Flow Cytometry in Hematopathology:

A Visual Approach to Data Analysis and Interpretation

D.N. and L.W.D.: With all of our love to Vladimir, Petrushka, Fidelio, Feivel, Amadeus, Cherubino, Tamino, Wolfgang ("Bum Jr."), Belmonte, Apollo, Sergei, "Post Auto," Paddington, "Cookie Jar," Sasha, Misha, Clipper, Beaker, Lanipo and Hiapo.

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A Visual Approach to Data Analysis and Interpretation

Second Edition

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HUMANA PRESS TOTOWA, NEW JERSEY

999 Riverview Drive, Suite 208
Totowa, New Jersey, 07512

www.humanapress.com

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ANSI Z39.48-1984 (American National Standards Institute) Permanence of Paper for Printed Library Materials.

10 9 8 7 6 5 4 3 2 1

eISBN: 978-1-59745-162-8

Library of Congress Control Number: 2007922048

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Preface to the Second Edition

During the time that has elapsed between the first edition and the second edition of this book, there has been considerable improvement in the incorporation of flow cytometry immunophenotyping into hematology and pathology laboratories, including institutions where previous practice had relied heavily on traditional morphology and paraffin-based immunostaining. In addition, flow cytometry (FCM) immunophenotyping has also gained acceptance as a useful diagnostic tool for the identification of not only acute myeloid leukemias but also other myeloid disorders, both malignant and premalignant. During the same time, advances have been made in terms of instrumentation and commercially available reagents. For instance, the introduction of the T-cell receptor (TCR)-Vβ eight-tube kit has greatly facilitated the evaluation of some difficult to diagnose mature T-cell disorders, and the implementation of the DNA dye DRAQ5 has improved the grading of lymphoma subpopulations present in heterogeneous samples. The role of FCM analysis has also progressed beyond that of establishing a diagnosis to that of monitoring disease and providing prognostic information.

The second edition of this book reflects the recent advances in the FCM analysis of hematopoietic disorders. To this end, the chapters have been revised to incorporate additional text and figures. The focus of the book and its organization remain unchanged, however. The availability of new software tools has made it possible to add more case studies to the new companion CD-ROM, as well as to render the disk easier to use without the need to install a database engine. The listing of the case studies (and their diagnoses) is provided at the beginning of the book. The reader will do well not to omit the case studies from consideration as they supplement the information provided in the book.

It is hoped that this book will bring a better appreciation of the important role of FCM analysis in the diagnosis and management of hematopoietic disorders. When FCM is applied systematically, the potential exists to reduce the confusion that still exists in the current classification of certain malignant lymphomas and lymphoproliferative disorders.

Preface to the First Edition

Flow cytometry immunophenotyping of hematopoietic disorders is a complex and demanding exercise that requires a good understanding of cell lineages, developmental pathways, and physiological changes, as well as broad experience in hematopathology. The process includes several interrelated stages, from the initial medical decision regarding which hematologic condition is appropriate for FCM assay, to the final step of diagnosis whereby the FCM data is correlated with other relevant clinical and laboratory information. The actual FCM testing involves three major steps: preanalytical (specimen processing, antibody staining), analytical (acquiring data on the flow cytometer) and postanalytical (data analysis and interpretation). The literature, including the latest FCM textbooks, provides ample information on the technical principles of FCM such as instrumentation, reagents and laboratory methods, as well as quality control and quality assurance. Similarly, correlations of morphologic findings and phenotypic profiles have been well covered in many publications. In contrast, much less attention has been given to the other equally important aspects of FCM immunophenotyping, especially data analysis. The latter is a crucial step by which a phenotypic profile is established.

To bridge this gap in the literature, the focus of this book is more on FCM data analysis than laboratory methods and technical details. For the reader to become familiar with our data analysis strategy, an overview of our approach to the preanalytical and analytical steps is also presented, with an emphasis on the preanalytical aspects, which have been rarely touched upon in the literature.

The process of data analysis follows a practical and systematic approach, utilizing the visual patterns of the dual parameter displays rather than calculating a "percent positive" for each individual antibody. The FCM graphic displays presented throughout the book, together with the clinical case studies contained in the companion CD-ROM should facilitate the readers to gain an in-depth appreciation of this visual approach to data analysis. Via the case studies, the topics discussed in the textbook can be illustrated in greater detail, and the FCM diagnostic subtleties will become more apparent.

The book is designed for all laboratory professionals involved in the immunophenotyping of hematologic disorders, including pathologists, PhDs, and technologists working in FCM laboratories, residents and fellows in pathology and hematopathology training programs, as well as clinical hematologists with a special interest in this subspecialty. In terms of organization, this book breaks away from the traditional mold used in other textbooks. The chapters are not arranged by specific diagnosis (i.e., the end point of a diagnostic workup) but by how the data presents at the time of the diagnostic consultation. This organization reflects the real-life problem-solving methods applied daily in the laboratory, whereby the strategies employed differ depending on whether the cell population in the sample analyzed is heterogeneous or nearly homogeneous.

The few available books covering FCM phenotypes in hematologic malignancies have tended to focus more on leukemias than lymphomas. In this book, equal emphasis is given to both categories of disease, thereby providing considerably more information on lymphomas and chronic lymphoproliferative disorders. Furthermore, DNA cell cycle analysis is also

included in the FCM study of mature lymphoid malignancies, in which the DNA data have been proven to be of prognostic significance, thus permitting a more objective and reproducible grading of these tumors.

The approach to the classification of hematologic neoplasms employed in this book also departs from that used in the various existing classifications. The antigenic profiles of leukemias and lymphomas have been incorporated into the more recent classification schemes. However, the phenotypes of many disorders, in particular malignant lymphomas, have been derived from paraffin-based immunostaining instead of FCM studies, thus not taking into consideration the large amounts of valuable information provided by FCM immunophenotyping (e.g., a better appreciation of the pattern of antigenic density distribution and coexpression). The approach taken in this book is to simplify the classification (which should facilitate the comparison of results between different institutions) by utilizing the graphical patterns of phenotypic expression and the results of DNA cell cycle analysis where appropriate, together with other relevant clinical/laboratory data including the morphology of the submitted specimen. A more detailed discussion on the morphology of the bone marrow and peripheral blood manifestations of hematologic disorders can be found in our previous textbook (and its companion CD-ROM) entitled *Diagnostic Hematology: A Pattern Approach* (Arnold Publishers; distributed in the United States by Oxford University Press; ISBN 0-7506-4247-5).

For practical reasons, most of the FCM graphics in the book are presented as black-and-white illustrations. The dot plots in many of the case studies contained in the CD-ROM are, on the other hand, presented in color to facilitate the viewing of the cell cluster(s) of interest, especially for educational purposes. The use of color dot plots is popular in some laboratories. In our opinion, laboratory staff involved in FCM data analysis should be familiar with both black-and-white and color FCM displays however, rather than relying solely on the color format.

The list of suggested readings is not meant to be exhaustive. Many of the references were chosen mainly for the readers to obtain more depth on certain topics, for example, the maturation and differentiation of hematopoietic cells.

Acknowledgments

The authors would like to acknowledge David Novo, president of De Novo Software (www.denovosoftware.com), for providing several copies of his FCS Express software to create the FCM illustrations in the book and the case studies contained in the CD-ROM. We would also like to thank our medical colleagues, in particular Wolfgang Huebl and Samer Al Quran, for the contribution of hematologic samples and/or unusual cases. The authors also thank the many FCM technologists we have been associated with for their excellent work, in particular Mikhail Mazharov and the crew at the University of Florida Shand's Hospital hematopathology laboratory, especially John B. Anderson, Heath I. Bailey, Sandra M. Campbell, Catherine R. Charleston, William D. Dixon, Gabriel R. Luchetta, and Darin J. Ryder.

List of Abbreviations

7-AAD	7-Amino-actinomycin D
AIDS	Acquired immunodeficiency syndrome
ALCL	Anaplastic large cell lymphoma
ALL(s)	Acute lymphoblastic leukemia(s)
AML(s)	Acute myeloid leukemia(s)
APC	Allophycocyanin
ATLL	Adult T-cell leukemia–lymphoma
BC	Blast crisis
CD	Cluster designation
cKappa	Cytoplasmic kappa
cLambda	Cytoplasmic lambda
CLL	Chronic lymphocytic leukemia
CLL/PL	Chronic lymphocytic leukemia with increased prolymphocytes
CML	Chronic myeloid leukemia
CMMoL	Chronic myelomonocytic leukemia
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DI	DNA index
DLCL	Diffuse large-cell lymphoma
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
ET	Essential thrombocythemia
FCC(s)	Follicular center cell(s)
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
FNA	Fine-needle aspiration
FRFH	Florid reactive follicular hyperplasia
FSC	Forward scatter
GCC(s)	Germinal center cell(s)
G-CSF	Granulocyte colony-stimulating factor
HCL	Hairy cell leukemia
H&E	Hematoxylin and eosin
HIV	Human immunodeficiency virus
HTLV	Human T-cell lymphotropic virus
IM	Infectious mononucleosis
LCL(s)	Large cell lymphoma(s)
LGL(s)	Large granular lymphocyte(s)
LL	Lymphoblastic lymphoma

LPC	Lymphoplasmacytoid
LPD(s)	Lymphoproliferative disorder(s)
MALT	Mucosa-associated lymphoid tissue
MBCL	Monocytoid B-cell lymphoma
MCL	Mantle cell lymphoma
MDS	Myelodysplastic syndrome(s)
M:E ratio	Myeloid:erythroid ratio
MF	Mycosis fungoides
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
MNC(s)	Mononuclear cell(s)
MoAb(s)	Monoclonal antibody (antibodies)
MPD(s)	Myeloproliferative disorder(s)
MPO	Myeloperoxidase
MRD	Minimal residual disease
MUD-BMT	Matched unrelated donor bone marrow transplant
MZBL	Marginal zone B-cell lymphoma
N/C	Nuclear/cytoplasmic
NHL(s)	Non–Hodgkin lymphoma(s)
NK	Natural killer
NOS	Not otherwise specified
NSE	Nonspecific esterase
PBSCT	Peripheral blood stem cell transplant
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinium chlorophyll protein complex
PLL	Prolymphocytic leukemia
PMT(s)	Photomultiplier tube(s)
PRV	Polycythemia rubra vera
PTCL(s)	Peripheral T-cell lymphoma(s)
RBC(s)	Red blood cell(s)
SLVL	Splenic lymphoma with villous lymphocytes

List of Case Studies

CHAPTER

1 Approach to flow cytometry: General considerations

The application of flow cytometry (FCM) in diagnostic hematopathology has gained much momentum since its introduction in the late 1970s. FCM immunophenotyping is now an established and necessary laboratory test in the clinical evaluation of any suspected hematologic malignancy. Several factors have contributed to the progress in FCM analysis, including the expanding repertoire and commercial availability of monoclonal antibodies and fluorochromes, as well as technological advances in both the hardware and software of FCM instruments. As a result, three- and four-color FCM studies have become the norm in hematopathology laboratories during this past decade.

Because it is a multiparameter analysis, FCM immunophenotyping offers the advantage of efficiency coupled with a high degree of sensitivity. Not only can an extensive array of markers be evaluated by FCM, but the expression of several antigens can also be assessed simultaneously on any given cell population. In contrast, the number of markers that can be used in immunostaining on tissue sections or smears is limited, and fewer cells can be evaluated using routine morphologic criteria. Although dual staining may be achieved by immunohistochemistry in selected situations, the technique is essentially limited to a single antibody per slide. Using FCM, the relative proportion of the population of interest within a sample can be quantitated, and its DNA content can be measured, when appropriate. The high level of sensitivity of FCM also allows for the detection of rare neoplastic cells (based on their specific characteristics) coexisting with other benign subpopulations.

The usefulness of FCM immunophenotyping is multifold, as it facilitates (1) the distinction between neoplastic and benign conditions, (2) the diagnosis and characterization of lymphomas and leukemias, (3) the assessment of other neoplastic and preneoplastic disorders such as plasma cell dyscrasias and myelodysplastic syndromes, and (4) the detection of minimal residual disease in patients with acute leukemia or chronic lymphoid malignancies. In some groups of lymphoid neoplasms, FCM study also provides prognostic information.

In many institutions, there is a tendency to perform immunological studies only when the lesion is considered difficult to diagnose by conventional morphology. It is preferable, however, not to delay FCM testing but to perform it routinely, even when the morphology is apparently typical, because the findings help to confirm the diagnosis and may provide prognostic or other useful biological information. In addition, the data are valuable for follow-up purposes, especially when samples of tumor recurrences are very small (e.g., cerebrospinal fluid [CSF], needle aspirates) where morphologic examination may fail to detect neoplastic cells.

Proper data analysis is a critical step in FCM immunophenotyping. In this process, the phenotypic profile of the cells of interest is derived from the light scatter and fluorescence intensity signals recorded from each individual cell on a cell-by-cell basis (the data thus collected is referred to as list mode data). Although the literature contains numerous publications on the characteristic immunophenotypes associated with different hematologic malignancies, few publications describe how the data analysis was performed.

1.1 Reasons for the necessity of proper data analysis

Even today, many laboratories still continue the less than desirable practice of reporting antigenic expression as the percentage of positive cells. In this approach to FCM analysis, (1) the cell population is gated first by light scatter, then the antibody fluorescence analyzed on single parameter histograms or dual parameter plots; (2) for each marker, a cursor is moved and set to measure the fraction of cells with fluorescence greater than that of the control sample (in which cells are exposed to an irrelevant immunoglobulin); (3) the results are then reported as percent positive per antibody tested. The origins of this approach to data reporting can be traced back to the microscopic evaluation of immunostaining performed on glass slides (smears, cytospin preparations) and the reporting techniques used for lymphocyte subset analysis (e.g., CD4 counts in human immunodeficiency virus [HIV]-infected patients).

In many laboratories, the larger share of the FCM workload is composed of T- (or other) cell subset determinations on peripheral blood samples that do not harbor malignant cells. In this setting, a change in the number of cells in each subset is clinically important. Furthermore, the cells analyzed are discrete subpopulations of normal lymphoid cells with relatively bright fluorescence. Therefore, it is appropriate to report each subset as a percent positive for each antibody and, where applicable, to calculate the CD4 : CD8 ratio. The numerical values thus generated are reminiscent of those obtained for chemistry tests, in which the abnormalities consist of altered levels of the normal components in the blood.

In samples suspected of harboring a hematopoietic malignancy, however, determining the exact number of neoplastic cells is less important than determining whether or not neoplastic cells are present and, if present, the type of hematopoietic neoplasm they represent. Unfortunately, this information is not always apparent from the "percent-positive" data format. The percent-positive format assumes, incorrectly, that within a leukemia or lymphoma, all of the tumor cells uniformly either lack or exhibit the same degree of clear-cut expression for a given antigen. However, in contrast with benign lymphocytes, neoplastic hematopoietic cells of the same clone often do not express the same amount of a given antigen on their cell surface and, therefore, display variability in the fluorescence intensity for that marker. The degree of variability depends on the particular surface antigen. For instance, reporting a case of leukemia as being 40% CD20 positive is ambiguous. This number could represent either (1) a case in which 40% of the cells formed a distinct population with a fluorescence intensity well above the negative control or (2) a single population in which 100% of the cells displayed a shifted fluorescence intensity, but only 40% of the cells were brighter than the background. The latter occurrence is frequently observed when the tumor cell expression for a surface antigen is weak.

1.1.1 The pitfalls of the FCM data format of "percent positive" per antibody tested

In the context of a leukemia–lymphoma workup, it is important to express the immunophenotyping data in ways that avoid ambiguity and offer the optimal information for correlation with other clinical and laboratory data. Expressing the FCM data as "percent positive" per antibody tested is rarely relevant and may even be misleading. Many institutions have used the 20% level as an arbitrary cutoff value for a marker to be considered positive. None of the publications has described how this number became established, however. The following real-life examples (obtained in 1997 in London, UK) illustrate why the approach of reporting FCM results as percent positive and omitting the fluorescence data is inappropriate in leukemia-lymphoma immunophenotyping, and can lead to erroneous interpretations.

Flow cytometry results on a bone marrow from a patient with suspected chronic myeloid leukemia in blast crisis (CML-BC), from an institution where the FCM laboratory is not part of the hematopathology laboratory, are shown below. The specimen was processed by Ficoll-Hypaque. Other procedure-related information was not made available.

CD19	31%	CD34	30%	CD2	11%
CD20	20%	CD14	7%	CD3	13%
CD10	29%	Kappa	8%	CD7	16%
CD13	32%	Lambda	10%	CD5	19%
CD33	26%	HLA-DR	29%		

Based on this format of data reporting and the 20% threshold, the case was interpreted as a biphenotypic blast crisis of CML (positive for CD19, CD10, CD13, CD33). However, when the list mode data was visualized on dual parameter dot plots, correlating the forward scatter (FSC) and antibody fluorescence, it became clear that (1) the neoplastic cells constituted 30% of the cell population in the FCM sample and (2) they were of medium cell size and had the following phenotype: CD19 moderate, CD20 dim, CD10 moderate, CD34 weak, HLA-DR moderate. Other antigens, (i.e., CD13, CD33, CD14, CD2, CD3, CD5, CD7, kappa, and lambda) were not expressed by the tumor cells. The CD13 (32%) and CD33 (26%) were present on mononuclear myeloid precursors (promyelocytes, myelocytes, and metamyelocytes) and not on the neoplastic population. The correct phenotype is that of a precursor B-cell acute lymphoblastic leukemia (ALL) and not biphenotypic leukemia. Correlation with the bone marrow aspirate morphology further confirmed a lymphoid blast crisis of CML.

A limited (follow-up) panel was performed on the peripheral blood of a patient with known chronic lymphocytic leukemia (CLL), to assess the efficacy of anti-CD20 therapy as part of a clinical trial. The lymphocyte count was 3.1×10^7/L. The blood film was unremarkable except for a mild increase in large granular lymphocytes. The FCM data were reported as follows:

CD2	75%	CD19	23%	Kappa	17%
CD3	62%	CD20	18%	Lambda	8%

Based on these results, it was concluded that there was no residual CLL in the patient's peripheral blood, especially as the kappa:lambda ratio was within the normal range. However, subsequent reevaluation of the list mode data, using simple correlated displays of FSC and antibody fluorescence, was sufficient to demonstrate the presence of a small population of monoclonal B-cells (CD19 moderate, CD20 weak) with weak kappa expression, in a background of benign T-cells and polyclonal B-cells. Contrary to the initial interpretation, residual CLL was present in the patient's peripheral blood.

It is apparent from the above examples that reporting FCM data as percent positive per antibody tested can negate the usefulness of FCM and easily lead to confusing or erroneous interpretations, which may impact therapeutic decisions.

Some laboratories do include fluorescence data in the FCM reports. However, the data may still be expressed in a suboptimal (and, therefore, inappropriate) manner, as shown in the following case example.

Below are the FCM results on a peripheral blood specimen studied (in the mid-1990s) at a teaching hospital:

CD2	48% moderate	CD19	47% moderate
CD3	45% moderate	CD20	26% moderate
CD4	21% moderate	CD22	47% moderate
CD7	47% moderate	sIgM	48% moderate
CD8	20% moderate	Kappa	3% moderate
CD13	3% moderate	Lambda	2% moderate
CD33	1% moderate	CD10	36% moderate
CD34	1% weak	CD45	100% strong
TdT	55% moderate	HLA-DR	55% moderate

The results indicated a proliferation of immature cells (TdT^+). The case was interpreted as ALL with a mixed (B-cell and T-cell) lineage. Because of the data-reporting format, it is unclear whether the immature cells are of B- or T-cell lineage, however. Although fluorescence intensities were mentioned, data interpretation in this particular laboratory was actually based on percent positive with an arbitrary 20% cutoff. When proper visual data analysis was subsequently applied to the raw data, it became apparent that the blood sample contained a clearly identifiable neoplastic population of precursor B-ALL, admixed with a high number of normal T-cells.

1.2 General aspects of FCM data analysis and interpretation

The above-described situations indicate the necessity of a comprehensive approach to FCM data analysis and interpretation. In the authors' experience, the optimal method is for the laboratory medical staff to apply a visual approach to FCM data analysis rather than relying on percentages. In other words, data interpretation is based on a visual appraisal of the FCM graphics, assessing the complex patterns formed by the shape and relative position of the cell clusters observed on various dot plots such as FSC versus fluorescence, side scatter (SSC) versus CD45, and correlated fluorescence dot plots. Any other approach to FCM data interpretation, using a scoring system or percent positive per antibody, underutilizes the full potential of FCM.

Laboratory professionals, as well as clinicians, should realize that visual FCM data analysis is a process reminiscent of the microscopic examination of morphologic material (e.g., bone marrow aspirate smears, lymph node sections) in which the data form a pattern and are reported in a qualitative and quantitative (where appropriate) format. Although microscopic examination encompasses all elements in the sample, reporting the data focuses only on the abnormal component. Similarly, the FCM interpretative report should be based on the cells of interest, even though the list mode data should be collected unselected (i.e., it includes all cells in the sample).

Collecting list mode data ungated ensures that no abnormal cells are lost, because in many instances, the nature of the abnormal population is not yet known at the time the specimen is run. Restricting the initial data collection to certain preset criteria (i.e., a "live gating" approach such as the use of a live light scatter gate) may easily result in "throwing the critical cells away." A specific example is missing a small number of circulating hairy cells when the analysis is live-gated on cells with the light scatter characteristics of normal lymphocytes. An additional advantage of the ungated approach is that the presence of other cells serves as internal positive and negative controls.

After the data have been acquired ungated, certain gating procedures can be applied during the analysis step. Some of the most useful gating strategies include (1) gating on B-cells, to determine clonality (Figure 1.1) and the coexpression of other critical antigens, and (2) gating

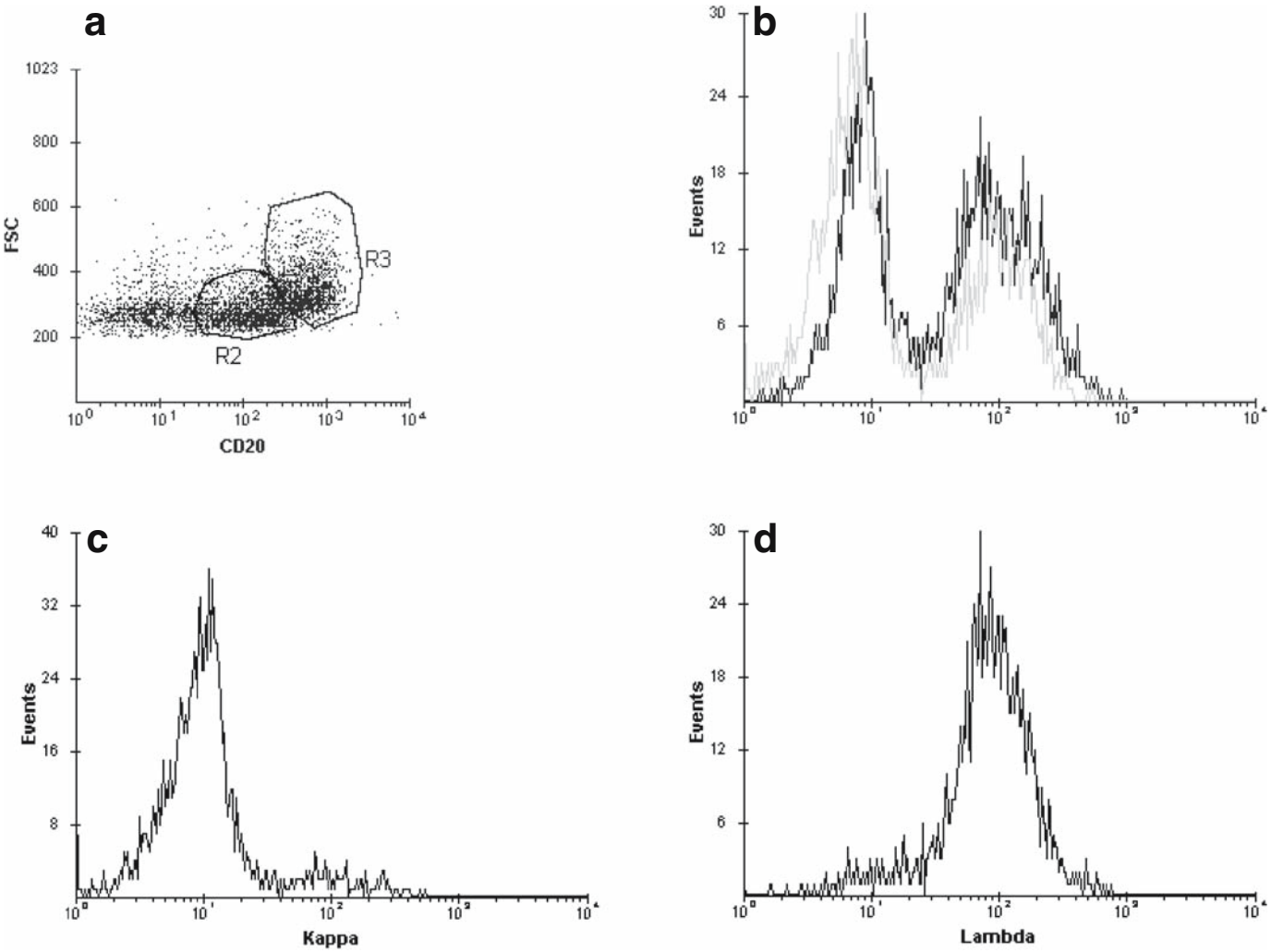

Figure 1.1 (a) Lymph node sample with two B-cell populations differing in FSC signals and CD20 intensities. (b) Overlay kappa/lambda histograms gated on R2: The B-cells with dimmer CD20 and lower FSC are polyclonal. (c, d) Gated on R3: The B-cells with brighter CD20 and higher FSC are monoclonal for lambda.

on CD45 to characterize leukemic blasts (Figure 1.2). These strategies require the use of multicolor (two-color, at the very least) antibody combinations. Following the recommendations by the U.S.–Canadian Consensus on the Use of Flow Cytometry Immunophenotyping in Leukemia and Lymphoma in 1997, the multiparameter approach to FCM testing has become a standard routine in clinical laboratories, taking advantage of more sophisticated instrumentation and a larger repertoire of fluorochromes.

Since the publication of the consensus recommendations, there has been an increase in the awareness of the visual approach to FCM data analysis. The literature contains very little information on this approach, however. The purpose of the first edition of this book was to fill this void. More recent developments in the field are added in this second edition.

The FCM dot plots and histograms displayed in this book, using FCS Express software, are derived from clinical samples analyzed primarily on Becton-Dickinson instruments, using commercially available antibody reagents (*see* Chapter 2). Other current state-of-the-art instruments are equipped with a similar capability for multicolor FCM testing and mechanisms for

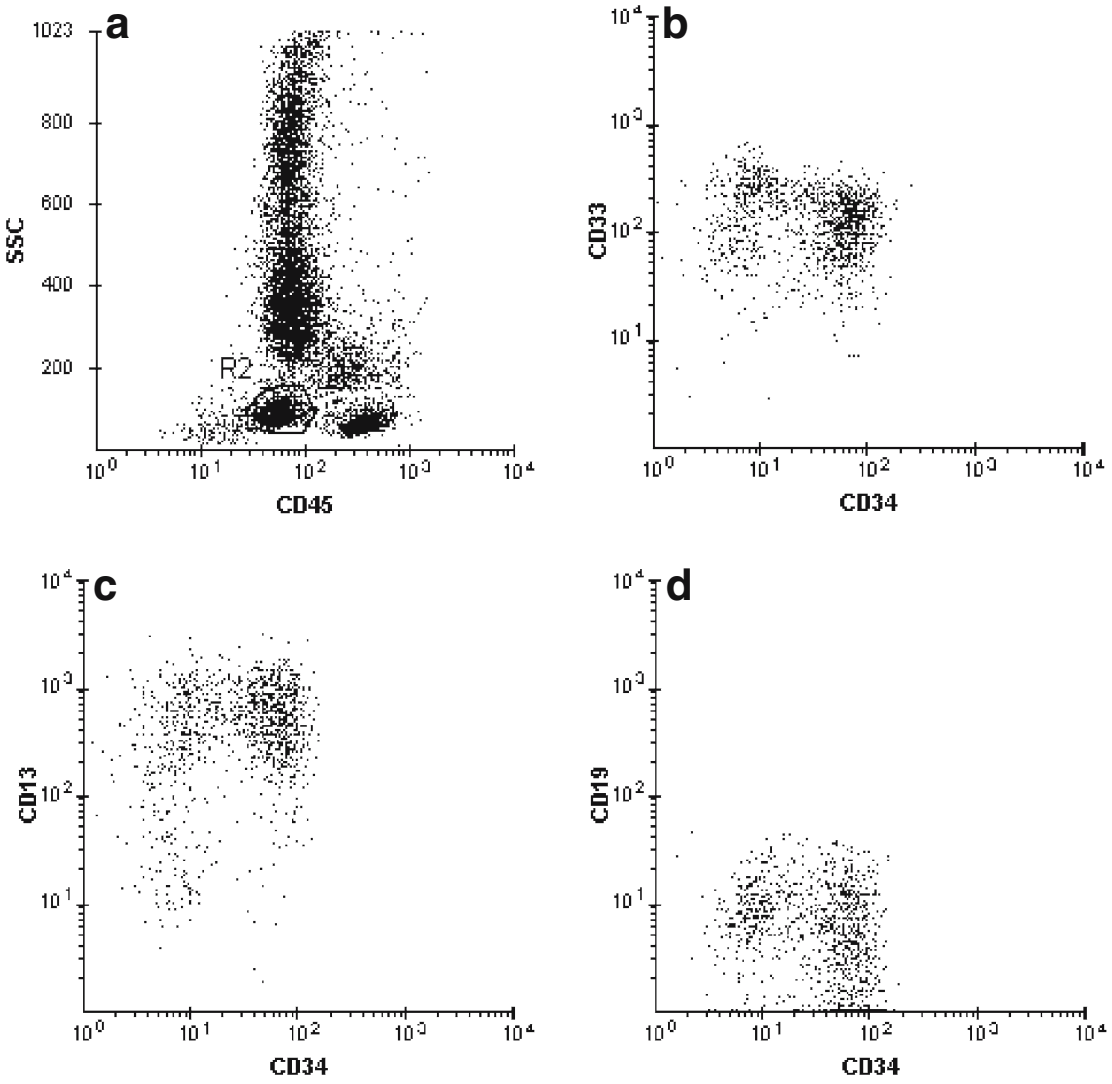

Figure 1.2 (a) Peripheral blood sample with a distinct cluster (R2) in the blast region. (b–d) Gated on R2: Blasts are positive for CD13 and CD33. CD19 is negative. CD34 is expressed with a bimodal distribution.

color compensation. The principles of FCM data analysis presented in this book are applicable to all brands of flow cytometers.

Interpretation of the FCM immunophenotyping results is one step in the diagnosis of malignant lymphoma and leukemia. Although, in many cases the diagnosis is apparent after a visual inspection of the FCM immunophenotyping data together with the DNA cell cycle histogram, in other instances the antigenic profile and the pattern of the cell clusters suggest only a differential diagnosis instead of a specific disorder. In such cases, it is critical that the diagnostic interpretation takes into account the other clinical and laboratory data, such as the hemogram findings and the cytologic/morphologic features. The synthesis of the pertinent results requires the responsible medical staff in the laboratory to be well versed in the different subdisciplines of hematopathology. Irrespective of whether a case is straightforward or complex, the authors advocate a routine systematic approach to FCM diagnostic interpretation. This will ensure that no relevant information is omitted.

A correlation between the FCM findings and the available morphologic data should be performed in all cases. Wright-Giemsa-stained cytospins made from the cell suspension of the

tissue or fluid submitted for FCM study must be reviewed, to correlate the findings with those derived from the FCM plots. This is especially helpful when abnormal (neoplastic) cells are few or the FCM data cannot be clearly interpreted.

For peripheral blood specimens, the FCM data are correlated with the hemogram and cytologic features from a fresh blood film. Similarly, FCM interpretation on bone marrow specimens should include a review of the hemogram, peripheral blood film, bone marrow aspirate smear or imprint, and cytochemistries, where appropriate. It cannot be emphasized enough that hemogram findings, along with fresh peripheral blood and bone marrow smears, must accompany the specimen when bone marrow is sent to a referral laboratory for immunophenotyping, so that a proper, thorough diagnostic evaluation of the case can be conducted. For interpretation on solid tissue (e.g., lymph nodes), the FCM data are correlated with the morphologic features on the imprints and hematoxylin and eosin (H&E) sections (where available).

In addition to the above-mentioned minimum correlation with the morphologic findings, it is also important to review immunoelectrophoresis results in suspected lymphoplasmacytoid neoplasms or plasma cell dyscrasias. Knowledge of the pertinent clinical history, especially the type of therapy (e.g., immunotherapy, growth factors), is also useful to further refine FCM diagnostic interpretation. This necessitates a dialogue between the medical staff caring for the patient and the FCM laboratory. Because of the time delay associated with molecular genotyping and cytogenetics, these techniques play a minimal role at the time of rendering the diagnosis. Correlation of those results with the FCM data is useful, however, for confirming the diagnosis or providing additional information.

1.3 Other applications of FCM in hematopathology

In addition to being a diagnostic tool, FCM analysis has also been used for prognostic purposes. The main caveat, when determining the prognostic significance of biological parameters of neoplastic cells, is that the validity of the results is influenced by various factors such as laboratory methodologies, clinical staging procedures, and therapeutic protocols. Despite such drawbacks, studies have shown that the DNA index may be of prognostic significance in childhood ALL and the S-phase fraction is useful in grading a lymphoproliferative disorder/non-Hodgkin lymphoma (LPD/NHL). The current classification of LPD/NHL according to the WHO scheme is partly based on cellular ontogeny and differentiation rather than on the biological behavior of the tumor. The choice of therapeutic regimens in lymphomas is still based on the grade of the tumor, however, because cell cycle-dependent drugs continue to feature prominently in the arsenal of chemotherapy for LPD/NHL. Therefore, it would be helpful that FCM testing on lymphomas includes DNA and cell cycle analysis, as the S-fraction gives an indication as to whether the tumor has a high growth-fraction (i.e., aggressive, high grade) or a low growth-fraction (i.e., indolent, low grade), which in turn influences the patient's response to therapy and survival. Determination of the S-fraction by multiparameter DNA analysis is preferable to paraffin-based immunostaining for Ki67 or PCNA (proliferating cell nuclear antigen), where it is not always possible to distinguish proliferating lymphoma cells from the intimately admixed proliferating benign T-cells.

The value of DNA ploidy in LPD/NHL as a prognostic factor still remains controversial. The presence of DNA aneuploidy is helpful, however, to identify those cases of suspected peripheral T-cell malignancies in which the hematologic and immunophenotyping data reveal no abnormalities. In Sézary syndrome, DNA ploidy by FCM has been shown recently to be useful for diagnosis and minimal residual disease (MRD) monitoring. Furthermore, the presence of aneuploidy is associated with increased numbers of large cells in the involved tissues.

In addition to cell cycle analysis, there have also been attempts to correlate certain antigenic features with the patient's response to therapy or survival. While there is only limited evidence that the expression of any particular antigen could serve as a reliable predictor of prognosis, it appears that the intensity of CD45 expression affects the outcome in pediatric ALL, and CD38 positivity in CLL is associated with an unfavorable clinical course. More recently, the advent of gene expression profiling by DNA microarray analysis has led to the identification of genes that may discriminate the subtypes of CLL. Most notably, the differential expression of the Zap-70 gene has been shown to correlate with the mutational status of immunoglobulin heavy-chain variable-region (IgVH) genes, the latter being an important prognostic factor in CLL. Because Zap-70 protein can be readily determined by FCM, in contrast with the more labor intensive and costly DNA sequencing analysis for IgVH mutations, Zap-70 is currently viewed as the best surrogate marker of IgVH mutational status in CLL. According to recent studies, the expression of Zap-70 protein identifies a subgroup of CLL with a more rapidly progressive clinical course and poorer outcome. Note that testing for Zap-70 is limited to peripheral blood samples only, however.

An important application of FCM analysis is MRD monitoring. With the emergence of new treatment protocols combining high-dose chemotherapy, autologous stem cell transplants and immunotherapy, MRD detection is becoming necessary in clinical laboratories. The applications of MRD monitoring are multifold, depending on the particular hematologic malignancy. For instance, in childhood ALL, the patient's MRD status during the initial phase of therapy is a powerful prognostic indicator based on which patients can be stratified into different risk groups. Routine monitoring of MRD during clinical remission in acute leukemias also facilitates early therapeutic intervention, so as to reduce the morbidity/mortality associated with overt clinical relapse. Furthermore, MRD assay is a helpful tool for assessing tumor response to new treatment modalities, such as humanized anti-CD33 conjugated to calicheamicin (gemtuzumab ozogamicin) for AML, and the tyrosine kinase inhibitor, imatinib, in Ph^1-CML.

The use of MRD detection in mature lymphoid malignancies differs from that in acute leukemia, in that it is usually not applicable at the time of front-line treatment because most of the patients, especially those with low-grade disease, still harbor MRD while in "complete" clinical remission after conventional therapy. MRD detection is therefore applied mainly to patients who, because of relapse, receive high-dose chemotherapy with stem cell transplant, and/or immunotherapy such as anti-CD20 (rituximab) or, in the case of CLL, anti-CD52 (alemtuzumab).

FCM was thought not to be as sensitive a technique for MRD detection as polymerase chain reaction (PCR)-based methodologies. However, this apparent lack of sensitivity is most likely due to the fact that the number of cells acquired in a standard FCM clinical assay is far less than that used in PCR analysis. Studies have shown that one leukemic cell in 10^4 to 10^5 bone marrow mononuclear cells can be detected by FCM when a large number of cells are analyzed, thus achieving a sensitivity level comparable with that of molecular analysis. These two techniques complement each other and are best applied in tandem to reduce any potential false-negative results. The FCM approach has the advantage of being less labor intensive and achieving a faster turnaround time. Furthermore, the ability of FCM to separate viable from dying cells permits a more accurate quantitation of MRD levels. Irrespective of the methodology, it appears that the clinically significant MRD level is 0.01% (i.e., 10^{-4}). The presence of residual leukemic cells above this level at the end of therapy or an increase in MRD levels in consecutive bone marrow samples during clinical remission has been shown to be associated with a higher risk of relapse and a poorer overall survival, and it tends to correlate with adverse cytogenetic abnormalities.

MRD studies have not been feasible in all patients with acute leukemia, however. Oligoclonality, clonal evolution, lack of specific leukemia sequences or absence of nonrandom

genetic abnormalities are some of the limiting factors to PCR-based MRD detection. Similarly, monitoring MRD status by FCM analysis can only be achieved if the leukemic blasts exhibit specific antigenic features differentiating them from their normal counterparts. In precursor B-ALL, it has been shown recently by DNA microarray analysis that a significant number of genes are overexpressed in leukemic cells in comparison with normal B-cell progenitors in the bone marrow. Of the several proteins encoded by the overexpressed genes, CD58 has become the marker of choice for MRD monitoring by FCM analysis because the protein is consistently overexpressed in a large number of patients with precursor B-ALL, and fluorochrome-conjugated anti-CD58 antibodies are commercially readily available.

1.4 Maturation and differentiation of hematopoietic elements: An overview based on the immunologic markers currently in use in the FCM laboratory

Adequate immunophenotyping of hematologic malignancies requires a large battery of cellular markers. These markers can be broadly categorized into the following groups: B-cell, T-cell, natural killer (NK) cell, myeloid/monocytic, erythroid, megakaryocytic, and non-lineage-associated markers (including activation markers such as CD38 and HLA-DR). The establishment of these markers was derived from studies on the differentiation and maturation of hematopoietic cells. Noncommitted hematopoietic stem cells express CD34. There is considerable heterogeneity within the population of $CD34^+$ progenitors, however, and those in the later stages also express HLA-DR and CD38. Another marker of immature cells is TdT, present predominantly although not exclusively, among lymphoid precursors.

The earliest B-cell precursors can be identified by cCD22, the hallmark of the B-cell lineage. Cytoplasmic CD22 is present even before any detectable rearrangement of the immunoglobulin (Ig) genes. Also found on early B-cell precursors are CD19 and CD10. The subsequent maturation and differentiation of the B-cell precursors in the bone marrow is characterized by a gradual decrease in CD10 together with a gradual gain of CD20. Cytoplasmic μ chain can be detected in the late stages of B-cell precursors. The appearance of surface Ig expression, along with the disappearance of immature markers (TdT, CD34), defines a mature B-cell. Mature naïve (resting) B-cells leave the bone marrow to enter the circulation and are characterized by the presence of well-expressed surface IgM, IgD and either kappa or lambda, along with CD20 and CD22. The further differentiation into different subtypes of mature B-cells (expressing IgA or IgG) and plasma cells occurs mainly in peripheral lymphoid tissues, where the cells home into different microenvironments depending on their stage of maturation.

The identifier of a T-cell lineage is cCD3, which appears in the earliest T-cell precursors in the thymus prior to rearrangements of the T-cell receptor (TCR) genes. Pre-T-cells are derived from a common T/NK progenitor coexpressing CD34, CD2 and CD5. Thymic maturation is characterized by acquisition of CD1a, and somatic recombination of the TCR genes to produce a diversity of TCR required for the recognition of a large variety of peptide antigens presented on major histocompatibility complex (MHC) molecules. The TCR molecule is part of a larger signaling complex that also includes the costimulatory molecule CD4 or CD8, and the signal transduction module formed by the various subunits of CD3. Until recently, studies of the TCR have been based on molecular techniques, either by Southern blot analysis of the β chain genes or PCR assay of the γ chain genes. The recent availability of a large panel of antibodies identifying 70% of the different variable (V) regions of the β chain has made it possible to study the TCR-Vβ repertoire by FCM, and thereby detect clonal expansions of abnormal T-cells.

The TCR molecule is a heterodimer composed of either α and β chains, or γ and δ chains. The structure of each chain is similar to that of Ig, consisting of constant and variable regions.

There are at least 65 Vβ segments on the β-chain gene. The TCR genes, located on chromosome 7 (β- and γ-chain genes) and chromosome 14 (α-chain gene, and δ-chain gene within the α-chain cluster) are rearranged in an orderly fashion. TCR-δ rearrangement occurs first as thymocytes progress from the $CD34^+CD1a^-$ to the $CD34^+CD1a^+$ stage. As these cells rearrange their γ genes, CD4 is upregulated producing CD4 (dim) single positive thymocytes. TCR-β gene rearrangements become detectable as the pre-T-cells become double positive for CD4 and CD8. The TCR-α gene is rearranged at the very late stage of pre-T-cell development. The prevailing thinking is that if γ and δ are productively rearranged first, then the T-cell precursor will become a γδ T-cell. Conversely, successful rearrangement of the β-gene will most likely be followed by recombination of the α-gene to produce an αβ T-cell. The subsequent maturation involves three processes: (1) precursor T-cells are exposed to thymic epithelial cells expressing either MHC class I or MHC class II molecules; (2) they undergo the process of negative and positive selection; and, (3) differentiate into either cytotoxic or helper T-cells, respectively, prior to entering the circulation. The selection process helps to prevent autoimmunity and to ensure that the mature T-cells are capable of recognizing foreign peptides. Only a very small number of mature T-cells remain double positive for CD4 and CD8.

Immunological recognition of a mature T-cell is based on the absence of TdT expression or other early thymic markers (e.g., CD1) and the overt expression of surface CD3. The great majority of mature T-cells are TCR-αβ positive, coexpressing CD2, CD5, CD7 and either CD4 or CD8. A minor population of T-cells express γδ TCR instead, accounting for about 3% to 5% of $CD3^+$ T-cells in the blood. The γδ T-cells emigrate from the fetal thymus to reside in cutaneous and mucosal (especially gastrointestinal) sites. Current knowledge about the function of γδ T-cells still remains limited. The majority of γδ T-cells are CD4- and CD8-negative; approximately one-third are CD8-positive.

Cells of the myeloid lineage can be identified by the expression of CD13, CD33, and CD117. The maturation process of myeloblasts into promyelocytes is accompanied by a loss of CD34 and HLA-DR, followed by the expression of CD15, CD11b, and CD16 at the myelocyte and metamyelocyte stages. Although CD13 and CD33 are expressed at all stages of granulocytic differentiation, the level of CD33 gradually decreases as the cells mature, whereas CD13 exhibits a bimodal distribution, being more brightly expressed on blasts and neutrophils, but less intensely on intermediate myeloid precursors. Intense CD14 is a characteristic of mature monocytes. An additional identifying feature of monocytic lineage is the expression of other myeloid markers (CD33, CD64), which differ in intensity and distribution from that on myeloid cells.

Erythroid precursors are characterized by downregulated CD45 and high expression of CD71 (transferrin receptor). CD71 is also present on cells of other lineages, however its levels are highest in erythroid cells, presumably because of the large amount of iron required for hemoglobin synthesis. According to studies performed on erythroid colonies, the levels of CD71 peak in the earliest erythroid precursors (prior to the proerythroblast stage), and decrease as the nucleated erythroid cells mature into reticulocytes. Erythroid maturation is accompanied by further loss of CD45 antigen and acquisition of glycophorin A (Gly-A), that is, CD235a. The levels of Gly-A become highest at the basophilic erythroblast stage, and remain unchanged from there onward. The maturation of reticulocytes into red blood cells (RBCs) is marked by a concomitant loss of reticulum and CD71 antigen. The evaluation of CD55 and CD59 is performed infrequently in the hematopathology laboratory, only when paroxysmal nocturnal hemoglobinuria (PNH) is suspected. These antigens are involved in the protection of cells by inhibiting the formation of membrane-attack complex. Decrease or loss of CD55 and CD59 in RBCs is characteristic of PNH.

Platelets and megakaryocytes can be identified by the presence of glycoprotein (Gp) IIb/IIIa, a heterodimer formed by integrin α2β (CD41) and integrin β3 (CD61). In addition, CD36,

known as GpIV and GpIIIb, is expressed. This antigen is also found on erythrocytes and monocytes, however.

It is important to be aware that some markers, although considered to be associated with a specific lineage, may be expressed by neoplastic cells of a different lineage. For example, CD15 is typically associated with myeloid processes, but it can also be present in a substantial number of T-ALL and precursor B-ALL, as well as monocytic leukemias. Similarly CD7, a T-cell-associated antigen, is often present in acute myeloid leukemia (AML). Such instances have given rise to the concept of aberrant immunophenotypes. The use of the label "lineage infidelity" warrants some caution, however, because not all lineage-associated antigens share the same degree of specificity. For example, antigens such as CD11b, CD16, and CD15 do not constitute sufficient evidence of myeloid differentiation. Furthermore, the concept of aberrancy is based on our current understanding of the normal sequence of phenotypic development, which may still be incomplete and evolving.

The great majority of currently known hematopoietic antigens are not lineage associated. For instance, CD10 is not only found on precursor B-cells and germinal center cells, but also on neutrophils, and may be expressed in some cases of precursor T-cell lymphoma/leukemia. Some of the non-lineage-associated antigens are activation markers that may be newly expressed on B- or T-cells after antigen stimulation and cellular activation. Common activation markers include HLA-DR, CD25, CD30, and CD38. Some non-lineage-associated antigens are adhesion molecules, which serve to facilitate cell-to-cell interaction. For instance, CD58, also known as lymphocyte function-associated antigen-3 (LFA-3) is an adhesion molecule widely expressed on hematopoietic and non-hematopoietic cells. The interaction of CD58 with CD2 (its only known ligand) mediates T-cell activation and cytokine production, as well as cytolytic activity in T-cells and NK cells. The value of CD58 in diagnostic FCM is, however, in the distinction between precursor B-ALL and benign B-cell progenitors in the bone marrow.

Another example of a non-lineage-associated marker routinely used in FCM immunophenotyping is CD56 (a neural cell adhesion molecule). Although CD56 is considered to be associated with the NK lineage, it is present in several other types of disorders including plasma cell tumors, small-cell neuroendocrine tumors, and a significant number of AMLs. Similarly, CD57, though expressed by most NK cells and a small subset of T-cells (also referred to as memory cytotoxic T-cells), is also present in many non-hematopoietic tissues or neoplasms. True NK cells and NK-like T-cells can be putatively identified based on the combined pattern of expression of one or more "NK markers" (CD16, CD56, or CD57) together with some of the T-cell-associated antigens.

More recently, the study of NK cells and NK-like T-cells has been further facilitated by the commercial availability of antibodies against NK receptors. NK receptors are membrane proteins involved in the recognition of MHC class I molecules (HLA-A, -B, -C, and -G) or their homologues. Functionally, NK receptors are either activating or inhibitory. The latter is involved in the recognition of class I MHC molecules with self-antigens, thus protecting healthy cells from NK cytotoxicity. Conversely, no inhibitory signal is generated in NK cells when the expression of MHC class I molecules is altered, which then leads to NK lysis of the target cells. Lysis is also mediated by the binding of activating NK receptors with specific class I ligands on the viral-infected cells or tumor cells. Structurally, NK receptors are subdivided into two broad categories: the killer cell Ig-like receptors (KIR) encoded by a family of 14 polymorphic genes on chromosome 19, and the C-lectin-like receptors encoded by a family of six conserved genes on chromosome 12.

The lectin-like NK receptors are composed of either heterodimers such as the CD94:NKG2 family of receptors specific for HLA-E, or homodimers such as CD161, which recognize MHC class I-like products. In contrast with the lectin-like receptors, KIR receptors are much more

complex structurally because they are involved in the recognition of the polymorphic determinants on MHC class I molecules. The KIR receptors are further subdivided into subfamilies according to the number of their Ig-like domains (two vs. three) and the length of their cytoplasmic tails (long vs. short). Each subfamily consists of one or more receptors. Receptors with long cytoplasmic tails have inhibitory function. KIR antigens are highly polymorphic. Their expression in NK cells is complex in that individual cell clones may express several members of the KIR family, and a given KIR receptor (for instance, CD158e) may be present only on a particular subset of NK cells. In a normal individual, the pattern of the usage of the KIR repertoire by NK cells remains stable over time. Currently, only a very small number of KIR antibodies (CD158a, CD158b, and CD158e) are commercially available, which does not yet permit adequate evaluation of the large KIR repertoire.

CHAPTER

2 FCM immunophenotyping and DNA analysis: Practical aspects that can affect data analysis and interpretation

In the optimal setting, the FCM lymphoma–leukemia immunophenotyping laboratory is an integral component of the diagnostic hematopathology service. Flow cytometric analysis involves three stages: preanalytical (specimen handling and processing, including antibody staining), analytical (running the sample through the flow cytometer and acquiring data), and postanalytical (data analysis and interpretation). The quality and performance of the preanalytical and analytical steps impact on the resulting fluorescence data and thereby the interpretation. Deficiencies such as suboptimal instrument performance, poor reagent quality (antibodies and/or fluorochromes), or poor specimen quality can all result in inadequate resolution of positive and negative immunofluorescence.

Integrating the FCM and hematopathology laboratories facilitates both the preanalytical steps (because the specimen can be processed simultaneously for other related technologies) and the postanalytical steps (during which the FCM results are correlated with other data prior to establishing a diagnosis). The methodologies used in the authors' laboratories follow closely the recommendations of the 1997 U.S.–Canadian Consensus on the Use of Flow Cytometry Immunophenotyping in Leukemia and Lymphoma. The general aspects of the methodologies for the preanalytical and analytical steps are presented in this chapter. Discussions on quality control are not included, however, as they have been presented at length in previous textbooks and manuals.

2.1 Sample selection

The laboratory has little control over certain factors, such as specimen collection and transportation, which can adversely affect the sample prior to its arrival. Although rigorous quality control applied to the various intralaboratory procedures can ensure the accuracy and reproducibility of the FCM results, poor specimen collection remains a major source of potential unsatisfactory FCM analysis. The time elapsed between specimen acquisition and delivery to the laboratory, and the environmental conditions during transport are critical factors affecting the viability of the cells in the sample. As a rule, specimens cannot be held for more than 48 hours in the fresh state after collection. This time window is much narrower for samples harboring a tumor with a high turnover rate (e.g., Burkitt lymphoma). For these reasons, specimen requirements and acceptance guidelines should be thoroughly communicated to the clinical services as well as to referring institutions. Exposure to extreme temperatures and the presence of blood clots (or gross hemolysis) are conditions that can render a blood or bone marrow specimen unacceptable for analysis.

Fresh specimens for FCM processing and analysis fall into two broad categories: liquid samples (peripheral blood, bone marrow, body fluids) and solid tissue (lymph nodes, tonsils/adenoids, spleen, bone marrow biopsies, and extranodal infiltrates). The size and cellularity of the sample, as well as the viability of the cells therein, are the main factors determining the final cell yield available for FCM study. Lower cell yield limits the number of markers that