Impact of Flow Cytometry on the Diagnosis and Characterization of Lymphomas, Chronic Lymphoproliferative Disorders and Plasma Cell Neoplasias

Raul C. Braylan*

Department of Pathology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida

Lymphocytes can become neoplastic at any stage of their development. When the neoplastic change occurs early on in their ontogeny, they act aggressively, producing a disease designated as lymphoblastic leukemia, invading the marrow, the blood, and sometimes, solid tissues (the latter are called lymphoblastic lymphomas). The antigen profile of these neoplastic cells recapitulates that of lymphoid precursors. When the oncogenic change takes place after lymphocytes have acquired maturation, the neoplastic progeny exhibit the phenotype of adult elements. If these more differentiated lymphocytes infiltrate lymph nodes or other lymphoid tissues or organs, we use the term lymphoma. When mature neoplastic lymphocytes circulate in the blood, they usually produce indolent ailments, generally designated as chronic lymphoproliferative disorders (CLPD). Differences between lymphomas and CLPD are not always distinct, since malignant lymphocytes may simultaneously involve tissues and blood. The term myeloma applies to systemic neoplasias of the bone marrow that are composed of plasma cells, the latest stage in the life of the B-lymphocyte.

The nomenclature of lymphoid neoplasias has evolved over time and this process is certain to continue, as new biological advances are made. Until recently, characterization of the lymphomas and lymphoproliferative disorders was largely descriptive and based exclusively on microscopic observations. While useful, this approach is very limited, since the identity of neoplastic cells cannot always be established, nor can their clonal nature be recognized. Descriptive terms, such as large or small, follicular or diffuse, granular, intravascular, anaplastic, plasmacytic, etc., which were prevalent in earlier classifications, are still being used to characterize malignant lymphoid cells, even in modern taxonomies, like the World Health Organization (WHO) classification (1). However, this classification scheme now incorporates more meaningful biological properties for their category definitions, including extensive descriptions of the immunophenotype of the neoplastic cells.

Flow cytometry (FCM) has been used in the analysis of human lymphomas since the late 1970s. The earlier studies focused mainly on the measurement of cell cycle phases and ploidy, exploiting the simplicity of measuring DNA content by this technology. In the 1980s, we witnessed a rapid increase in the use of FCM analysis of lymphoid tumors, as the understanding of the biology of the immune cells became much clearer and the production of specific markers such as monoclonal antibodies expanded rapidly. Today, FCM analysis is an accepted and essential medical practice in the clinical evaluation of lymphoid neoplasia. This technology assists in the diagnosis and characterization of lymphomas and CLPD, and in the detection of low-level disease. In patients with plasma cell dyscrasias, FCM is playing an ever-growing role, providing diagnostic support and prognostic information.

WHAT MAKES FCM A UNIQUE METHOD OF ANALYSIS FOR NEOPLASTIC LYMPHOID CELLS?

FCM offers important advantages over competing laboratory technologies in the detection of lymphoid and plasma cell neoplasias. In addition to being a fast procedure, FCM: 1) can analyze a broader array of antigens than those detectable by conventional, fixed tissue-based immunohistology (IH); 2) allows a clear-cut correlation of multiple measurements (antigen expressions, DNA content, light scatter) in individual cells; 3) has the ability to quantitate both population frequencies and level of antigen expression in individual cells; and 4) facilitates the analysis of cells within discrete subpopulations defined and selected (gated) based on other parameters.

The above are unique properties of FCM that can be extremely helpful in the recognition and characterization of lymphoid and plasma cell neoplasias, even when corresponding tissue sections or other morphologic preparations are not available. The following are some examples:

Demonstration of Aberrant Phenotypes

The cell lineage of lymphoid populations can easily be established by FCM, even in heterogeneous samples. In

*Correspondence to: R.C. Braylan, M.D., Department of Pathology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL 32610.
E-mail: braylan@ufl.edu

addition, FCM can determine the level of expression and coexpression of multiple antigens on individual cells. Abnormal overexpression, underexpression, or coexpression of cell antigens represent some of the aberrant phenotypes that are commonly observed in lymphoid and plasma cell neoplasias, and are helpful for their diagnosis and classification.

Assessment of B-cell Clonality

In normal lymph nodes or peripheral blood, virtually every B-cell expresses cell surface light-chain immunoglobulins, and the fraction of kappa to lambda immunoglobulin-expressing B-cells is approximately 1.5. The lack of expression of surface immunoglobulins in mature B-cells, or a significant increase or decrease in this normal ratio (light chain restriction), supports the presence of a monoclonal B-cell population. Immunohistochemistry does not usually provide a clear evidence of surface light-chain restriction due to the large amount of background immunoglobulin in the interstitial spaces of tissues, which obscures the relatively weak monoclonal immunoglobulins on the surface of B-cells. The cell isolation and washing required for the FCM analysis allows an unambiguous assessment of the expression of cell surface immunoglobulin in most instances. The presence of a monoclonal B-cell population, particularly when it is associated with changes in light scatter signals and/or additional abnormalities of antigen expression, is a characteristic feature of mature B-cell neoplasias. This "B-cell clonal expansion" assessment has been one of the most valuable contributions of FCM. Considering the additional information that one gains by simultaneously evaluating the coexpression of other antigens on the clonal B-cells, this analysis remains unmatched by any other existing laboratory technique.

Assessment of T-cell Clonality

In contrast to the straightforward B-cell clonality determination based on single immunoglobulin light chain expression, clonality assessment of T-cells is not possible with FCM using conventional marker analysis. Molecular analysis of T-cell receptor (TCR) gene rearrangements is indicated in cases of suspected T-cell malignancy to determine the clonal status of the T-cells. However, many antibodies against V domains of TCRαβ molecules have been developed, and it is now possible to recognize approximately 70% of all individual Vβ domains. Thus, FCM analysis of the Vβ repertoire of TCRαβ molecules may be useful in the detection of T-cell clonality, since T-cell clonal expansions are expected to express a single Vβ domain. In fact, reference values on healthy controls and numerous studies demonstrating the feasibility of this approach in the detection of T-cell lymphoproliferative processes have already been published (2,3), supporting the notion that the FCM analysis of the Vβ repertoire may be a useful alternative to nonquantitative molecular clonality studies. By combining Vβ domain antibodies with other T-cell markers, the FCM approach offers the additional advantage of being able to quantitate Vβ-expressing cells within T-cell subsets or selected cells with aberrant T-cell phenotypes. Furthermore, once identified, the Vβ restricted T cell population can be quantitatively monitored following therapy.

The requirement for a large antibody panel to cover the majority of the Vβ repertoire has been facilitated by the availability of a commercial eight-tube kit (IO Test Beta Mark; Beckman Coulter, Miami, FL). This kit contains three different Vβ antibodies, labeled with only two fluorochromes in each of the tubes, thus allowing for additional antibodies to be used in the same tube. As long as the T-cell population analyzed expresses Vβ determinants (as is expected in the majority of mature T-cell proliferations), this technology promises to greatly facilitate the diagnosis and monitoring of peripheral T-cell malignancies.

Diagnosis of Lymphoma in Small Samples

As concerns for cost and invasiveness of surgical biopsies intensify, procedures for obtaining samples of potential lymphomas using fiberscopes or needles for tissue aspirations and biopsies are becoming commonplace. As a consequence of this practice, pathologists are increasingly confronted with small amounts of tissue samples, or just cytology preparations, for diagnosis, which limits the interpretative capabilities of microscopic observations. In these situations, the added phenotypic information provided by modern FCM instruments may allow an accurate recognition and classification of neoplastic cells, even in specimens with extremely low numbers of cells. This ability has been documented in numerous studies using fine-needle aspirations or small endoscopic biopsies obtained from mucosa-associated lesions (4). Likewise, accurate diagnosis may be attained on fluids that contain small numbers of cells, such as vitreous humor, cerebral spinal fluid, or pericardial fluid.

Detection of Low-Level Neoplastic Involvement

FCM analysis is a sensitive method for revealing small populations of abnormal lymphoid cells that may not be otherwise recognized using conventional diagnostic technologies. FCM is frequently used in the staging of non-Hodgkin lymphoma, and should be useful in the detection of circulating neoplastic cells, even in leukopenic patients (e.g., hairy cell leukemia) (5,6). The demonstration of peripheral blood involvement may circumvent the need for bone marrow examination. Also, the detection of residual disease may impact on treatment decisions.

Evaluation of Plasma Cell Disorders

Most plasma cell neoplasias such as multiple myeloma are diagnosed without the need for FCM. The clinical manifestations, routine laboratory data, serum or urine protein studies, and the microscopic examination of a bone marrow aspirate, generally provide sufficient diagnostic information. However, FCM may be useful in certain patients who reveal hematologic abnormalities and

increased plasma cells in the marrow, but have no specific clinical manifestations of myeloma. FCM may not be the best method to quantitate plasma cell load in marrow aspirates due to hemodilution or plasma cell fragility, but the phenotypic features of normal and malignant plasma cells are usually distinct. Normal plasma cells are polyclonal (heterogeneous with regard to the type and class of immunoglobulin they carry), and express CD38 (intense), CD19, variable CD45, and no CD56. In contrast, malignant plasma cells are monoclonal, they usually have different scatter properties, and they express CD38 less intensely than normal counterparts; CD45 is generally dim; and CD19 is not detectable, while CD56 is well expressed (7). Thus, the simultaneous determination of CD38, CD45, CD56, and CD19 expression, together with scatter analysis, can distinguish normal from malignant plasma cells in most patients. These phenotypic characteristics can also help in differentiating multiple myeloma from monoclonal gammopathy of undetermined significance (MGUS) (8), and predict disease progression (9).

Rapid Cell Cycle Phase Analysis of Neoplastic Subpopulations

Growth fraction measurements in lymphoma and myeloma have proven to be of prognostic value. Also, numerous studies in lymphoma repeatedly demonstrated a relationship between the fraction of cycling cells and histologic grade (10). Thus, an assessment of proliferation would serve the same function as conventional pathological grading. FCM has been one of the technologies most often used in the quantitation of proliferative fractions in lymphoma. There was, however, some variation among the multiple studies presented in the literature. There was also overlap in the range of values for S-fractions observed in aggressive and low-grade lymphomas. These results were most likely influenced by the different methods used in performing the analysis. Indeed, one should question the accuracy of measuring S-fractions in whole lymphoma samples, since there is usually a variable, and often significant, number of normal lymphocytes intimately admixed with the neoplastic cells present in these samples. If the normal cells are not identified and excluded from the measurements, the results of the "neoplastic cell" analysis would be inaccurate, a problem that affected many of the retrospective studies that used cell nuclei extracted from paraffin-embedded tissues.

Previous studies used anti-immunoglobulin light-chain antibodies in combination with propidium iodide to measure tumor-specific S-phase by FCM. However, the simultaneous analysis of propidium iodide–stained DNA and surface antigen requires cell fixation and/or permeabilization. This treatment frequently alters the physical and antigen labeling properties of the cells, hampering identification of discrete cell subpopulations. It is also very difficult to use more than a single antibody in the preparations. A new dye, DRAQ5, is a deep red fluorescing synthetic anthraquinone, excitable with 488 nm lasers, which rapidly binds DNA in unfixed cells, thus preserving light-scatter and antigen-labeling properties (11). As such, DRAQ5 is ideal for DNA analysis of selective (e.g., neoplastic) cell subpopulations identified by light scatter and more than one surface antigen in heterogeneous samples (12).

WHAT IS NEXT?
Better Instrumentation

FCM instruments are becoming more sophisticated. Although three-color and four-color measurements are the norm in clinical laboratories, manufacturers are now offering cytometers with more detectors, taking advantage of increasingly available fluorochromes with useful color spectra. The ability to simultaneously detect a larger number of markers should facilitate the analysis of small samples and decrease costs, by reducing the number of redundant reagents, sample preparation, and analysis time. The simultaneous use of five or more colors should also help in the detection of minimal disease, which usually requires the analysis of a large number of cells. Instruments that allow faster cell analysis should make this task easier.

A number of devices that automate certain steps in sample preparation have become available commercially. Ideally, we should also expect to see capable programmable devices (i.e., robotics) that can handle diverse samples, cell preparations, and cell injection into the cytometer with minimal human participation. The increased use of automation will undoubtedly improve results, allow faster analysis, and reduce error and cost.

Software Improvements

Data analysis of lymphoid and hematologic neoplasias is not a simple task. It is an interactive exercise that requires experience and ample knowledge of normal and pathological cell phenotypes. Currently, data interpretation is one of the most difficult and time-consuming aspects of the entire analytical process. Furthermore, it has not been standardized and the expression of results is highly variable. Better software may be developed that could facilitate this complex data analysis and result in more consistent interpretations.

Quantitation of Antigen Expression

With the advent of specific monoclonal antibody–based therapies for lymphoma and CLPD, oncologists are increasingly interested in learning whether neoplastic cells express certain antigens that could be targeted by the specific reagents. Furthermore, since the therapeutic effectiveness of a monoclonal antibody may be related to the density of membrane antigen on the tumor cells, there has been a growing interest in the FCM quantitation of surface antigen expression. As antibody-based therapies become more prevalent, it is possible that quantitative FCM analysis may develop into a routine component of the analysis of lymphoid neoplasia. Unfortunately, this methodology suffers from lack of standardization, and

more work will be necessary before optimal analytical procedures are in place in the clinical laboratory.

New Markers

Many antibodies used in diagnosis are useful because they recognize cell lineage and differentiation stages, but only a few provide information on cell behavior. Reagents that are directed against molecules involved in specific cell functions are frequently used in research laboratories and will undoubtedly become increasingly common in the clinical laboratory. It is likely that in the near future antibodies against critical proteins involved in cell activities such as cell growth, growth arrest, apoptosis, adhesion, immune activation and signaling, DNA damage and repair, drug resistance, or angiogenesis may be incorporated to our routine diagnostic panels, particularly in combination with cell lineage and differentiation markers in the setting of multiparametric measurements.

Assessment of Cell Death and Proliferation

The current WHO classification of lymphoid neoplasias is based on the origin and differentiation of the neoplastic cells (1). However, tumors of similar origin and maturation stage, as assessed by their immunophenotypic features, may follow very different clinical courses due to different growth capacity. We now better understand some of the reasons for these dissimilar behaviors. Lymphomas can be broadly classified as either low-grade (low growth) or high-grade (high growth) tumors. Over the past 20 years we have learned that low-grade lymphomas are the result of an accumulation of neoplastic cells due to suppression of apoptosis and prolonged cell survival. On the other hand, high-growth fraction lymphomas are characterized by high cell proliferation, mostly due to the deregulation of oncogenes that play a role in cell cycle control, usually in association with apoptotic defects. The ultimate behavior of the lymphoma is dictated by cell production and survival, modified to a certain extent by host influences, such as growth factors and cell-to-cell interactions.

It is unfortunate that despite the wide variation in growth potential exhibited by lymphomas, very little emphasis has been placed on incorporating kinetic or apoptotic information in lymphoma classifications. The concept of grade that was critical in previous classifications and provided some notion of tumor aggressiveness is no longer emphasized in the present WHO scheme. However, the treatment of lymphomas is still largely based on this parameter, and will remain the same, as long as cell cycle–dependent therapeutic drugs continue to be used. Given the importance of cell proliferation and cell death, accurate measurements of cell cycle phases and apoptotic rates of neoplastic elements should prove beneficial in predicting tumor growth and even in predicting response to therapy. In this regard, FCM has unique abilities that no other laboratory techniques can offer.

Critical Genes

The use of modern analytical techniques allowed us to understand the changes occurring in normal lymphocytes as they undergo differentiation and maturation. These techniques have also provided us with a better appreciation of the genetic abnormalities that characterize many of the lymphocyte neoplasias, the relationship between lymphoma cells and their normal counterparts, and the genetic events that lead to malignant transformation of lymphoid cells. The development of the technology based on "gene chips" or DNA microarrays has greatly facilitated the simultaneous study of the expression of many genes at the mRNA level. With the advent of gene microarray–based expression profiling, differentially expressed genes are being discovered at an unprecedented rate. Systems that are investigated include normal versus neoplastic cells, aggressive versus indolent tumors, therapy responsive and nonresponsive cancer cells, tumors of different origins, and others. The newly identified genes may play important roles in the pathogenesis of lymphoma and potentially represent novel diagnostic genetic markers, but the avalanche of information generated by this technology will have to be sorted out before it is utilized clinically. Even if this is accomplished, the analysis and quantitation of the expression of informative genes at the RNA level in heterogeneous clinical samples may not be an easy task. However, once critical genes and their products are recognized, it may be possible to identify or generate antibodies that can easily detect and quantitate these products or their surrogates using FCM. This course of action has already begun (13,14).

LITERATURE CITED

1. World Health Organization classification of tumours: pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2001.
2. van Den BR, Boor PP, van Lochem EG, Hop WC, Langerak AW, Wolvers-Tettero IL, et al. Flow cytometric analysis of the Vbeta repertoire in healthy controls. Cytometry 2000:40:336–345.
3. Langerak AW, van Den BR, Wolvers-Tettero IL, Boor PP, van Lochem EG, Hooijkaas H, et al. Molecular and flow cytometric analysis of the Vbeta repertoire for clonality assessment in mature TCRalphabeta T-cell proliferations. Blood 2001:98:165–173.
4. Almasri NM, Zaer FS, Iturraspe JA, Braylan RC. Contribution of flow cytometry to the diagnosis of gastric lymphomas in endoscopic biopsy specimens. Mod Pathol 1997:10:650–656.
5. Wells DA, Hall MC, Shulman HM, Loken MR. Occult B cell malignancies can be detected by three-color flow cytometry in patients with cytopenias. Leukemia 1998:12:2015–2023.
6. Cornfield DB, Mitchell Nelson DM, Rimsza LM, Moller-Patti D, Braylan RC. The diagnosis of hairy cell leukemia can be established by flow cytometric analysis of peripheral blood, even in patients with low levels of circulating malignant cells. Am J Hematol 2001:67:223–226.
7. Kawano MM, Mihara K, Tsujimoto T, Huang N, Kuramoto A. A new phenotypic classification of bone marrow plasmacytosis. Int J Hematol 1995:61:179–188.
8. Ocqueteau M, Orfao A, Almeida J, Blade J, Gonzalez M, Garcia-Sanz R, et al. Immunophenotypic characterization of plasma cells from monoclonal gammopathy of undetermined significance patients. Implications for the differential diagnosis between MGUS and multiple myeloma. Am J Pathol 1998:152:1655–1665.
9. Rawstron AC, Davies FE, DasGupta R, Ashcroft AJ, Patmore R, Drayson MT, et al. Flow cytometric disease monitoring in multiple my-

eloma: the relationship between normal and neoplastic plasma cells predicts outcome after transplantation. Blood 2002;100:3095–3100.

10. Braylan RC. Flow-cytometric DNA analysis in the diagnosis and prognosis of lymphoma. Am J Clin Pathol 1993:99:374–380.

11. Smith PJ, Blunt N, Wiltshire M, Hoy T, Teesdale-Spittle P, Craven MR, et al. Characteristics of a novel deep red/infrared fluorescent cell-permeant DNA probe, DRAQ5, in intact human cells analyzed by flow cytometry, confocal and multiphoton microscopy. Cytometry 2000:40:280–291.

12. Yuan CM, Douglas VK, Ahrens KP, Luchetta GR, Braylan RC, Yang L. DRAQ5-based DNA content analysis of hematolymphoid cell sub-populations discriminated by surface antigens and light scatter properties. Cytometry 2004; (In press). DOI: 10.1002/cyto.b.20000.

13. Chen JS, Coustan-Smith E, Suzuki T, Neale GA, Mihara K, Pui CH, et al. Identification of novel markers for monitoring minimal residual disease in acute lymphoblastic leukemia. Blood 2001:97: 2115–2120.

14. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101:4944–4951.