

# Flow Cytometric Analysis of Lymphomas

## Current Status and Usefulness

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• **Context.**—Immunophenotyping has become a routine practice in the diagnosis and classification of most cases of non-Hodgkin lymphoma, and flow cytometry is often the method of choice in many laboratories. The role that flow cytometry plays, however, extends beyond just diagnosis and classification.

**Objective.**—To review and evaluate the current roles of flow cytometry in non-Hodgkin lymphoma, to compare it with immunohistochemistry, and to discuss its potential future applications in the molecular diagnostic era.

**Data Sources.**—The information contained herein is derived from peer-reviewed articles on the subject published in the English-language medical literature during the years 1980 to 2005 that were identified using PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>, 1980–2005) search, various books and other sources on flow cytometry, and the author's personal experience of more than 10 years with flow cytometric analysis of lymphomas and leukemia using Becton-Dickinson (San Jose, Calif) and Beckman-Coulter (Miami, Fla) flow cytometers.

**Study Selection.**—Studies were selected based on adequate material and methods, statistically significant results, and adequate clinical follow-up.

**Data Extraction.**—The data from various sources were compared when the methods used were the same or similar and appropriate controls were included. Most of the

studies employed 2-color, 3-color, or 4-color flow cytometers with antibodies from Becton-Dickinson, Beckman-Coulter, or DakoCytomation (Carpinteria, Calif). Results were evaluated from studies utilizing the same or similar techniques and flow cytometers. Only objective data analyses from relevant and useful publications were included for reporting and discussion.

**Data Synthesis.**—Flow cytometry serves a variety of roles in the field of lymphoma/leukemia including rapid diagnosis, proper classification, staging, minimal residual disease detection, central nervous system lymphoma detection, evaluation of prognostic markers, detection of target molecules for therapies, ploidy analysis of lymphoma cell DNA, and evaluation of multidrug-resistance markers. It offers many advantages in comparison to immunohistochemistry for the same roles and provides uses that are either not possible or not preferable by immunohistochemistry such as multiparameter evaluation of single cells and detection of clonality in T cells.

**Conclusions.**—By virtue of its ability to evaluate not only surface but also cytoplasmic and nuclear antigens, flow cytometry continues to enjoy widespread use in various capacities in lymphoma evaluation and treatment. Additional roles for flow cytometry are likely to be invented in the future and should provide distinctive uses in the molecular era.

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The laboratory diagnosis of non-Hodgkin lymphoma (NHL) is usually accompanied by immunologic evaluation by immunohistochemistry (IHC) and/or flow cytometry (FCM). In some cases, however, other ancillary techniques may also be employed, such as in situ hybridization and gene rearrangement studies for the immunoglobulin heavy chain gene or the T-cell receptor  $\gamma$  or  $\beta$  chain genes. It should be clear, however, that FCM and other ancillary techniques serve an adjunctive role in the diagnosis and classification of hematopoietic neoplasms and must be reconciled with morphology for such diagnoses. Although FCM offers many advantages in comparison to IHC in the diagnosis and classification of NHLs,

it can also be used as a complementary rather than a competitive technique for this role. Despite its widespread use, however, proper use of antibodies and interpretation of flow cytometric data can be challenging not only for individuals with limited experience but also for flow technologists and pathologists well versed in this technique. This review is focused on the uses and current status of FCM in lymphoma evaluations and includes a brief review of CD antigens commonly used in these cases. The review also discusses the relative merits and limitations of FCM in comparison to IHC for this role.

### CD ANTIGENS

For proper interpretation of FCM results, it is helpful to have a sound understanding of the normal expression pattern of CD antigens on various hematopoietic cell types. Although 339 CD antigens have been defined and characterized, only a handful are routinely utilized for lymphoma and leukemia analysis.<sup>1</sup> Brief comments on com-

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monly used antigens will facilitate better interpretation of these antigens.

### **B-Cell–Associated Antigens**

Among the B-cell–associated surface markers, CD19 is the most sensitive and as specific as other B-cell–specific antigens such as CD20 and CD22 for lymphocytes.<sup>2</sup> The expression of CD19 on lymphocytes defines their B-cell lineage. It appears before CD10, CD20, CD22, and CD23 on B-cells and is expressed by all B-cell subsets and at all stages of maturation except terminally differentiated plasma cells. CD20, in contrast, is not expressed on the very early B-cells (pro B-cells) and is expressed at low levels on pre B-cells. It is expressed by all mature B-cell subsets and at all stages of maturation except terminally differentiated plasma cells.<sup>3</sup> CD10 is expressed by both B-cell and T-cell lymphoid progenitor cells and on follicular germinal center B-cells.<sup>4</sup> CD22 is another B-cell–specific antigen expressed in the cytoplasm of pro B-cells and pre B-cells and on the surface of mature B-cells.<sup>5</sup> It appears in the cytoplasm of B-cells before CD20 does and hence its cytoplasmic expression is more helpful for determination of B-cell lineage in very early cells, unlike CD20, which is absent in pro B-cells. Its expression pattern on B-cell lymphomas is similar to that of CD20; both usually have dimmer expression on small lymphocytic lymphoma/B-cell chronic lymphocytic leukemia.<sup>6</sup> This dim expression of CD20, however, is exploited for diagnostic purposes. CD79a was initially claimed to be exclusively expressed on B lineage cells but has also been reported in T-lymphoblastic lymphomas,<sup>7,8</sup> and dim expression has also been observed in acute myeloid leukemia (personal observation). In precursor B-cell acute lymphoblastic leukemia detection of cytoplasmic CD79a for B-cell lineage may not provide any additional benefits if evaluation of CD19 and CD20 is undertaken at the same time. However, in acute leukemia of uncertain lineage or minimal differentiation, CD79a plays an important role in lineage identification.

### **T-Cell–Associated Antigens**

Among the T-cell–associated antigens, CD3 is the most sensitive and specific for mature T-cells.<sup>9</sup> Its cytoplasmic expression is seen in all precursor and mature T-cell lymphomas and leukemia, but its surface expression may be lacking in some cases.<sup>10</sup> The  $\zeta$  chain of the CD3 complex is also expressed by natural killer (NK) cells and can be detected using TIA-2 antibody, but a polyclonal antibody raised against the CD3 $\epsilon$  subunit can also react positively with NK cells.<sup>11</sup> The other commonly used T-cell markers such as CD1, CD2, CD4, CD5, CD7, and CD8 have their selective usage and expression patterns. In general, all the above T-cell–associated markers should be run in addition to CD16, CD56, and CD57 for evaluation of T/NK-cell lymphomas. CD5 is also expressed on a small subset of normal mantle zone B-cells and thus cannot be used as a pan-T-cell marker.<sup>12</sup> In peripheral T cells, CD4 and CD8 have mutually exclusive expression patterns, although a very small fraction of peripheral T cells may show expression of both antigens.<sup>13</sup> CD7 is not only the earliest of all T-cell–associated antigens (it appears before cytoplasmic expression of CD3) and expressed at all stages of T-cell maturation but it is also expressed by a subset of pluripotent hematopoietic stem cells.<sup>14</sup> CD2, like CD7, is also expressed by NK cells but also is seen on a very small population of mature B cells in the blood (usually clini-

cally insignificant).<sup>15</sup> Aberrant expression of CD2, CD4, CD7, and CD8 has been observed on B-cell NHL.<sup>16</sup> Loss of surface CD3 is not unusual in T-cell lymphomas, but cytoplasmic CD3 expression is consistently seen in these cases.<sup>17</sup> In peripheral T-cell lymphomas, not otherwise specified, loss of CD7, CD4, or CD8 is observed, but lack of CD5 is less commonly seen.

### **NK-Cell–Associated Antigens**

Among the commonly used markers that NK cells express are CD2, CD7, CD8, CD11, CD16, CD56, and CD57—markers that are also expressed by all T cells or a subset of T cells.<sup>18</sup> Only CD3 and CD4 are not expressed by NK cells, except for the CD3 $\zeta$  chain.

### **FCM**

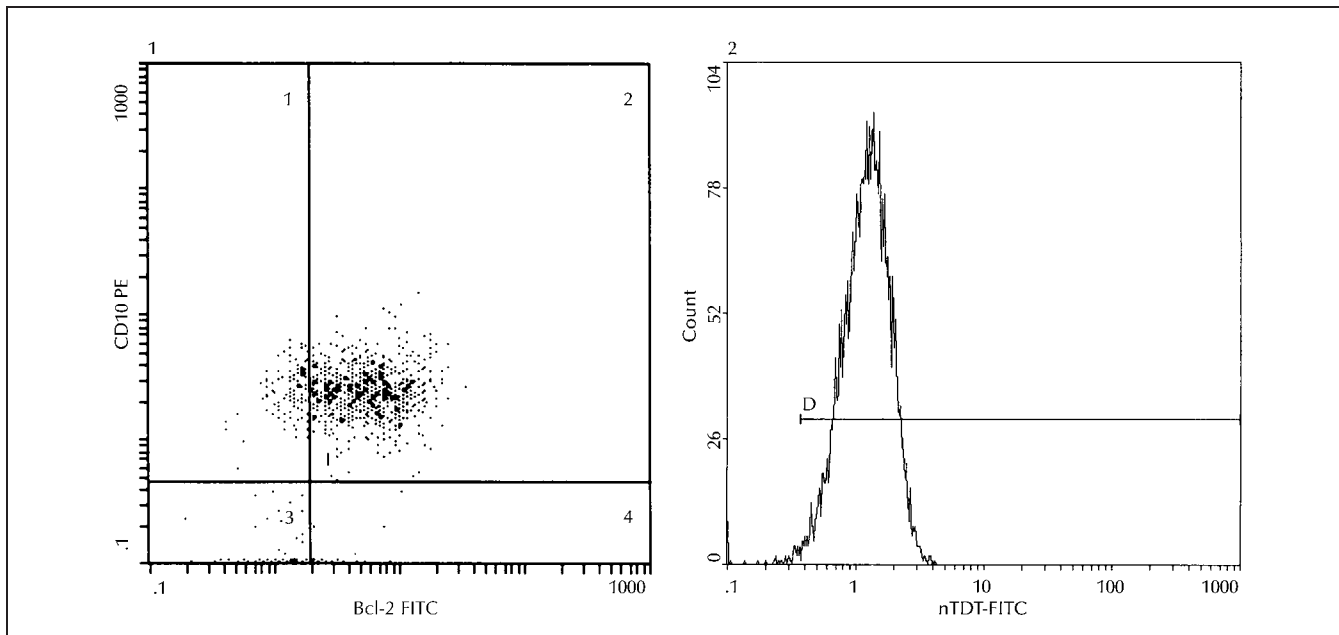
A very brief review of the principles of FCM will also facilitate the understanding of the applications and uses of this technique. A flow cytometer is an instrument that evaluates the physical and/or chemical characteristics of single cells and other particles (in the same size range) as the cells/particles pass individually through a measuring device and sensing point in a fluid stream and are illuminated by an incident light.<sup>19</sup> It is important to remember that the measurements and evaluations are done on each individual cell/particle and not as the average analysis of the aggregate.<sup>20</sup> The ability of various components of the cells, such as surface receptors, intracellular molecules, and DNA, to bind with fluorescent dyes allows their detection and evaluation. Fluorescence is produced when an incident light of one wavelength excites electrons of certain chemicals from their ground state to higher energy levels with subsequent return of electrons to lower energy levels by emitting light of a longer wavelength.<sup>21</sup> Flow cytometers detect the characteristics of cells/particles by measuring the amount of incident light reflected by these particles and by detecting the fluorescence produced by fluorochromes conjugated either directly with cell components or conjugated to antibodies directed against various cell components.

## **CLINICAL USEFULNESS OF FCM**

### **A. Diagnosis of Lymphoma**

The diagnosis of several categories of B-cell NHL can often be made on morphology alone if ample tissue is available for review. Immunophenotypic analysis of such cases by FCM merely confirms what is already known by morphology and plays an adjunctive rather than a diagnostic role. There are situations, however, where FCM plays the diagnostic role and proves more informative and useful than IHC.

Clonality analysis in B-cell NHL is usually done by evaluating the expression of immunoglobulin (Ig)  $\kappa$  and  $\lambda$  light chains in distinct populations and is generally identified when the  $\kappa$ : $\lambda$  light chain ratio is greater than 4:1 or less than 1:2.<sup>22</sup> Exceptional cases of B-cell NHL, however, may show a lower  $\kappa$ : $\lambda$  ratio ( $\sim$ 3:1) and some investigators have used a lower cutoff ratio,<sup>23</sup> but in general ratios in the gray-zones ( $\kappa$ : $\lambda$  3–4:1 and  $\lambda$ : $\kappa$   $\sim$ 1:1) must be interpreted carefully in the right clinicopathologic context. Clonality analysis is most useful when interpretation is done for immunophenotypically abnormal discrete populations such as CD10-expressing B cells. Figure 1 shows a case of follicular lymphoma with a  $\kappa$  to  $\lambda$  light chain ratio of 2.7:1 but shows Bcl-2 expression permitting iden-



**Figure 1.** A flow cytometry histogram showing coexpression of Bcl-2 by CD10+ B cells in a case of follicular lymphoma. The coexpression of Bcl-2 indicates the clonal nature of CD10+ follicular B-cells.

**Figure 2.** Expression of TdT by a Burkitt lymphoma with t(8;14)(q24;q32). This case was initially misclassified as precursor B-cell acute lymphoblastic leukemia because of TdT expression and lack of surface immunoglobulin light chains.

tification of neoplastic CD10+ B cells. A small number of B-cell NHL cases lack surface expression of both Ig- $\kappa$  and Ig- $\lambda$  light chains but show rearrangement of the *IgH* chain gene, thus indicating their B-cell clonality.<sup>24,25</sup> It must be remembered, however, that a subset of normal follicle center B cells also exhibits this phenomenon, and rare cases of florid follicular hyperplasia, especially in patients with acquired immune deficiency syndrome, have shown a lack of light chain expression of either type. Until recently, clonality determination in T-cell lymphoproliferations was limited to molecular techniques using Southern blot analysis or polymerase chain reaction to determine the status of T-cell receptor genes. With the availability of a broad panel of antibodies against the variable region of the *TCR $\beta$*  gene, it has become possible to elucidate clonality in most T-cell proliferations by FCM.<sup>26</sup> Beck et al<sup>27</sup> reported a sensitivity of 89% and a specificity of 88% for the rapid diagnosis of T-cell lymphoma/leukemia.

Aberrant expression of antigens or marked expansion of a subset of lymphocytes can also signal clonality in both B-cell and T-cell lymphoproliferative disorder. Examples include expression of Bcl-2 by CD10+ B-cells in follicular lymphoma,<sup>28</sup> aberrant expression of T-cell-associated antigens (CD2, CD4, CD7, and CD8) on B-cells,<sup>29</sup> clonal expansion of CD5+, CD19+ B cells in B-cell chronic lymphocytic leukemia and mantle cell lymphoma, marked expansion of CD4-, CD8-,  $\gamma/\delta$  T cells (>50% of all T-cells) of spleen in  $\gamma/\delta$  hepatosplenic T-cell lymphoma, aberrant total/near-total loss of CD3 in T-cell NHL, and marked expansion of CD3-, CD8+, CD56+ cells (>50% of lymphocytes in blood) in NK-cell lymphoma/leukemia. Although clonality in NK-cell proliferations cannot be determined by FCM, this question arises only rarely, and NK-cell lymphomas involving node or other tissues do not pose any diagnostic dilemma if a monomorphic population of NK cells with a typical phenotype comprises the majority of

the proliferation. The question of clonal versus reactive NK-cell proliferation most commonly occurs in patients with a moderate increase in NK cells in the blood without any lymphadenopathy or organomegaly. Most of these patients have allergies, autoimmune disorders, viral infections, or drug reactions. However, an early involvement by an NK-cell leukemia cannot be excluded entirely, and follow-up with detailed clinical evaluation is the key in differentiating benign versus neoplastic NK-cell proliferations.

Flow cytometry is especially useful in determining clonality in posttransplant B-cell lymphoproliferative disorders, which may be monomorphic (m-PTLD) or polymorphic (p-PTLD).<sup>30,31</sup> All m-PTLDs are monoclonal, but p-PTLDs can be monoclonal or polyclonal, and morphology with or without IHC may not be diagnostic of clonality in polymorphic cases. Furthermore, PTLDs frequently show dim or low-level expression of CD20, which may be important in therapeutic decisions regarding the use of anti-CD20 antibody.<sup>31</sup>

## B. Classification of Lymphoma

In the current World Health Organization classification, all NHLs are distinct clinicopathologic entities defined based on their morphology, immunophenotype, genetic alterations (in several cases), and clinical features. Thus, determination of immunophenotype is an integral component for proper classification except in those cases where morphology alone can be diagnostic of a particular lymphoma type, such as follicular lymphoma and small lymphocytic lymphoma, thus making determination of the immunophenotype a secondary exercise except for the determination of specific surface molecules for treatment purposes, such as the expression of CD20. Most new cases, however, undergo the ritual of immunophenotypic analysis either by FCM or IHC as part of routine handling of

The Immunophenotypes of B-cell Non-Hodgkin Lymphoma With Respect to CD5, CD10, and CD23*				
Lymphoma	Typical Pattern	Less Common Pattern	Unusual Pattern	Incompatible
SL/CLL	CD5 <sup>+</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup> CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>+</sup>
MCL	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>
FCC	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>+</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>	CD5 <sup>+</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>
DLBCL	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>+</sup> CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>
MZL	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>
B-PLL	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup> CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup> CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>+</sup>
LpCyL	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>		CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>
HCL†	CD5 <sup>-</sup> , CD10 <sup>-</sup> , CD11c <sup>+</sup> , CD25 <sup>+</sup> , CD103 <sup>+</sup>	CD5 <sup>-</sup> , CD10 <sup>-</sup> , CD11c <sup>+</sup> , CD25 <sup>-</sup> , CD103 <sup>+</sup>	CD5 <sup>-</sup> , CD10 <sup>-</sup> , CD11c <sup>+</sup> , CD25 <sup>-</sup> , CD103 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup> CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>
BL	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>+</sup>	CD5 <sup>-</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>	CD5 <sup>-</sup> , CD23 <sup>+</sup> , CD10 <sup>-</sup>	CD5 <sup>+</sup> , CD23 <sup>-</sup> , CD10 <sup>-</sup>

\* SL/CLL indicates small lymphocytic lymphoma/chronic lymphocytic leukemia; MCL, mantle cell lymphoma; FCC, follicle center cell lymphoma; DLBCL, diffuse large B-cell lymphoma; MZL, marginal zone lymphoma; B-PLL, B-cell prolymphocytic leukemia; LpCyL, lymphoplasmacytoid lymphoma; HCL, hairy cell leukemia; BL, Burkitt lymphoma.

† A few hairy cell leukemia variant cases have been reported to express CD5 or CD10.

suspected lymphoma cases and for documentation for future analyses and records. The use of FCM permits simultaneous evaluation of several surface antigens, with clonality analysis providing objective and quantitative results even on very small samples. This use of FCM is clearly superior to IHC, where analysis depends on evaluation of single antigens, usually one at a time, with a dose of subjectivity in equivocal antigen expressions. Most cases can be properly classified and do not pose any significant challenges in classification using FCM, but there undoubtedly are cases where aberrant antigen expression patterns must be reconciled with morphology. It is incumbent upon the pathologist to know the existence of aberrant expression patterns and rectify such discrepancies, thus avoiding diagnostic pitfalls. The immunophenotypes of lymphomas are widely known, but atypical patterns, as shown in the Table, do occur and pose significant diagnostic difficulties.<sup>22</sup> In atypical and unusual cases, all clinicopathologic data must be interpreted using a holistic approach rather than interpreting individual bits of information without regard to the context. It should be remembered that different pieces of information carry different weights for different diagnostic categories; for example, the immunohistochemical expression of cyclin D1 is much more specific than the expression pattern of CD5 or CD23 antigens by FCM for mantle cell lymphoma (MCL), and the expression of Bcl-6 antigen has more weight than the knowledge of CD10 expression by FCM in the case of follicular lymphoma. Cyclin D1 expression, however, may be absent from MCL. Fu et al<sup>32</sup> described 6 cases of MCL that did not express cyclin D1 but expressed cyclin D2 and cyclin D3. Also, identification of specific translocations rules out certain entities, irrespective of the immunophenotype. Figure 2 shows a case of TdT+ Burkitt lymphoma that was initially misclassified as precursor B-cell lymphoblastic leukemia based on the immunophenotype but later properly reclassified as an example of TdT-expressing Burkitt lymphoma/leukemia with the identification of t(8;14)(q24;q32) and necessitating major modifications in the treatment plan. Thus, although FCM immunophenotyping is an integral component of proper classification, it is by no means the most definitive modality for this purpose in all cases. As opposed to B-cell NHL, the classification of peripheral T-cell lymphomas as a group requires more in-

put of clinical data, and except for certain categories such as Sézary syndrome and T-cell prolymphocytic leukemia, the immunophenotype may not show gross aberrancies and may not be diagnostic of any one distinct entity. Hepatosplenic  $\gamma/\delta$  T-cell lymphoma has a distinctive immunophenotype that permits its proper classification as such, however.<sup>33</sup>

### C. Fine-Needle Aspiration Biopsies for Lymphoma

With advancement in minimally invasive surgical techniques and widespread use of fine-needle aspiration biopsies (FNABs), the tissue sample size for evaluation has been reduced. Pathologists are expected not only to make a diagnosis but also to provide proper classification in small specimens. The diagnosis and classification of lymphoma on FNAB is one such example and is becoming an ever-growing practice. The technique of FCM has played an indispensable role in this regard. Numerous recent articles have evaluated and established the utility of flow cytometric immunophenotyping of FNAB specimens for lymphoma workup.<sup>34-38</sup> The most valuable and indisputable use of FCM is in detecting clonality in B-cell proliferations, where it permits identification of clonality in almost 100% of cases. Meda et al<sup>39</sup> reported 95% and 85% diagnostic sensitivity and specificity with diagnoses suggestive of lymphoma or positive for lymphoma, respectively. Specificity was 100% when only definitive diagnoses of lymphoma were considered. Thus, patients with benign reactive nodes, as identified using FNAB and FCM, can be saved from having an excisional lymph node biopsy. Zardawi et al<sup>37</sup> reported 99% sensitivity of diagnosis of malignancy, 0% false-negative rate, and 100% positive predictive value of malignant diagnosis with combined FNAB cytology. From the reported data, a consensus appears to be growing among pathologists that some but not all cases of B-cell NHL can be classified using both cytomorphology and FCM. The lymphomas that can be classified properly on FNAB with FCM include small lymphocytic lymphoma and diffuse large B-cell lymphoma (DLBCL). The grading of follicular lymphoma is not possible on cytology specimens; thus, an excisional biopsy must be performed. The diagnosis of MCL, lymphoplasmacytoid lymphoma, and Burkitt lymphoma can be made in classic cases by experienced cytopathologists in consul-

tation with hematopathologists well versed in FCM. Extranodal marginal zone B-cell lymphoma can be suspected in sites such as thyroid and parotid glands, but a definitive diagnosis requires open biopsies of these organs. The question whether a lymph node shown to have lymphoma involvement and classified on a FNAB with FCM should or should not be excised is yet to be settled, but a few clinicians still insist on having an excisional node biopsy even with a complete diagnosis and classification done on a FNAB specimen. A correlation, however, exists between the diagnosis on FNAB and subsequent surgical excision of the node.<sup>40-42</sup> Nodal involvement by peripheral T-cell NHL is very uncommon and often requires gene rearrangement studies in cases with less-than-compelling morphologies. A definitive diagnosis of such cases may not be possible on FNAB with FCM, but a provisional diagnosis can be made pending gene rearrangement studies.

#### D. Staging for Lymphoma

Because of its sensitivity, FCM is often utilized as part of the staging procedure for newly diagnosed NHL. Morphology of the trephine marrow biopsy specimen alone may yield a definitive diagnosis, but FCM has been shown to improve the sensitivity of detection of lymphoma cells in the marrow, with the reported gain in sensitivity ranging from slight to moderate.<sup>43-45</sup> The literature, however, is limited on this role of FCM. The greatest value of FCM is in selected cases where the bone marrow biopsy specimen shows only small and few aggregates nondiagnostic by morphology alone or with IHC. Lymphoma involvement in the marrow is usually patchy, and hence in a few cases the material from small trephine core biopsies may not contain any lymphoid aggregates but the specimen obtained by aspiration contains enough lymphoma cells, permitting detection by FCM. This added sensitivity translates into proper staging for another ~5% of individuals who would have been understaged by morphologic evaluation alone. On the other hand, some core biopsy specimens contain benign lymphoid aggregates, especially in older individuals, and may be mistaken for lymphoma involvement in staging procedures. Flow cytometry plays an invaluable role in these cases by determining the clonality status of any B-cell population. The redundancy achieved with FCM for staging may not be economical for some patients, but it improves the staging accuracy for many other patients.

#### E. Minimal Residual Disease Detection

The definition of minimal residual disease is dependent on the sensitivity of the method used for detection of the disease. The absence of lymphoma in the bone marrow by morphologic evaluation is not synonymous with the absence of disease in the specimen. It merely implies lack of detection by morphologic analysis. Clonal cells in the bone marrow can be identified by polymerase chain reaction or FCM in cases where no clonal cells could be identified by morphologic analysis.<sup>46</sup> However, for most cases of minimal residual disease analysis, routine analysis by polymerase chain reaction is either not available or not possible. The detection sensitivity that FCM offers appears to correlate well with the presence or absence of clinical disease. Patients treated for NHL may have benign lymphoid aggregates that may pose a diagnostic challenge for minimal residual disease evaluation. Douglas et al<sup>47</sup> reported

in their series 11 of 13 posttherapy bone marrow specimens that were interpreted as positive or suggestive of lymphoma based on routine hematoxylin-eosin-stained sections, but immunohistochemical and/or flow cytometric immunophenotyping showed that 6 of the 11 cases were negative for lymphoma; the lymphoid infiltrates were composed entirely of T cells without B cells. Flow cytometric evaluations of such cases prove invaluable in determining their clonality. Detection of plasma cells in patients with myeloma can be technically challenging but often yields satisfactory results if proper gating methods are used.<sup>48,49</sup> The advantages of FCM in comparison to methods like polymerase chain reaction or fluorescence in situ hybridization are its availability in most laboratories and rapid results.

#### F. Detection of Central Nervous System Lymphoma

Patients with acquired immune deficiency syndrome can develop ring-enhancing lesions in the brain that may represent toxoplasma infection or B-cell lymphoma, typically DLBCL. Cytologic examination of the cerebrospinal fluid is often performed, but a definitive diagnosis may not be rendered, requiring an invasive biopsy of the brain. Immunophenotypic evaluation of the cerebrospinal fluid by FCM has substantially improved the diagnostic accuracy by determining the clonality of B cells.<sup>50-54</sup> Hegde et al<sup>55</sup> reported the detection of occult central nervous system disease in 11 of 51 patients with newly diagnosed NHL as opposed to only 1 case detected by cytologic examination alone. Other patients with secondary involvement of the brain may also benefit from FCM analysis of the cerebrospinal fluid.<sup>55</sup> If a sufficient cerebrospinal fluid specimen can be acquired, a portion of it must be submitted for flow cytometric analysis. Intraocular involvement by B-cell NHL is very rare, but FCM has successfully been applied in such cases as well.<sup>56-60</sup>

#### G. Detection of Target Molecules for Therapy

In the last several years, identification and targeting of various surface or intracytoplasmic molecules in tumor cells has added a new dimension in the treatment of malignancies.<sup>61</sup> This molecular targeting offers selective binding of antitumor agents to cells of interest, thereby reducing untoward effects and maximizing efficacy. Research investigations and clinical studies have shown antitumor effects with antibodies against an array of CD antigens including CD19,<sup>62</sup> CD20,<sup>63-66</sup> CD22,<sup>67,68</sup> CD25,<sup>69</sup> CD45,<sup>70,71</sup> CD52,<sup>72-74</sup> and CD74,<sup>75</sup> and multiparameter FCM has been used to evaluate the expression pattern of these antigens before and after treatment with a particular anti-CD antibody. Indeed, FCM is unique and an ideal instrument because it offers detection and quantification of more than one antigen on lymphoma cells rather than staining lymphocytes indiscriminately for a given antigen whether or not they are clonal, as occurs with immunohistochemical analysis.

#### H. Prognostic Information

The expression or the lack thereof of certain molecules in neoplastic cells has been correlated with prognosis and outcomes in many hematopoietic and nonhematopoietic malignancies. Not surprisingly, FCM has been used to identify the expression of such prognostic markers in B-cell NHL. The most widely utilized markers for prognosis in lymphomas include expression analysis of CD38

and Zap-70 in B-cell chronic lymphocytic leukemia (CLL),<sup>76-80</sup> and less commonly the expression analysis of CD10, CD21, and Bcl-2 in DLBCL. Although evaluation of CD38 expression is part of the reporting for CLL in many laboratories, the expression analysis of Zap-70 is not yet widely available because of technical difficulties in obtaining consistent staining and expression. The expression of Zap-70, which acts as a surrogate marker, generally indicates an unmutated configuration of the *IgV(H)* genes and an overall shorter survival and a short time to disease progression.<sup>81-86</sup> With the identification of any such new prognostic markers, the use of FCM for this role may continue to rise.

### I. Ploidy Analysis

Although analysis of DNA ploidy and S-phase fraction (SPF) is easily done using FCM, its applications in lymphoma evaluation are limited to a very few uses. As opposed to cases of lymphoblastic leukemia, DNA ploidy analysis for NHL does not provide any independent prognostic information.<sup>87</sup> Many early studies correlated S-phase fraction with grade and outcome but the value of S-phase fraction, as an independent prognostic marker, was never established.<sup>88</sup> Earlier studies also indicated the usefulness of ploidy determination for plasma cell myeloma, and this remains true after more than 20 years of experience with myeloma ploidy.<sup>89-92</sup> Bunn et al<sup>93</sup> showed ploidy to be an independent prognostic marker in myeloma at the time of presentation, and developing aneuploidy in a patient with an initially diploid tumor is correlated with disease progression and relapse. Similar studies have also been conducted for Waldenstrom macroglobulinemia and monoclonal gammopathy of undetermined significance, with variable results. DNA ploidy analysis has been used to help detect Sèzary cells in blood for diagnosis and minimal residual disease detection.<sup>94,95</sup>

### J. Multidrug Resistance Assays

The ability of FCM to detect intracellular molecules has also been exploited to determine the levels of proteins conferring resistance to chemotherapeutic drugs.<sup>96-99</sup> Jakab et al<sup>100</sup> used a calcein assay to determine the activity of multidrug resistance protein (P-glycoprotein) and multidrug resistance associated protein in B-cells as identified using antibodies against CD19 and CD20. This method is very useful since the cells of interest can be isolated and evaluated using specific antibodies. However, the use of FCM for this purpose is not widely applied yet.

### DIAGNOSTIC LIMITATIONS

With all the uses and advantages that FCM offers, it is equally important to acknowledge the limitations of this technique as well. One of the main drawbacks of immunophenotyping by FCM is the requirement for fresh, unfixed tissue for analysis. Although prospective handling of specimens for lymphoma diagnosis often yields ample tissue for immunophenotyping, such analyses cannot be performed on archival fixed tissues.

Flow cytometry has had little success in the evaluation of Hodgkin lymphoma and is not used for making this diagnosis. Likewise the diagnosis of T-cell-rich B-cell lymphoma, which is often similar to Hodgkin lymphoma, is often made based on morphology and IHC. Cases of T-cell-rich B-cell lymphoma evaluated with FCM may only show a large number of reactive T-cells and may not

detect clonality in B-cells. Most cases of peripheral T-cell lymphoma show aberrant expression of one or more T-cell-associated antigens, thereby suggesting presence of an aberrant T-cell population. Although this is not diagnostic of T-cell malignancy by itself, it is often a feature of clonal T-cell populations. With the development and availability of a broad array of antibodies against the variable region of the TCR beta (V beta) chain, it is now possible to detect T-cell clonality directly by FCM, and with trial and error the detection of T-cell clonality may no longer be a limitation. The diagnosis of NK-cell lymphoma/leukemia, however, must be made on morphology and immunophenotype in conjunction with clinical data since detection of clonality is not possible by FCM.

### FCM VERSUS IHC

The applications of FCM and IHC in the diagnosis and classification of lymphomas can both be complementary and competitive. The preference to use either FCM or IHC for the same role (competitive use) is a matter of ease of use, experience with the technique, personal preference, and laboratory resources and expertise in the techniques. There are distinctive uses and limitations of each technique, however. The biggest advantage that FCM offers is the multiparameter evaluation of single cells and the ability to work with very small samples, including fluid samples, whereas the major edge of IHC as compared to FCM is its ability to work on fixed, paraffin-embedded tissues and provision of morphologic analysis at the same time. The recent availability of antibodies against the variable region of the TCR beta (V beta) chain allows determination of T-cell clonality by FCM; such an evaluation is not possible currently for diagnostic purposes using IHC. Most commonly used antibodies are available for both FCM and IHC, but a few antibodies work better for flow cytometric analysis than paraffin-based use, such as antibodies to immunoglobulin  $\kappa$  and  $\lambda$  light chains, whereas antibody to cyclin D1 for mantle cell lymphoma works well with IHC and not with FCM. Certain evaluations work better with FCM than IHC and vice versa.

- Competitive use where FCM analysis works better than IHC
  1. Determination of B-cell clonality by analysis of immunoglobulin  $\kappa$  and  $\lambda$  light chains
  2. Evaluation of coexpression profiling of antigens on the same cells, such as co-expression of CD5 and CD23, aberrant loss of antigens, and simultaneous evaluations of cytoplasmic and surface antigens
- Competitive use where IHC analysis works better than FCM
  1. Evaluation of nuclear antigens such as cyclin D1 and TdT

Following is a list of clinical conditions and specimen types where FCM, in addition to morphology, plays either an exclusive, preferable, complementary, or limited role in the diagnosis of lymphoma (in comparison with IHC).

1. Evaluations where only FCM and not IHC plays the diagnostic role
  - a. Blood specimens for lymphoma/leukemia involvement
2. Evaluations where FCM is often diagnostic and more informative than IHC
  - a. Needle core biopsies of lymph nodes, especially with scant tissue

- b. Lymph node FNAB
  - c. Diagnosis of central nervous system lymphoma in the spinal fluid in patients with acquired immune deficiency syndrome
  - d. Pleural, peritoneal, and pericardial lymphocytic effusions
  - e. Orbital lymphoid proliferations and intraocular fluid analysis
  - f. Suspected T-lymphoblastic lymphoma of the thymus, core biopsies
  - g. Minimal nodal involvement by Sèzary cells in patients with known mycosis fungoides
  - h. Splenic and nodal involvement by hepatosplenic  $\gamma/\delta$  T-cell lymphoma
3. Evaluations where FCM and IHC are almost equally informative and diagnostic
    - a. Excisional lymph node biopsies
    - b. Splenectomy specimens
    - c. Resection of extranodal masses for suspected lymphoma
    - d. Bone marrow for NHL staging
  4. Evaluations where IHC is often diagnostic and more informative than FCM
    - a. Skin biopsies for suspected B-cell, T-cell, or NK-cell lymphoma
    - b. Small biopsies of stomach, intestine, lung, liver, kidney, and brain

Not only that FCM is preferable to IHC in most situations for lymphoma analysis, it can identify conditions that could potentially be missed if IHC were to be used instead, such as partial involvement of the node by marginal zone B-cell lymphoma and composite B-cell and T-cell lymphomas. Furthermore, analysis by FCM provides a better and more informative immunophenotypic evaluation for certain diagnoses such as hepatosplenic  $\gamma/\delta$  T-cell lymphoma and hairy cell leukemia. Distinction of a lymphocyte-rich thymoma from T-cell lymphoblastic lymphoma can be very challenging on morphology with or without IHC. This is another example where FCM has successfully identified the correct diagnosis in most cases.

#### FUTURE OF LYMPHOMA FCM

Flow cytometry currently enjoys widespread use and a well-established role in lymphoma and leukemia diagnosis in most clinical laboratories throughout the United States and many European countries, but it has come a long way to earn that status. The clinical use of FCM evolved from its research use some 4 decades ago, as is true for many other technologies before their widespread clinical use. Immunophenotyping is one of the integral components in the current World Health Organization lymphoma classification scheme, and FCM has proven value in the diagnosis and proper classification of most categories. The multiple roles that FCM plays in lymphoma/leukemia diagnosis, classification, and prognostication serve the current management plans and treatment protocols based on our current understanding of the biology of the disease. With advances in our understanding of the molecular basis of the disease, however, we are delving more and more into tying and correlating various components of the normal and deranged molecular machinery and specific genetic findings with specific disease categories and predicting clinical outcomes. Examples include determining the mutation status of the *IgV(H)* genes in B-cell CLL to predict the clinical course of the disease. The

biggest move, however, is toward gene expression profiling of various lymphomas and leukemia to understand the molecular derangements and biology of the disease.<sup>101</sup> The dissection of the deranged molecular machinery will undoubtedly facilitate better understanding, diagnosis, and ultimately treatment of these malignancies.

Numerous publications have appeared in the past 5 to 6 years studying the gene expression profiling of benign and malignant lymphocytes. Alizadeh et al,<sup>101</sup> using gene expression profiling, identified 2 molecularly distinct forms of DLBCL with gene expression patterns indicating different stages of B-cell differentiation. One type expressed genes characteristic of germinal center B cells; these cells were called 'germinal center B-like DLBCL.' The second type expressed genes normally induced during *in vitro* activation of peripheral blood B cells; these cells were termed 'activated B-like DLBCL.' Patients with germinal center B-like DLBCL had a significantly better overall survival than those with activated B-like DLBCL. Murakami et al<sup>102</sup> described a case of primary cutaneous CD30-negative large T-cell lymphoma where gene expression profiling using complementary DNA microarrays indicated significantly increased expression of an apoptosis-inhibitory protein and certain cytokines and cytokine receptors (eg, MCP-1, MCP-2, IP-10, and IL-2R gamma) in the tumor-indurated skin. Recently, Rizzatti et al<sup>103</sup> showed that several genes related to apoptosis and to the PI3K-Akt, WNT, and TGF- $\beta$  signaling pathways were altered in MCL when compared with naive B cells. These and many other recent publications underscore the importance of a shift toward developing and using molecular tests to identify various molecules implicated and pertinent for a specific lymphoma/leukemia entity.<sup>104-106</sup> It is not the availability of gene chips or the costs of these tests that impedes the establishment of these tests for routine diagnostic use; rather, it is largely the lack of sufficient meaningful data to correlate specific proteins identified by these expression profiling studies. The plethora of expression profiling data might be intimidating for now, but with more clinical studies only a handful of molecules will be identified as playing a central rather than peripheral role in specific malignant categories. Sooner or later, molecular tests will be offered by most clinical labs for routine diagnosis, molecular classification, prognostication, and identification of target molecules for the treatment of leukemia and lymphoma. This is not to say that the use of FCM is likely to cease in the near future but to emphasize the natural evolution in the armamentarium available for the diagnosis, classification, prognostication, and treatment of lymphoma. Flow cytometry will continue to enjoy its use in diagnostic labs in addition to any new molecular technologies for years to come, as long as separating distinct lymphoma categories is one of the primary objectives of treatment plans. With targeting of specific molecules as the primary objective in treatment plans, however, molecular tests will take a lead in comparison to other diagnostic laboratory techniques.

It would be a conjecture to predict the future of lymphoma FCM in the next 20 years, but the ability of FCM to detect not only surface but also intracellular and intranuclear molecules such as receptors, enzymes, nucleic acid, and protein products of specific genes is likely to lead to reinvention of the diagnostic and prognostic applications of this technique for several possible future uses in lymphoma management. Such applications might range

from detection of novel specific surface, intracellular, or intranuclear protein products relevant in diagnosis, classification, or prognostication to evaluating enzyme activities for cell kinetics and tumor cell metabolism and response to drugs. Thus, with the identification of each new molecule critical in lymphoma/leukemia management, new potential for FCM is created.

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