotypic markers from PCD (to be discussed below in the ''Primary BM Disorders'' section). Of importance, cyclin D1 staining has not clearly been detected in B-CLL.

To address further the differential of MCL versus HCL, as mentioned previously, cyclin D1 staining is generally more intense in MCL. However, in addition, there is a particularly useful paraffin IHC marker: DBA.44 (Figure 2). This marker stains follicle mantle cells in routinely fixed and decalcified paraffin-embedded tissues. It is expressed by only scattered small lymphocytes in normal BM. It is positive in greater than 90% of HCL cases. Positivity of

DBA.44 in combination with the morphology is highly specific for an initial diagnosis of HCL.[74]

Paraffin IHC may be helpful in subtyping B-cell lymphomas composed predominantly of small cells, such as FLs, particularly in cases where a nodular pattern is not readily apparent and also in cases where there is a consideration of an SLL with a nodular pattern or nodular MCL. As mentioned previously, CD10 is expressed by 80% of all types of FL and thus is not highly sensitive. Additional monoclonal antibodies available for paraffin IHC that may aid in establishing a diagnosis of FL include CDw75 and Bcl-6. CDw75 and Bcl-6 both stain normal germinal center cells. CDw75 variably stains B-cell lymphomas.[75] Most FLs are positive, and most SLLs and MCLs are negative. Bcl-6 is restricted to B cells of germinal center origin and 10% to 15% of CD3/CD41$^+$ intrafollicular T cells.[76] It is commonly expressed in low-grade FL and is rare in other indolent B-cell lymphoid malignancies.[77] The combination of these 3 stains (CD10, CDw75, and Bcl-6) in conjunction with CD5 are extremely useful in establishing a specific diagnosis of FL.[78,79]

Monocytoid B-cell lymphoma has a somewhat characteristic immunophenotype by FCI; however, there may be a minor to significant component of monoclonal plasma cells that are best appreciated by paraffin IHC. The monoclonal plasma cells may be identified by CD138, κ, and λ.[80] Lymphoplasmacytic lymphoma is also best diagnosed by a combination of identifying the monoclonal B cells by FCI and the monoclonal plasma cells by paraffin IHC.

Paraffin IHC may be applied not only to immunophenotyping and subtyping of B-cell lymphomas composed predominantly of small cells but also to immunophenotyping B cells composed of intermediate-sized to large cells, to determine their cell of origin and biologic potential. MUM1 expression, which was previously discussed, in combination with evaluation of CD10, Bcl-6, and CD138 may allow for the distinction of 4 immunophenotypic profiles in diffuse LBCLs: germinal center-CD10$^+$ (GC-CD10$^+$; CD10$^+$/Bcl-6$^+$/MUM1$^-$/CD138$^-$), germinal center-CD10$^-$ (GC-CD10$^-$; CD10$^-$/Bcl-6$^+$/MUM1$^-$/CD138$^-$), post–germinal center (pGC; CD10$^-$/Bcl-6$^\pm$/MUM1$^+$/CD138$^-$) and plasmablastic (CD10$^-$/Bcl-6$^-$/MUM1$^+$/CD138$^+$).[81]

Large cell lymphomas of follicle center cell origin (LCL-FCC) generally have a similar immunophenotype to that of FL that have previously been described; LCL-FCC more frequently have absence of CD10. There are controversial reports in the literature regarding the prognostic impact of CD10$^+$ and Bcl-2$^+$ in diffuse LBCLs. Xu et al[82] reported that CD10$^+$ correlated with a lower complete remission (CR) rate; Bcl-2$^+$ also correlated with a lower CR rate and poorer overall survival (OS). Dual CD10/Bcl-2$^+$ correlated with a poorer CR rate to initial therapy and a strikingly worse OS. However, a subsequent study by Chang et al[83] revealed CD10$^+$ cases, particularly those cases with low international prognostic index (IPI) scores, had a better prognosis. Subsequent studies have supported the association of Bcl-2$^+$ with advanced stage and poor OS in diffuse LBCL, including those of non-GC origin[81,84] and of CD10$^+$, Bcl-6$^+$, and MUM1$^-$ (GC-LBCL) with increased OS and event-free survival (EFS).[84] These prognostic markers are summarized in Table 2.

Burkitt lymphoma is also an NHL of follicle center cell origin and typically reveals strong expression of CD10 (90% of BL cases) and Bcl-6 (100% of BL cases). In contrast to LCL-FCC, Bcl-2 is typically not expressed in BL, as previously discussed. The differential diagnosis of BL includes diffuse LBCL with morphological high-grade features, including those with a c-*myc* rearrangement. Ki-67 (MIB-1) has been claimed as a useful marker by paraffin IHC to distinguish BL from LBCL with morphological high-grade features. The MIB-1 index should be greater than 98% and not less than 95% in BL. In contrast, the MIB-1 index in diffuse LBCL with c-*myc* rearrangement ranges between 48% and 90% (mean, 66%); a MIB-1 index greater than 95% was not observed in this group. Another immunophenotypic difference is the presence of Bcl-2 in a higher percentage (75%) of these cases.[85] However, a difference in prognosis between BL and diffuse LBCL with a c-*myc* rearrangement has not been evaluated or established.

Of interest, Bcl-6 protein expression has also been observed in diffuse LBCL of the stomach and small intestine.[86] In a study by Kwon et al,[86] two distinct patterns of Bcl-6 expression were recognized in this group of lymphomas: diffusely dense (>75% cells positive), seen in those of germinal center (GC) B-cell deviation; and sporadic (<75% cells positive with lack of consistently dense positivity), seen in non-GC LBCL, including high-grade MALT lymphoma. In addition, CD10 expression was observed in 3 of 20 (67%) of the high-grade MALT lymphomas analyzed. Thus, Bcl-6 and CD10 may be expressed in diffuse LBCL of non-GC origin; however, the pattern of Bcl-6 protein expression is distinctly different.

Another appropriate group of lymphomas to discuss at this point include primary central nervous system (CNS) diffuse LBCLs among immunocompetent individuals. In a study by Chang et al,[87] expression of p53, c-Myc, or Bcl-6 correlated with poorer overall survival (p53, c-Myc, and Bcl-6) and increased mortality rate (p53, c-Myc, and Bcl-6). MUM1 was not analyzed in this study. In addition, this same group of authors reported expression of p53 or c-Myc in non-CNS diffuse LBCLs correlated with an adverse clinical outcome.[88] Another recent study by Braaten

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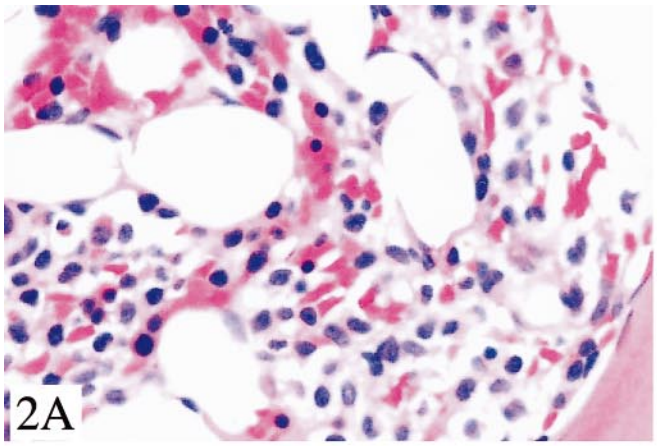
Figure 2. *This case of hairy cell leukemia (HCL) was originally diagnosed as lymphoma of follicular origin due to CD10 coexpression by flow cytometric analysis. However, when the patient did not respond to therapy, review of the follow-up marrow revealed (A) a pattern of bone marrow involvement, highly suggestive of HCL. This diagnosis was confirmed by (B) intense staining of the cells with DBA.44 (A, hematoxylin-eosin stain, original magnification ×600; B, DBA.44 stain, original magnification ×600.)*

Figure 3. *This case of lymphocyte-rich classical Hodgkin lymphoma (LRCHL) may be morphologically similar to nodular lymphocyte-predominant Hodgkin lymphoma (LPHL) due to (A) the nodular pattern containing large atypical cells. However, on higher power (B), classic Reed-Sternberg cells are identified that reveal (C) intense cytoplasmic membrane staining with CD20 and intense Golgi staining with (D) CD15 and (E) CD30 as well as staining with (F) fascin (A, hematoxylin-eosin stain, original magnification ×200; B, hematoxylin-eosin stain, original magnification ×400; C, CD20 stain, original magnification ×400; D, CD15 stain, original magnification ×400; E, CD30 stain, original magnification ×400; F, fascin stain, original magnification ×400).*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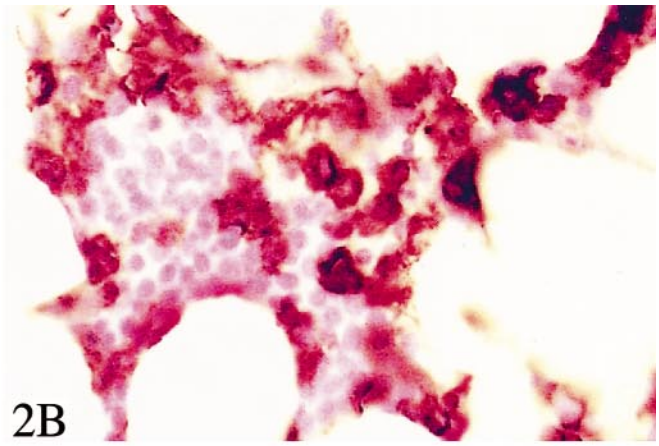
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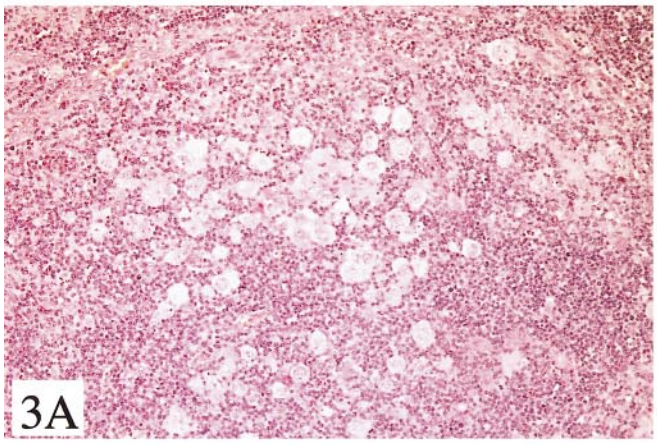
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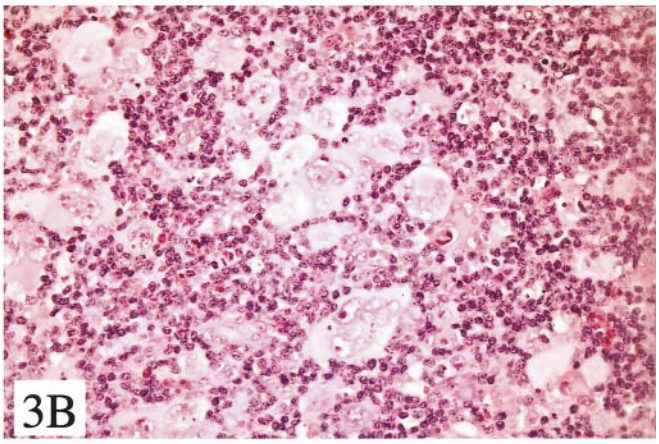
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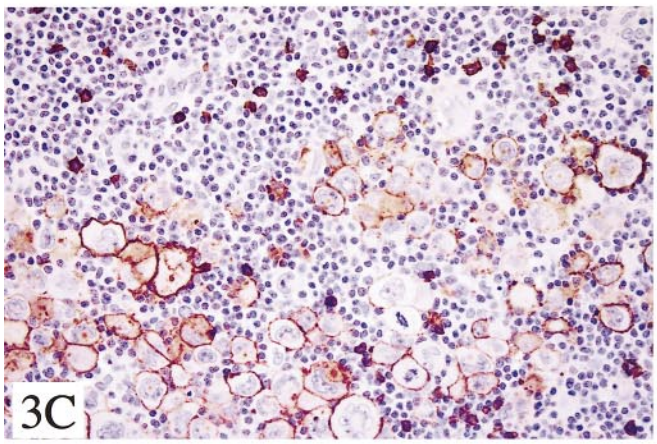
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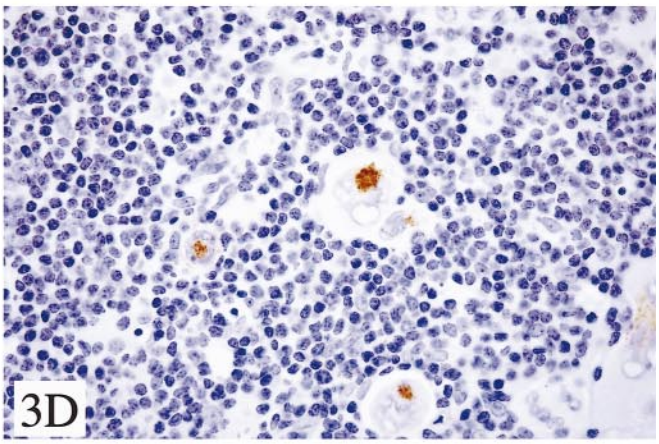
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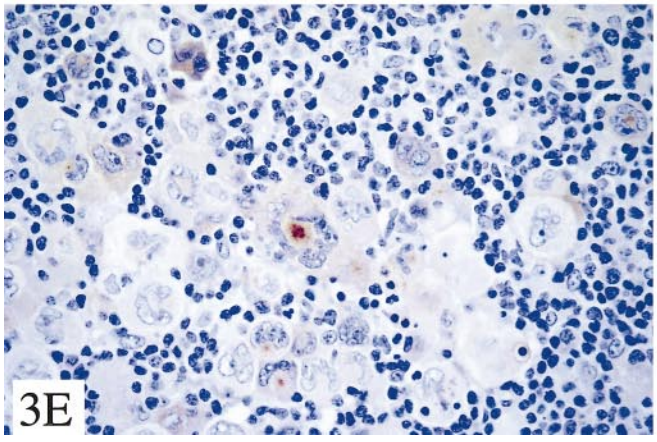
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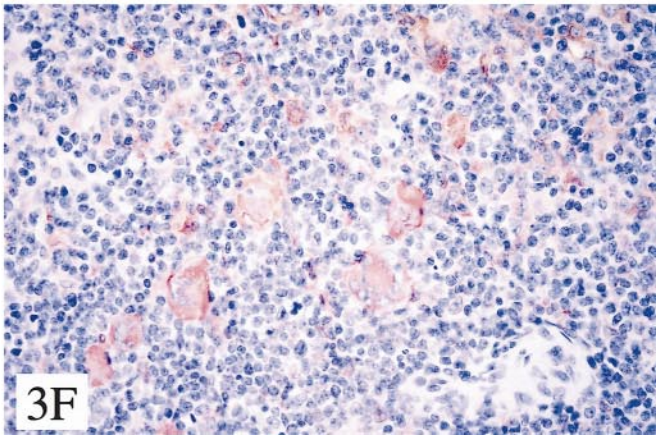
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et al[89] of non-HIV associated primary CNS lymphomas revealed that Bcl-6 expression predicted longer OS. This group was also associated with MUM1 expression, indicating a late GC stage of differentiation.

It is clearly important to distinguish $CD5^+$, $CD23^-$ LCLs of B-cell origin. These may represent a large cell variant (blastic or pleomorphic) of MCL or de novo $CD5^+$ LCL. These 2 are distinguished based on the association of t(11;14) in the large cell variants of MCL. Cyclin D1 may thus be evaluated by paraffin IHC; however, the sensitivity is not particularly high. Nevertheless, this distinction may not be clinically relevant because the general group of $CD5^+$, $CD23^-$ LCLs have an aggressive clinical course, characterized by very poor treatment outcome associated with frequent relapses.[90] It should also be recognized that de novo $CD5^+$ diffuse LBCL may represent a heterogenous group of LBCLs, including an unusual form of splenic lymphoma with diffuse, red pulp involvement.[91] There are even rare reported cases of $CD5^+$ BL.[92]

Another LCL of B-cell origin important to recognize is primary mediastinal B-cell lymphoma (PMBCL), due to its morphology and clinical presentation, which raises the differential diagnosis of a syncytial variant of nodular sclerosing HL; CD30 positivity in this group of LBCLs may raise the differential diagnosis of an anaplastic $CD30^+$ LCL. PMBCL is typically associated with a moderate to marked degree of tumoral sclerosis, often yielding too few cells for FCI or a relatively low number of tumoral cells in the sample for FCI. In addition, by FCI, the malignant B cells not only are few in number but also typically lack any sIg or light-chain expression. By paraffin IHC, PMBCL typically has the following immunophenotype: $CD20^+$, $CD45^+$, $CD5^-$, $CD10^{-/+}$ (32% of cases), $CD30^+$ (weak-strong, focal-extensive), $Bcl\text{-}6^+$, $CD15^-$, and $ALK\text{-}1^-$. The findings of Bcl-6 positivity and variable CD10 positivity in this group suggests evidence for derivation from germinal center B cells, at least in a subset of these lymphomas.[93] However, thymic B cells have also been proposed as the putative normal counterpart of PMBCL. This proposal has been supported by the identification of MAL protein expression in 70% of PMBCL in a study reported by Copie-Bergman et al.[94] A recent large IHC study of PMBCLs disclosed the following immunophenotype: $CD45^+$, $CD20^+$, $CD79a^+$, $Pax\text{-}5/BSAP^+$, $BOB.1^+$, $Oct\text{-}2^+$, $Bcl\text{-}2^+$, $CD30^+$, MAL $protein^{\pm}$, $Bcl\text{-}6^{\pm}$, $MUM1/IR4^{\pm}$, $CD10^{-/+}$, $CD15^-$, and $CD138^-$. Immunoglobulins were negative both by IHC and in situ hybridization (ISH). This study supports the concept that a sizable fraction of this group of LBCLs are from activated germinal center or post–germinal center cells. Of interest, it shows defective immunoglobulin production despite the expression of BOB.1 and Oct.2 transcription factors.[95] The characteristic immunophenotypes of CHL and anaplastic $CD30^+$ LCL will be discussed later for comparison.

Another type of LBCL that may be morphologically considered in the differential diagnosis of HL is a TCRBCL or LHRBCL. These LBCLs are characterized by a background rich (>80% background cells) in lymphocytes, with or without a histiocytic component. These lymphomas typically do not reveal a monoclonal B-cell population by FCI due to the low number of malignant cells relative to the background cells. This group of diffuse LBCLs represents a heterogenous group, and by paraffin IHC, the scattered large malignant B cells have the following immunophenotype: $CD20^+$, $CD10^{-/+}$ (25%–50%), CD5 (10%), $Bcl\text{-}2^+$ (30%–50%), and $Bcl\text{-}6^+$ (majority of cases). The distinction of TCRBCL from a pure, diffuse type of LPHL may not be possible and has been a matter of controversy.[96–98] Areas within NLPHL may have a diffuse pattern characterized by scattered L&H cells set in a diffuse background of reactive T cells with a loss of $CD57^+$ T cells as well as a loss of the follicular dendritic cell meshwork. The distinction of LPHL with a diffuse pattern from TCRBCL requires the presence of a nodular component of LPHL existing in association with the diffuse areas in the same biopsy. In the absence of a nodular component of LPHL, a purely diffuse pattern would be regarded as TCRBCL. In the report of NLPHL by Fan et al,[64] the diffuse pattern of LPHL (TCRBCL-like) was significantly more common in cases that recurred than in those without recurrence, and the predominance of a diffuse pattern was even a stronger prediction of recurrence. Thus, although the distinction between TCRBCL and pure, diffuse LPHL has been controversial, a predominant diffuse pattern seems important to recognize.

Anaplastic morphology may occasionally be observed in DLBCL, and these cases may be morphologically difficult to differentiate from anaplastic $CD30^+$ LCL and/or anaplastic plasmacytoma. In addition, there are rare cases of $CD30^+$ diffuse anaplastic lymphomas of B-cell origin associated with t(2;5)(p23;q35) or anaplastic lymphoma kinase (ALK) positivity.[99] These rare neoplasms are considered an anaplastic variant of DLBCL, as the WHO classification reserves the terminology of anaplastic $CD30^+$ LCL for those of T-cell origin, defining a distinct, separate entity. To complicate further these differential diagnoses, plasmablastic lymphomas have recently been described in the new WHO classification.[100] This type of lymphoma typically presents in the oral cavity and occurs in an immunocompromised setting (ie, human immunodeficiency virus [HIV] syndrome) and has the following immunophenotype: $CD45\ (LCA)^-$, $CD20^-$, $VS38C^+$, $CD138^+$, EMA^+, monoclonal light chain, and IgH. In addition, there have been recent case reports of diffuse LBCL of plasmablastic type (ALK^+, $CD30^-$, $CD20^-$, EMA^+, $CD38^+$ and $CD138^+$ or $VS38C^+$, and monoclonal cytoplasmic immunoglobulin or light chain) associated with the t(2;5)(p23;q35) chromosomal translocation or other abnormalities involving 2p23.[101,102] These patients were not reportedly HIV^+. One of these cases was associated with a monoclonal gammopathy. Anaplastic plasmacytoma, on the other hand, has an identical immunophenotype but is ALK^-. Table 10 compares the immunohistochemical immunophenotype profiles of these entities. Another diffuse LCL that may represent a heterogenous disease of post-GC origin and be difficult to differentiate morphologically from diffuse LBCL, plasmablastic lymphoma, anaplastic plasmacytoma, and anaplastic $CD30^+$ LCL is primary effusion lymphoma (PEL). As the name implies, PEL usually presents as a serous effusion, and it is most often associated with HIV^+ and universally associated with human herpes virus 8. The lymphoma cells in PEL are usually LCA^+, $CD20^-$, $CD138^+$, $CD30^+$, sIg^-, and $MUM1^+$.[103] However, there are cases that are $CD138^-$, $CD20^+$, $CD30^-$, and sIg^+. In addition, there may be aberrant expression of T-cell markers (CD3, CD7) and CD56 in PEL.[104]

Anaplastic $CD30^+$ LCL, as mentioned previously, is defined by the WHO classification as of T-cell origin. They are defined by their morphology and CD30 positivity and may occur as several well-described variants (common,

small cell, lymphohistiocytic, sarcomatous, and so forth). They are defined by expression of a T-cell antigen (CD2, CD3, CD4, CD5, or CD8) or a null cell immunophenotype (ie, lacking expression of T, B, histiocytic, and plasmacytic markers) with a T-cell gene rearrangement by molecular analysis. They may have variable expression of LCA (CD45) and EMA. In addition, there may be rare cases of $CD30^+$ lymphomas with anaplastic morphology and an associated t(2;5) in which the malignant cells are of histiocytic derivation, based on immunoreactivity with CD68 and no additional markers.[105]

$CD30^+$ ALCL may occur as a primary cutaneous form or as a systemic form. The primary cutaneous form of ALCL is characteristically ALK negative and has a more favorable prognosis than systemic ALCL. However, systemic $CD30^+$ ALCL, which are associated with the t(2;5) (p23;q35) chromosomal translocation and express ALK, are associated with a better survival. Because systemic ALCL may be ALK^- and primary cutaneous ALCL is characteristically ALK^-, there may be occasional cases in which the distinction of these 2 forms is unclear. Initially, expression of clusterin was reported to be characteristic of systemic ALCL and usually negative in cutaneous ALCL as well as other lymphoma types.[106] However, subsequent studies have revealed that clusterin is positive in a substantial subset of cutaneous ALCL,[107] as well as a smaller subset of diffuse LBCLs and, rarely, in cases of peripheral T-cell lymphoma and nodular sclerosing HL (3%).[108] No cases of mixed cellularity HL or nodular LPHL were $clusterin^+$. However, subsequently, CD44v6 (a variant form of CD44—a multifunctional cell surface adhesion molecule) has been shown to be an independent marker of the systemic form of ALCL.[109]

The typical staining pattern of CD30 in ALCL is uniform intense Golgi staining. It should be noted at this point that non-ALCLs may reveal CD30 staining, but the staining is generally not as intense or diffuse. CD30 staining may also be observed in benign immunoblastic proliferations[110] as well as RS cells and their variants in HL.[108] Of interest, another marker, BLA.36, may also be expressed by RS cells, their mononuclear variants, and ALCL of T-cell origin as well as lymphomatoid papulosis (LyP) of T-cell origin. This finding has been suggested to support a relationship between HL, ALCL, and LyP and has led to the suggestion that RS cells are histogenetically related to both B and T lymphocytes.[111] On the other hand, ALK-1 staining is much more specific with expression currently only described in ALCL and a nonhematopoietic entity, inflammatory myofibroblastic tumor.[112,113]

Composite lymphomas represent simultaneous involvement by 2 types of lymphoma and may be composed of 2 distinct types of NHL but more frequently an NHL and a concomitant CHL. Classical HL is uniquely defined by paraffin IHC. Classical HL is composed of 4 subtypes: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-rich (LR), and lymphocyte-depleted (LD). The immunophenotype of LPHL has been previously described. Although it has been well established that LPHL represents a B-cell lymphoma, there has been more controversy in the origin of the RS cell and variants in CHL. In CHL, CD20 may be expressed in 10% to 20% of cases in paraffin material and in 87% of cases in frozen material.[114–116] In addition, another B-cell marker, CD79a (mb.1), expression has been described in 20% of CHL cases.[117] Also, as mentioned previously and recently described, Pax-5 expression has been detected in 97% of CHL. However, other B-cell transcription factors commonly expressed in NLPHL and DLBCL, including Oct-2 and BOB.1, are not expressed in CHL. In a recent study by Browne et al,[118] a BSAP $(Pax\text{-}5)^+$, $Oct\text{-}2^+$, $BOB.1^+$ immunophenotype was predictive of NLPHL and diffuse LBCL, whereas a $BSAP^+$, $Oct\text{-}2^-$, $BOB.1^-$ immunophenotype was predictive of CHL. In the study by Rudiger et al,[97] all cases of CHL were $CD30^+$ in at least a proportion of the tumor cells, but CD15 expression was identified in only 80% of cases. Although CD15 is not extremely sensitive, it may be used in combination with other markers in the most common differential diagnoses of CHL: NLPHL and ALCL. CD15 is apparently present on the L&H cells of NLPHL but is usually not detected due to its presence as a sialylated form.[119] The incidence of $CD15^+$ in ALCL ranges from 0% to 20% in 3 large series.[120–122] Expression of EMA or CD45 (LCA) is rare (less than 5%) in CHL.

LRCHL (Figure 3) may be morphologically most confused with NLPHL and the other subtypes of CHL and with NHL, including ALCL. Table 11 compares the immunophenotypes of CHL with NLPHL and ALCL. Classical HL may be differentiated from ALCL if ALK is overexpressed. In ALK^- cases, expression of T-cell–related antigens (CD3, CD43, CD45RO, CD5, CD4, CD8), EMA, or CD45 strongly supports ALCL, whereas expression of CD20, Pax-5, EBV-latent membrane protein (LMP), or CD15 supports CHL. Initially, fascin expression was considered possibly useful in differentiating CHL from ALCL. However, although fascin shows strong positivity in all cases of CHL, fascin has also been shown to be expressed in high percentages of ALCL (59% T-cell ALCL, 77% null cell ALCL, 91% $ALK\text{-}1^+$ ALCL).[123]

In addition, Barry et al[124] have recently reported the coexpression of both CD30 and CD15 in at least a subset of the neoplastic cells in cases of peripheral T-cell lymphoma (PTCL). Two distinct groups were identified based on morphologic and immunophenotype features. The first group had histologic features mimicking CHL with $CD30^+$, $CD15^+$ RS-like cells in an inflammatory background of varied extent and composition. The background lymphoid cells showed minimal cytologic atypia. The RS-like cells were negative for CD20 and CD79a, and CD45

Table 10. Comparison of Immunohistochemical Immunophenotypic Profiles of Anaplastic Large Cell Lymphoma (ALCL), Diffuse Large B-Cell Lymphoma (LBCL) (Anaplastic and Plasmablastic Variants), Anaplastic Plasmacytoma, and Plasmablastic Lymphoma*

Marker	ALCL	DLBCL, Anaplastic	DLBCL, Plasma-blastic	Anaplastic Plasma-cytoma	Plasma-blastic Lymphoma
CD45 (LCA)	+/−	+	NR	−	−
CD20	−	+	−	−	−
T-cell markers	+/−	−	−	−	−
CD30	+	+	−	−/+	NR
CD138	−	−	+	+	+
VS38C	−	−	+	+	+
EMA	+	+	+	+	+
clg	−	+	+	+	+
ALK	+	+	+	−	−

* DLBCL, diffuse LBCL; NR, not reported; EMA, epithelial membrane antigen; clg, cytoplasmic immunoglobulin; and ALK, anaplastic lymphoma kinase.

Table 11. Comparison of Immunophenotypic Profiles of Classical Hodgkin Lymphoma (CHL), Nodular Lymphocyte Predominant Hodgkin Lymphoma (NLPHL), and Anaplastic Large Cell Lymphoma (ALCL)*

Type of Lymphoma	T-Cell Markers (CD3, CD5, CD4, CD43, CD45RO)	CD20	CD79a	Pax-5	Oct-2	BOB.1	CD15	CD30	CD45	EMA	LMP	ALK
CHL	−	+(10%–20%)	+(20%)	+(97%)	−	−	+ (80%)	+(100% Strong	− (<5%+)	− (<5%+)	+	−
NLPHL	−	+	+	+	+	+	− (rare+)	+(19% Focal	+	+(44%)	−	−
ALCL-O	− (CD43v+)	−	−	−	−	−	− (0%–20%+)	+	Variably+	+(80%)	−	+/−
ALCL-T	Variably+	−	−	−	−	−	− (0%–20%+)	+	Variably+	+(80%)	−	+/−

* EMA indicates epithelial membrane antigen; LMP, latent membrane protein; ALK, anaplastic lymphoma kinase; O, null cell type; and V, variably.

expression was absent in 80% of cases. The RS-like cells expressed CD25 and at least one T-cell–associated marker in all cases. The background T-cell population showed convincing subset predominance in 80% of cases, loss of T-cell–associated antigens in 60% of cases, and coexpression of CD30 and CD15 in 20% of cases. The second group had morphologic features more in keeping with PTCL than CHL. The proportion of neoplastic cells coexpressing CD30 and CD15 varied. Loss of T-cell antigens was noted in all cases, and CD4 predominated in 80% of cases. Fifty percent of cases expressed CD45. Polymerase chain reaction analysis revealed clonal T-cell receptor gamma (TCR-γ) chain gene rearrangements in 82% of cases but no IgH rearrangements. In situ hybridization studies for EBV were negative in all cases. In some PTCL cases, the overlap with CHL may be striking, and combined immunophenotypic and molecular studies are often necessary to confirm the diagnosis.

The remainder of T-cell lymphomas may be immunophenotyped based on the profiles discussed in the ''Flow Cytometry'' section. One unique use of paraffin IHC in T-cell lymphomas is in evaluating expression of cytotoxic molecules, including granzyme B, perforin, and T-cell intracellular antigen (TIA-1). These molecules are variably expressed in the following types of T-cell lymphoproliferative disorders: nasal NK/T-cell lymphoma (all 3+), subcutaneous panniculitis-like T-cell lymphoma (all 3+), hepatosplenic T-cell lymphoma (TIA-1$^+$, perforin$^-$), and enteropathy-type T-cell lymphoma (all 3+). TIA may be expressed in 60% to 70% of ALCL as well as in most cases of T-cell LGL leukemias.[125,126]

Posttransplant lymphoproliferative disorders may be categorized as outlined in Table 12.[127] The various types generally immunophenotype as the entities already discussed (ALCL, HL, and so forth). Infectious mononucleosis-like early lesion shows a mixture of small lymphocytes, plasma cells, histiocytes, and scattered immunoblasts. It is EBV$^+$ and polyclonal. Polymorphic PTLDs typically show a mixture of B and T cells; cytoplasmic immunoglobulin may be either polytypic or monotypic.

Paraffin IHC may also be useful in differentiating a large cell NHL with high-grade morphologic features from LLs and granulocytic/monocytic sarcomas, particularly if fresh tissue is not available for FCI. Typically, LL is of T-cell lineage but may rarely be of B-cell lineage. The application of paraffin IHC to diagnose LL and granulocytic/monocytic sarcoma will be discussed in the ''Primary BM Disorders'' section, devoted to acute leukemias.

Table 12. Categories of Posttransplant Lymphoproliferative Disorder (PTLD) Recognized at the Society for Hematopathology Workshop[127]*

- Early lesions
  - Reactive plasmacytic hyperplasia
  - Infectious mononucleosis-like
- PTLD—polymorphic
  - Polyclonal (rare)
  - Monoclonal
- PTLD—monomorphic
  - B-cell lymphoma
    - Diffuse large B-cell lymphoma (immunoblastic, centroblastic, anaplastic)
    - Burkitt/Burkitt-like lymphoma
  - T-cell lymphoma
    - Peripheral T-cell lymphoma, unspecified type (usually large cell)
    - Anaplastic large cell lymphoma (T or null)
    - Other types (eg, T-NK)
- Other
  - T-cell–rich/Hodgkin disease–like large B-cell lymphoma
  - Plasmacytoma-like
  - Myeloma

* NK, natural killer.

Primary BM Disorders

Acute Leukemias and Differential Diagnoses.—Although FCI is the preferred method of immunophenotypically differentiating acute leukemias, paraffin IHC may be useful in situations in which FCI is not able to be performed. Because many of the markers used by FCI in differentiating acute leukemias are not available for paraffin IHC, markers unique to paraffin IHC will be discussed.

In a study by Horny et al[128] evaluating the diagnostic usefulness of 6 antibodies (anti-lysozyme, MAC387, Ki-M1p, KP1, Ki-My2p, and anti-neutrophil elastase), the combination of anti-lysozyme and KP1 was recommended for use in routine diagnostics for the differentiation of AML from ALL on the basis of paraffin IHC of EDTA-decalcified, formalin-fixed BM specimens. Staining with both anti-lysozyme and KP1 (CD68) was encountered in a relatively large percentage (anti-lysozyme, 56%; KP1, 83%) of cases of AML but in none of ALL.

A subsequent large study by Arber et al addressed the usefulness of additional available markers for immunophenotyping acute leukemias by paraffin IHC, including

CD3, CD20, CD34, CD43, CD68, CD79a, HLA-DR, myeloperoxidase (MPO), and TdT, in predominantly Bouin-fixed, decalcified (s/p decal) BM specimens. Myeloperoxidase and CD68 were the most specific myeloid markers (96% and 77% of AML cases positive, respectively). In fact, MPO^+ AML cases even included all studied cases of M0 AML. However, 11% of precursor B-ALL cases were also MPO and $CD68^+$, but generally weaker staining than in the AML cases. CD79a and TdT expression were the most frequently detected antigens in precursor B-ALL cases (89% $CD79a^+$; 100% TdT^+). However, CD79a was also detected in 11% of AML cases, most frequently of M3 subtype (90% of M3 cases, $CD79a^+$), and TdT, in 13% of AML cases. An $MPO^+/CD79a^+/HLA\text{-}DR^-$ immunophenotype was only seen in M3 AML. In comparison to the FCI results in this study, CD3 and CD20 were more reliably determined by paraffin IHC; CD34 and HLA-DR were more reliably determined by FCI. However, CD20 was present on only 33% of precursor B-ALL cases. Nevertheless, 72% precursor B-ALL cases were identified by a $CD79a^+/TdT^+/MPO^-/CD3^-$ immunophenotype. All T-ALL cases were either $CD3^+/CD79a^-/MPO^-/TdT^+$ or $CD3^+/CD79a^-/MPO^-/TdT$ uninterpretable. Thus, this study recommended a panel of CD3, CD79a, MPO, and TdT as the most useful approach to paraffin immunophenotyping of acute leukemias.[129] A simultaneous study by Chuang et al[130] on B-5-, subsequently formalin-fixed, formic acid decalcified BM specimens supported the usefulness of these markers and the expected expression of CD20 in the more differentiated B-ALL cases (strongest in L3). This study also described the usefulness of hemoglobin in M6 AML (100% positive) and factor VIII–related antigen in M7 AML (75% positive). These markers may also be applied to extramedullary presentations of acute leukemia, keeping in mind that in AML, the classification is determined on a BM specimen. In addition, a relatively recent study by Chang et al[131] revealed that the immature myeloid cells in GS are frequently $HLA\text{-}DR^+$ but uncommonly $CD34^+$ (except in underlying MDS and CML cases). Thus, immunophenotyping of GS is useful in differentiating it from other malignancies, including LCL, and if the BM is not involved. However, a BM specimen is necessary for classification of AML.

The French-American-British (FAB) classification of AML is based on morphology, enzyme cytochemical (EC) staining, and FCI. Because occasionally BM aspirates are not obtainable for FCI and EC staining, various studies have attempted to classify AML by applying paraffin IHC.[132] The relatively recent large study of AMLs by Manaloor et al[133] compared immunophenotypic data by FCI with paraffin IHC of formalin or B-5 fixed, acid decalcified BM specimens using antibodies to CD34, MPO, hemoglobin, factor VIII–related antigen, and CD68 (HAM56, KP1, and PG-M1). They reported a high concordance rate of CD34 results by both methods, excellent correlation of CD14 and CD64 results by FCI with CD68 (PG-M1), staining of the dominant erythroid population with antihemoglobin in the 2 M6 AML cases, and staining of the 1 M7 AML with antibody to factor VIII–related antigen. In addition, there was concordance of CD13 and CD33 results by FCI with MPO (except in M0 AML); MPO was negative in 7 of 8 M0 AML cases. However, this study did not compare the FCI data or paraffin IHC results with EC staining, which is required for FAB subtyping. As mentioned previously, CD14 by FCI is not a sensitive marker of AML with a monocytic origin or component, and CD64 by FCI is not entirely specific for these AML cases; MPO by paraffin IHC has also been described in ALL. A later study of AMLs (B-5 fixed, decalcified BM specimens) compared EC staining results with FCI data and paraffin IHC using antibodies to CD34, CD15, CD117, and MPO.[134] Immunodetection of CD34 expression in AML had a similar sensitivity by FC and IHC techniques. Immunodetection of CD15 and CD117 had a higher sensitivity by FC analysis than by IHC analysis. Detection of MPO by IHC analysis was more sensitive than by EC analysis. There was no correlation of FAB subtype of AML with CD34 or CD117 expression. Expression of CD15 was associated with AMLs with a monocytic component. Myeloperoxidase reactivity by IHC analysis was observed in AMLs originally FAB subtyped as M0, as in the previous study.

Most recently, another paraffin IHC marker, vascular endothelial growth factor (VEGF), has been demonstrated to be restricted to certain stages of differentiation and maturation of myeloid cells (not maturing granulocytic cells) and megakaryocytic cells and to correlate with the FAB category of AML.[135] VEGF is expressed by the vast majority of myeloblasts (>90%) in all FAB subtypes (M1–M7, except M0) and in megakaryocytic cells. The percentage of $VEGF^+$ blasts was clearly lower in M0 AML, and the erythroblast component of M6 AML was clearly $VEGF^-$. Vascular endothelial growth factor was expressed in the M7 AMLs. Thus, this marker deserves consideration as a useful marker in subtyping AMLs by paraffin IHC, in combination with additional markers previously described and in distinguishing myeloblasts from erythroblasts. The application of paraffin IHC to the evaluation of BM specimens for acute leukemia may also be useful in follow-up marrow specimens. If a distinct immunophenotype has been established at initial diagnosis, a tailored panel may be applied to evaluate for residual/relapsing acute leukemia, especially if a BM aspirate is not attainable. A summary of immunohistochemical immunophenotyping of acute leukemias is outlined in Table 13.

Paraffin IHC is not only useful in differentiating AML from ALL and subtyping AML but also in differentiating acute leukemia from high-grade lymphoma and nonhematopoietic malignancies, including small blue cell tumors. In children, the differential diagnosis includes neuroblastoma, PNET/ES, medulloblastoma, and rhabdomyosarcoma; and in adults, small cell undifferentiated carcinoma. High-grade NHL may be differentiated from ALL by the evaluation of MIC2 (CD99), TdT, Bcl-2, and CD34 by paraffin IHC. Expression of CD99, TdT, Bcl-2, and CD34 support the diagnosis of ALL over small noncleaved cell lymphomas (SNCL).[136,137] Neuroblastomas typically express chromogranin and somatostatin; rhabdomyosarcomas typically express desmin and muscle-specific actin. There may be immunophenotypic overlap between acute leukemia and PNET/ES in that they may both express CD99, and CD99 expression is not restricted to TdT^+ hematologic malignancies.[138] However, CD56 and CD57 are typically expressed in PNET/ES and negative in ALL; MPO is generally expressed in AML (except M0 AML). Small cell undifferentiated carcinomas generally express low-molecular-weight keratins as well as CD56. In addition, a high-grade MDS or accelerated phase of a chronic myeloproliferative disorder (CMPD) may be in the differential diagnosis. These may be distinguished from ALL by the markers previously described as helpful in

Table 13. Immunohistochemical Immunophenotyping in Acute Leukemia*

Type of Acute Leukemia	Most Useful Markers	Reason	References
AML	Anti-lysozyme, KP-1	Negative in all cases of ALL	128
	MPO	96% of AML cases+	129
	Hemoglobin	M6 AML	130
	Factor VIII	M7 AML	130
	VEGF	VEGF—in erythroblasts	135
T-ALL	$CD3^+/CD79a^-/MPO^-/TdT^+$ or $CD3^+/CD79a^-/MPO^-$/TdT indeterminate	All cases with one of these two phenotypes	129
B-ALL	$CD79a^+/TdT^+/MPO^-/CD3^-$	72% of cases identified	129

* AML indicates acute myelogenous leukemias; MPO, myeloperoxidase; VEGF, vascular endothelial growth factor; and ALL, acute lymphoblastic leukemia.

differentiating ALL from AML. However, more likely is the distinction of AML from these disorders. Evaluation of CD34 may be helpful in determining a percentage of blasts for these purposes; however, one must keep in mind that a subset of myeloblasts are $CD34^-$. Anti-hemoglobin and VEGF may be helpful in differentiating erythroid cells from myeloblasts in the differential of AML and high-grade MDS, as VEGF does not stain erythroid cells.

Other Primary BM Disorders.—Because quantitation and determination of clonality of plasma cells (PCs) are essential in establishing a diagnosis of PCD and FCI is not a useful method for these purposes, paraffin IHC of BM specimens may be applied. CD138 is a highly sensitive and specific marker for identifying and quantifying normal and neoplastic PCs in paraffin-embedded BM and lymph node samples (Figure 4). In human BM, CD138 only reacts with PCs, and in NHL, CD138 reacts with the mature PCs of lymphoplasmacytic lymphoma and the neoplastic cells of plasmablastic lymphoma, DLBCL of plasmablastic type associated with ALK positivity, PTLDs with a plasmacytic component, and in plasmacytomas.[139,140] Clonality of the PCs may be determined by κ/λ paraffin IHC. These stains may also be applied in follow-up marrows to determine the presence or absence of residual/relapsing PCD.

Another BM disorder in which paraffin IHC is particularly useful is mast cell disease, namely systemic and malignant mastocytosis. There has been evidence that mast cells (MCs) are derived from multipotential hematopoietic stem cells (HSCs); they are clonally increased in systemic mastocytosis (SM). Recent results have demonstrated the multipotential HSCs of SM give rise to B cells and monocytes in addition to MCs.[141] Also, MCs have previously been shown to be closely related to cells of the mononuclear phagocyte system but not to those of macrophage derivatives belonging to the immune accessory cell compartment. Horny et al[142] have shown normal MCs stain with the macrophage-associated antibodies KP1 (CD68), Ki-M1p, and PG-M1 (CD68) but not with 3 other antibodies (HAM56, MAC387, LN5) or CD35 and S100 protein. Of interest, KP1 stained all normal/reactive and neoplastic MCs, whereas Ki-M1p and PG-M1 did not stain normal/reactive MCs. Ki-M1p stained nearly all cases with neoplastic MCs; PG-M1 stained approximately half of the cases with neoplastic MCs.[142]

Although the multipotential HSCs of SM give rise to B cells, and BM lesions often have admixed lymphocytes by IHC, the lymphocytes represent an equal mixture of mature T and B cells. In addition to expressing the monocyte-associated antigens previously described, MCs of SM constantly coexpress tryptase, CD25, and CD117 protein.[143] The CD117 protein expression is due to the fact that SM is associated with a point mutation of the c-*kit* gene. MCs of SM also may be identified with the toluidine blue special stain.

Evaluation of BM for Lymphomatous Involvement.—Lymphomas may involve the BM primarily or, more frequently, secondarily. Because the applications of IHC to primary lymphoma diagnostics has been previously discussed, this section will primarily focus on the evaluation of BM for secondary lymphomatous involvement. However, prior to embarking on this discussion, the diagnostic usefulness of 1 IHC stain, $p27^{Kip1}$, will be discussed. $p27^{Kip1}$ staining detects a cyclin-dependent kinase inhibitor protein, which is expressed at high levels in most small B-cell NHL, inversely correlated to the proliferation rate. A study by Kremer et al[144] supports the usefulness of this stain in BM biopsies for the differential diagnosis of small B-cell NHL infiltrates. Chronic LL, FL, and marginal zone lymphoma revealed strong $p27^{Kip1}$ nuclear staining, whereas MCL and HCL revealed absent (78% MCL, 77% HCL) or weak staining.

Secondary lymphomatous involvement of the BM may most often be determined reliably by morphologic examination combined with FCI; however, there may be occasional cases that require IHC due to the presence of small cell lymphoid aggregates (SCLAs) in the BM. In a large study by Bluth et al,[145] L-26 (CD20) was determined to be useful in differentiating reactive from neoplastic SCLAs. With L-26, 3 distinct patterns of lymphocyte marking were identified within aggregates: (1) homogenous—uniform marking of almost all lymphocytes, (2) mixed—even distribution of marking and nonmarking lymphocytes, and (3) focal homogeneous—collections of uniformly marking lymphocytes either surrounding or surrounded by nonmarking lymphocytes. A homogeneous pattern was the predominant type in 73% of neoplastic SCLAs.[145] A smaller percentage (8%) of reactive SCLAs showed homogeneous staining; however, these were always associated with aggregates with other staining patterns. The size and number of aggregates with a homogeneous pattern further helped discriminate between reactive and neoplastic SCLAs. Those BMs revealing greater than 4 homogeneously staining aggregates were all associated with neoplastic SCLAs. Furthermore, no reactive SCLAs were associated with more than 1 large homogeneous aggregate. Another IHC stain that may be useful in distinguishing reactive from neoplastic SCLAs is Bcl-2. Although rare reactive SCLAs (4%) may show $Bcl\text{-}2^+$, all cases of neoplastic SCLAs studied by Ben-Ezra et al[146] revealed weak to in-

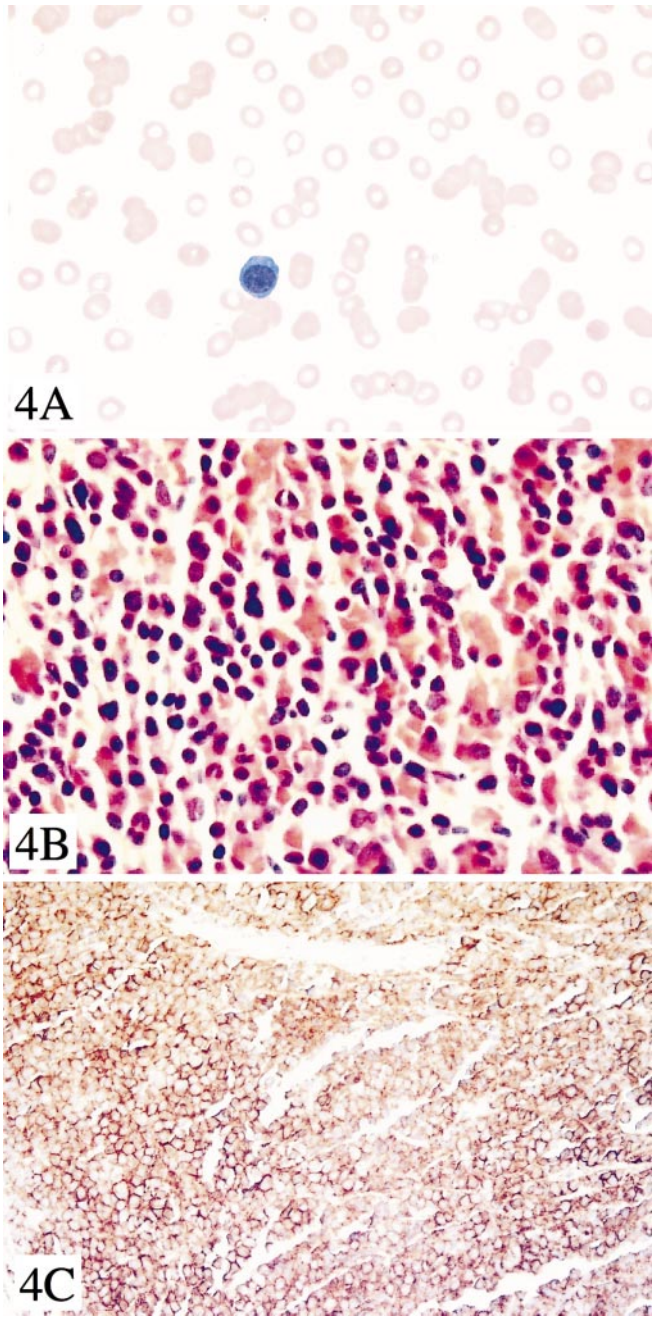

Figure 4. *Occasional cases of plasma cell dyscrasia (PCD) may have a more lymphocytic morphology, as seen in (A) the peripheral blood smear and (B) bone marrow hematoxylin-eosin–stained section of this case of plasma cell leukemia. The plasmacytic origin of the neoplastic cells is confirmed by (C) intense staining of the cells with CD138. (A, Wright's stain, original magnification ×600; B, hematoxylin-eosin stain, original magnification ×600; C, CD138 stain, original magnification ×400).*

tense staining. Thus, lack of Bcl-2$^+$ in SCLAs support a benign SCLA.

SUMMARY

There are extensive, constantly developing applications of FCI and IHC to diagnostic hematopathology. These applications are useful in diagnosis and in determining prognosis of hematologic malignancies as well as in evaluating residual/relapsing disease. Additional molecular studies will likely result in increased applications of these techniques in diagnostic hematopathology.

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