

Pan B-Cell Markers Are Not Redundant in Analysis of Chronic Lymphocytic Leukemia (CLL)

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Background: The classic immunophenotype for chronic lymphocytic leukemia (CLL) is CD19⁺, restricted dim surface expression of kappa or lambda light chain, CD5⁺, CD23⁺, dim CD20⁺, negative FMC7, and negative CD79b. However, the necessity of assaying for all 3 pan B-cell markers (CD20, FMC7, and CD79b) by flow cytometry has not been definitively documented for CLL.

Methods: Qualitative patterns and semi-quantitative assessment of staining intensity for CD20, FMC7 and CD79b were performed in 70 cases with a current or prior diagnosis of CLL or CLL with increased prolymphocytes leukemia (CLL/PL). The concurrent morphology in 66 of 70 specimens was classified as typical CLL in 53 cases, CLL/PL in 10 cases, and large cell lymphoma in 3 cases.

Results: Forty percent of the cases varied from the characteristic immunophenotype by having moderate or bright staining of CD20 (36%), FMC7 (7%), and/or CD79b (18%). Discrepant qualitative staining patterns were found between FMC7 and CD20 (21%), CD20 and CD79b (15%), and CD79b and FMC7 (10%). Semiquantitative measurement of staining intensity showed little correlation between CD79b and CD20 or FMC7. Moderate correlation was seen between CD20 and FMC7. No correlation was observed between morphology and intensity of marker expression.

Conclusions: Variable patterns and intensity of staining were seen for FMC7, CD20, and CD79b in this cohort of CLL samples. Dim or negative staining was most consistently seen for FMC7 (93% of specimens). Although FMC7 staining intensity was moderately correlated with CD20, CD79b intensity was poorly correlated with either CD20 or FMC7, and thus, may provide some independent information. *Cytometry Part B (Clin. Cytometry) 56B:30–42, 2003.* © 2003 Wiley-Liss, Inc.

Key terms: chronic lymphocytic leukemia; immunophenotype; flow cytometry; CD20; FMC7; CD79b

Immunophenotypic analysis is a key adjunctive tool for diagnosis of chronic lymphocytic leukemia (CLL). The characteristic immunophenotype for CLL is a monotypic B cell (CD19⁺) with dim expression of surface immunoglobulin light chain (sIg), dim CD20⁺, CD5⁺, and CD23⁺ (1–3). Dim expression of CD20 is one feature that supports the diagnosis of CLL (4–6). Other pan B-cell markers useful in analysis of B-cell lymphoproliferative disorders include FMC7 and CD79b (1,6,7). FMC7 is directed toward an antigen on certain subgroups of neoplastic and normal B cells that belong to intermediate to late stages of maturation (8–11), but it is usually not expressed in CLL. FMC7 has not been clustered and may represent an epitope on the CD20 antigen (12). Matutes et al. (1) proposed a scoring system based on the expression and staining intensity of five markers (CD5, CD22, CD23, FMC7, and sIg) and reported that the absence of expression of FMC7 is one of the most reliable markers that differentiates CLL from other B-cell neoplasms. Another pan B-cell marker, CD79b, is a transmembrane glycopro-

tein that forms a heterodimer with CD79a and mediates antigen specific signal transduction through a non-covalent association with immunoglobulin (7,13). Most cases of CLL are CD79b negative (7,13). Reportedly, the further addition of CD79b to the panel of five standard markers (CD5, CD22, CD23, FMC7, sIg) improves the accuracy of diagnosing CLL (14). McCarron et al. (7) reports that assessment of the intensity of CD79b expression is more valuable in improving diagnostic accuracy than CD20 when added to a panel that includes CD5, CD19, CD23, and sIg.

In the current environment of cost containment and reimbursement ceilings, laboratories are frequently under

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pressure to reduce the size of immunophenotypic panels. However, the number of markers needed in a panel to optimize diagnostic accuracy for B-cell chronic lymphoproliferative disorders requires careful evaluation. Results from a recent study imply that the intensities of expression of FMC7 and CD20 are not independent and that the inclusion of FMC7 in an immunophenotyping panel adds little information when the intensity of CD20 is already considered (6). Therefore, the purpose of this study is to assess the independence of staining intensity of CD20, FMC7, and CD79b in cases of CLL. Both qualitative and semiquantitative assessments of staining intensity are performed. In addition, the relationships between the variations in staining intensity of these pan B-cell markers and the morphologic categories of typical CLL, CLL with prolymphocytic leukemia (CLL/PL), and transformation to large cell lymphoma are investigated.

MATERIAL AND METHODS

Patient Samples

Eighty-seven specimens from patients with CLL were identified from the files of the Flow Cytometry Laboratory of Northwestern Memorial Hospital between 1998 and 2000. Initially, cases were identified based on the presence of a clonal light chain restricted B-cell population that was CD19⁺, CD5⁺, and CD23⁺. Cases were excluded if subsequent review of morphology revealed findings that were inconsistent with the diagnosis of CLL, CLL/PL, or transformation to large cell lymphoma with a previous or concurrent diagnosis of CLL (9 specimens). Cases were also excluded if material for morphologic review was not available (4 specimens) or if quality of morphologic material was inadequate for evaluation (4 specimens). Six of the patients had 2 samples from different times evaluated. The 70 specimens from 64 patients included peripheral blood (37 specimens), bone marrow aspirates (25 specimens), lymph node tissue (7 specimens), and pleural fluid (1 specimen). Thirty-three of the specimens were from initial diagnosis and the patients were untreated. Thirty-seven specimens were from patients with a prior diagnosis of CLL with or without various forms of treatment.

Morphologic Evaluation

Review of morphology concurrent with the immunophenotypic analysis by flow cytometry was done in all cases by 2 pathologists (L.C.P. and S.A.M.). For confirmation of the diagnosis of current or prior CLL or CLL/PL, all additional material available throughout the course of disease for each patient was also evaluated morphologically. Peripheral blood smears, bone marrow aspirate smears and the pleural fluid were stained with Wright-Giemsa. Peripheral blood smears were prepared from ethylenediamine-tetraacetic acid anti-coagulated blood. Sections of paraffin embedded, B5-fixed bone marrow biopsies and B5-fixed or formalin fixed lymph node biopsies were stained with hematoxylin and eosin.

The guidelines of Bennett et al.(15) were used to categorize cases as typical CLL or CLL/PL based on review of peripheral blood smears and/or bone marrow aspirates. In

summary, typical CLL was characterized by small cells with scant cytoplasm, round nuclei, condensed chromatin and inconspicuous nucleoli. Cases with rare cells having small indentations of the nuclear contour and occasional larger cells comprising <10% of total lymphocytes were designated as typical CLL. Cases with prolymphocytes comprising >10% and <55% of the lymphocytes were designated as CLL/PL. A distinction was not made between cases designated as CLL/PL with a stable number of prolymphocytes and cases with temporally increasing numbers of prolymphocytes. Cases were categorized as transformation to large cell lymphoma if the large cell was present in the sample that was immunophenotyped, regardless of the morphology of any other concurrent peripheral blood smear or bone marrow aspirate.

Immunophenotyping by Flow Cytometry

Peripheral blood and bone marrow aspirates were collected in heparin tubes and lymph node specimens were transported in normal saline solution. Three-color immunophenotyping was performed using combinations of antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanin 5.1 (PC5). Specimens were stained and analyzed within 24 hours of collection.

Reagents. Direct immunofluorescence staining was performed with the following monoclonal antibodies: CD19 (J4.119, Beckman-Coulter, Miami, FL) IgG1 conjugated with PC5, CD20 (B9E9/HRC20, Beckman-Coulter) IgG2a conjugated with FITC, CD5 (SFC124T6G12, Beckman-Coulter), IgG2a conjugated with PE, FMC7 (FMC7, Beckman-Coulter) IgM conjugated with FITC, CD79b (B3-1, Beckman-Coulter) IgG1 conjugated with PE, CD23 (HD50, Beckman-Coulter) IgG2b conjugated with PE, CD11c (KB90, Dako, Carpinteria, CA) IgG1 conjugated with FITC, and CD19/lambda/kappa (Dako) IgG1/IgG1/IgG1 conjugated with PC5/PE/FITC, respectively. In some cases, additional antibody clones may have been used and include the following: CD5 (BL1a, Beckman-Coulter) IgG2a, CD19/lambda/kappa (Beckman-Coulter), IgG1/F(ab')₂/F(ab')₂, kappa (polyclonal, Beckman-Coulter,) IgG, and lambda (polyclonal, Beckman-Coulter) IgG. During the course of this study, 5 different lot numbers of CD20, 6 of CD79b, and 7 different lots of FMC7 antibodies were used. All different lot numbers of antibody were tested on a common sample before being used comparing geometric mean fluorescence intensity of the CD19⁺ and CD19⁻ cells for each antibody as well as the signal to noise (S/N) as described below and the percentage of positive cells.

Sample preparation. Fresh lymph node specimens were washed twice in phosphate buffered saline (PBS) containing 0.2% sodium azide. The peripheral blood or bone marrow specimens were prepared by lysis of 100 µl of sample using a modified protocol of the red blood cell lysis technique, QPrep (Beckman-Coulter). The modification involves adding lysis reagent, followed by stabilizing reagent and washing twice in PBS containing 0.2% sodium azide. The cells were then resuspended in PBS with 30%

newborn bovine calf serum (BRL Life Sciences, Gaithersburg, MD) and stained with appropriate antibody panels. The B-lymphocyte panel included the following antibody combinations: CD19/CD23/FMC7, CD19/CD5/CD20, CD19/CD79b/CD11c and CD19/lambda/kappa. After the addition of the appropriate antibodies, the cells were incubated for 30 min at room temperature. Following incubation, the cells were centrifuged, the supernatant aspirated and resuspended in PBS/0.5% paraformaldehyde.

Data collection and analyses. Analysis was performed on a Coulter XL/MCL flow cytometer (Beckman-Coulter). Instrument alignment was confirmed, and graphed, daily using an alignment control bead (Flow-Check, Beckman-Coulter). Instrument linearity was confirmed using multiple fluorescence level calibration beads (Immuno-Brite, Beckman-Coulter). Instrument sensitivity was confirmed daily using a fluorescence standard bead (Flow-Set, Beckman-Coulter). Sensitivity was maintained within 1% (median bead fluorescence channel number kept at $\pm 1\%$ of target value). If a high voltage change was needed to maintain the target bead channel number then any change was transferred to all analysis protocols in order to maintain approximately the same instrument sensitivity throughout the course of the study. Initial instrument set-up (high voltage, target channels, etc.) was established based on both positive and negative cells, keeping both positive and negative populations on scale. This established the high voltage, gain settings, and target ranges for the fluorescence standard beads. This target range was then maintained for the entire study period. Further, relative staining intensity as defined below was monitored for all antigens in normal control peripheral blood specimens for both the negative and positive staining populations throughout the study. Instrument compensation was established using a CD19-PC5/CD4-PE/CD8-FITC stained normal control specimen and gating on the lymphoid population (positive populations with mutually exclusive staining in normal peripheral blood lymphocytes). Proper compensation was confirmed by ensuring that the geometric mean fluorescence level for both the positive and negative populations in each fluorochrome were the same in the other fluorochrome detection regions (i.e., the FITC fluorescence level in the CD19⁺ cells (PC5⁺ population) is the same as for the CD19⁻ cells (PC5⁻ population), PE fluorescence level in the CD19⁺ cells is the same as for the CD19⁻ cells, etc.) (16). For each specimen, 2,500 or more events (range: 2,500–59,000) were collected within the forward and side scattered light intensity region of lymphocytes to slightly greater than lymphocytes. In all cases, a CD19⁺ B-cell selected region was analyzed with a minimum of 2,500 events (range: 2,500–58,000). CD19⁺ lymphocytes were further analyzed for staining intensity of CD20, FMC7, and CD79b. In each specimen, the geometric mean fluorescent intensity for CD20, FMC7, and CD79b of the CD19⁺ cells was compared as a ratio with the geometric mean fluorescent intensity for the CD19-negative cells in the same sample contained within the same selected scat-

tered light region. This was considered a semiquantitative measure of fluorescence intensity in that no fluorescence equivalent calibration curves were used. The reproducibility of this relative, semi-quantitative, fluorescence intensity measure was assessed in two ways. One, determination of the fold increase in staining intensity (S/N) for CD20, CD79b, or FMC7 in the CD19⁺ cells as compared to the CD19⁻ cells was determined on five replicate aliquots (stained and measured independently) for three separate, independent samples. The coefficient of variation of these replicate measurements in each independent sample ranged from 3% to 5%. Further, this same S/N measure of staining intensity for each of the assessed pan B-cell antigens was also monitored in 100 independent, normal donor (control) specimens analyzed through out the course of this study. The coefficient of variation of the S/N measurements for CD20, FMC7, and CD79b ranged from 35% to 40% for these 100 different normal donors over the study period. Within the study group, differences of less than 2-fold were not considered significant.

In addition, a qualitative assessment of fluorescent staining intensity for each relevant antigen in the CD19 antibody combinations was graded by visual inspection as negative, dim, moderate or bright based on comparison and degree of overlap in the staining for each relevant antigen in the CD19⁺ and CD19⁻ population clusters. Negative antibody staining for a relevant antigen in the CD19⁺ cellular population was defined as demonstration of approximately equivalent fluorescent staining as compared to the same antigen in the CD19⁻ cellular population from the same gated region (generally, less than 2-fold difference in geometric mean intensity of the relevant antigen staining in the CD19⁺ cells compared to the geometric mean for the same antigen in the negative population (non-B lymphocytes) within the same tube). Dim antibody staining was defined as greater intensity of the relevant antigen in the CD19⁺ cells than in the CD19⁻ population, but with significant overlap of the intensity distribution for the 2 populations (generally representing a 2- to 5-fold difference in geometric mean intensity). Moderate antibody staining was defined to be only a slight overlap of the intensity distribution for the relevant antigen staining in the CD19⁺ cells and the CD19⁻ population (generally representing a 5- to 10-fold difference in geometric mean intensity between the 2 populations). Bright positive antibody staining for a relevant antigen in the CD19⁺ cells showed minimal or no overlap in the distribution of intensities for the relevant antigen in the CD19⁺ and CD19⁻ cells and represented at least a >10-fold increase in geometric mean intensity of the relevant antigen staining in the CD19⁺ cells as compared to the geometric mean intensity of the CD19⁻ within the same tube. Because of the broad range of staining intensities within any one qualitative intensity category (negative, dim, moderate, and bright) and the inherent subjective assessment of these categories, a difference in staining intensity was only considered significant if the difference was separated by more than one grade (i.e., negative versus moderate, dim versus bright, or negative versus bright). Thus, nega-

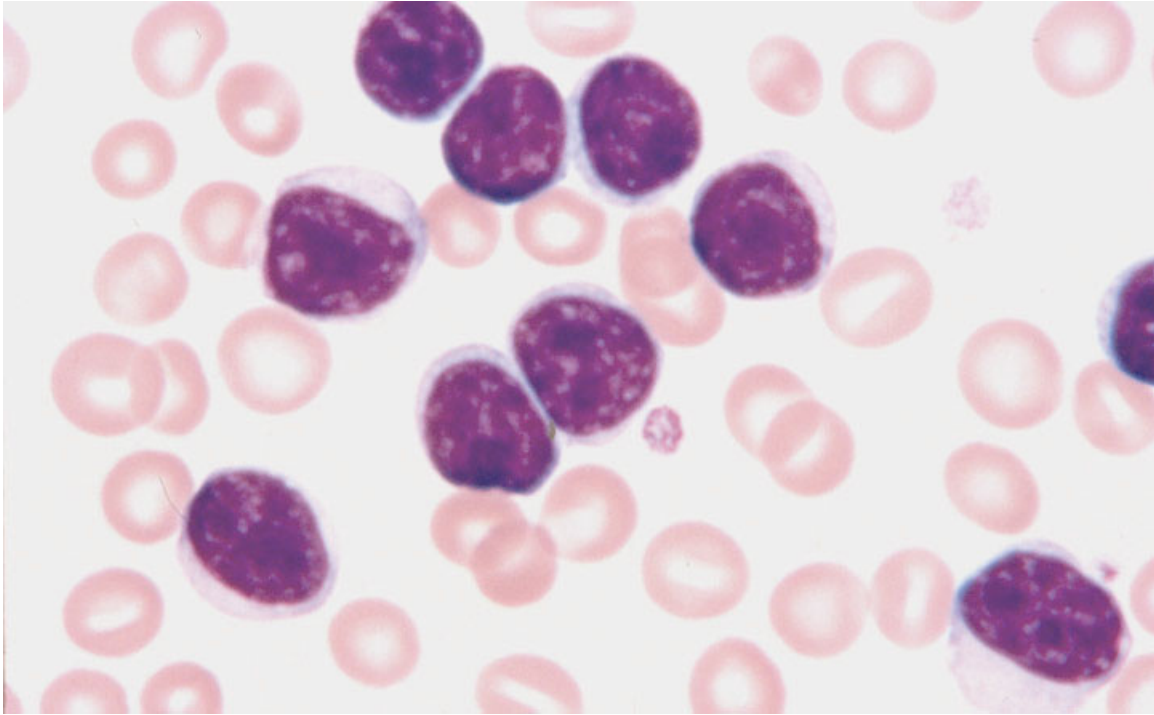


FIG. 1 Most of the lymphocytes in this peripheral blood film are small with scant cytoplasm and condensed chromatin in this case classified morphologically as typical CLL. Immunophenotyping of a concurrent bone marrow aspirate in this case showed a $CD5^+$, $CD23^+$ monocytic B-cell population with moderate staining intensity of CD20 and CD79b. FMC7 was dimly expressed (Wright-Giemsa, 1,000 \times). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tive and dim staining was considered equivalent and typical for CD20, FMC7, and CD79b in CLL.

Statistics

Comparison of the semi-quantitative relative geometric mean fluorescence intensities for each antigen (geometric mean fluorescence intensity of the relevant antigen in the $CD19^+$ cells minus the geometric mean fluorescence intensity in the $CD19^-$ cells in the same tube from the same scattered light selected region) was done using standard linear least square analysis.

RESULTS

Morphologic Evaluation

Morphologic categorization as typical CLL, CLL/PL, or transformation to large cell lymphoma was determined in 66 cases. Morphologic categorization in 65 cases was based on review of the concurrent peripheral blood smears and/or bone marrow biopsy. Two lymph node biopsies and a pleural fluid sample without transformation to large cell lymphoma did not have a concurrent peripheral blood smear or bone marrow aspirate, precluding classification as either typical CLL or CLL/PL, and were excluded from the comparison between morphology and immunophenotype. One peripheral blood smear had too few lymphocytes for morphologic categorization, although immunophenotyping of the peripheral blood and morphologic review of prior periph-

eral blood smears with absolute lymphocytosis confirmed the diagnosis of CLL.

Fifty-three cases were classified as typical CLL, 10 cases as CLL/PL, and 3 cases as transformation to large cell lymphoma. Predominantly small, mature-appearing lymphocytes were found in 32 cases that had immunophenotyping performed on peripheral blood and 18 cases with immunophenotyping performed on bone marrow aspirates (Fig. 1). Three additional cases with typical morphology for CLL in the peripheral blood had immunophenotyping performed on lymph node biopsies. The morphology of the lymph node biopsies in these three cases was in concordance with the peripheral blood findings and showed predominantly small lymphocytes with occasional proliferation centers.

Ten cases had increased circulating prolymphocytes (>10% to <55% of lymphocytes) in the peripheral blood and were categorized as CLL/PL (Fig. 2). Samples that were immunophenotyped in these cases included 3 peripheral blood samples, 6 bone marrow aspirates and 1 lymph node biopsy. Sections of the lymph node in the latter case showed sheets of prolymphocytes.

Three cases from 2 patients were categorized as transformation to large cell lymphoma. In one patient, numerous large, immunoblast-like lymphocytes were in the peripheral blood and bone marrow. The other patient had immunophenotyping of a lymph node biopsy.

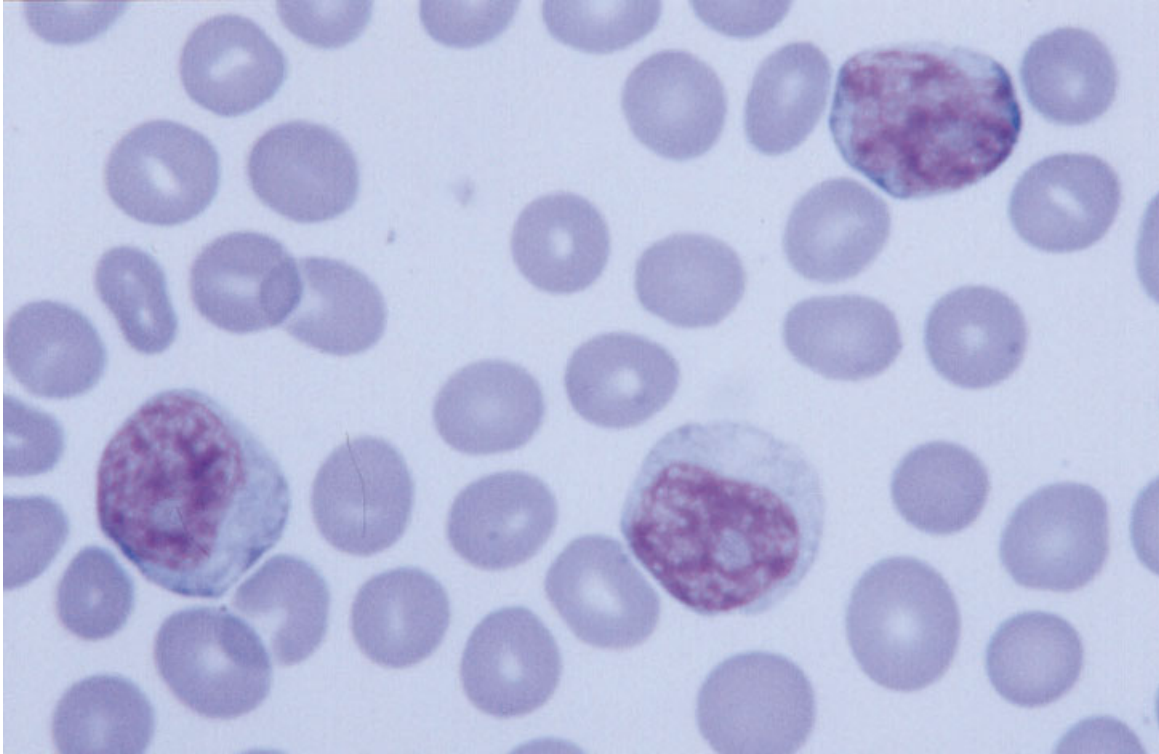


FIG. 2 Larger lymphocytes with prominent nucleoli comprise 10–55% of the lymphocytes in the peripheral blood in this case of CLL/PL. Flow cytometry of the peripheral blood demonstrated a characteristic immunophenotype for CLL with a $CD5^+$, $CD23^+$ monotypic B-cell population showing dim staining intensity of $CD20$ and $CD79b$. The lymphocytes lacked expression of $FMC7$ (Wright-Giemsa, 1,000 \times). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

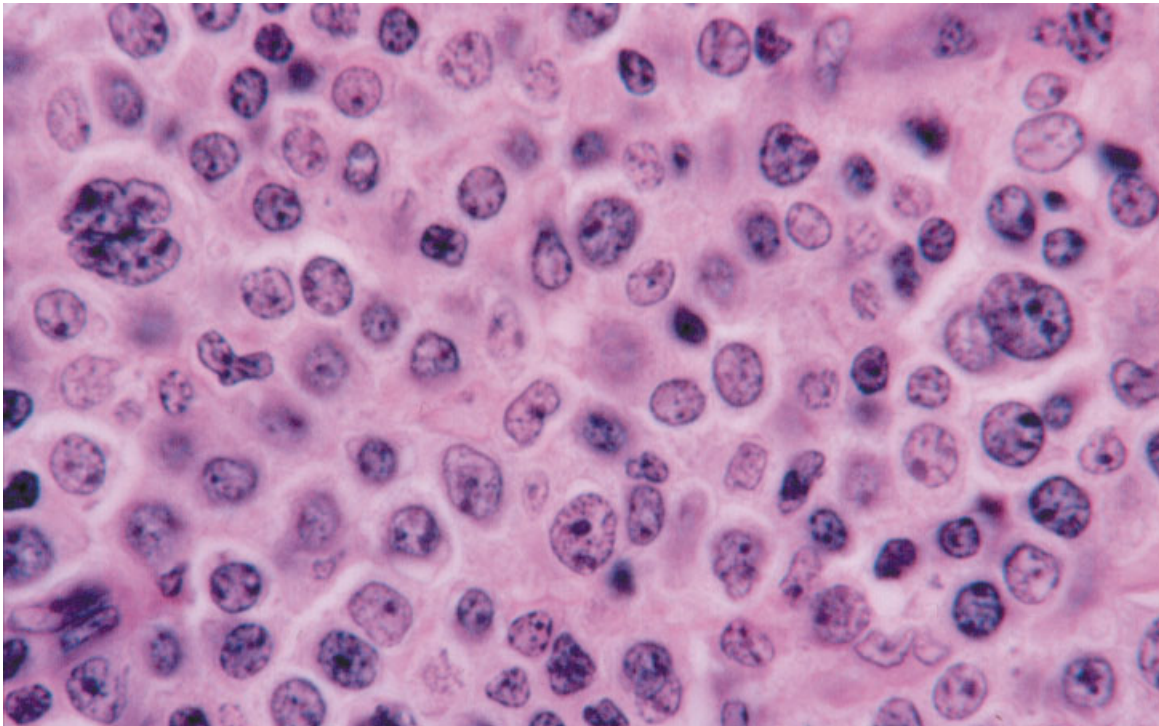


FIG. 3 This lymph node biopsy shows effacement by large cell lymphoma. The lymphocytes have a moderate amount of basophilic cytoplasm, vesicular chromatin and prominent nucleoli while some cells are multinucleated. The pan B-cell markers had characteristic staining intensity for CLL with dim $CD20$ and $CD79b$ expression. $FMC7$ was negative (hematoxylin and eosin stain, 400 \times). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Morphologic review of the lymph node revealed bizarre, pleomorphic lymphoma cells with some multinucleation (Fig. 3).

Immunophenotyping by Flow Cytometry

Seventy samples from 64 patients were immunophenotyped by flow cytometry. All cases demonstrated a population of B lymphocytes (CD19⁺) with kappa or lambda light chain restriction and coexpression of CD5 and CD23. Forty percent (28/70) of the cases varied from the characteristic immunophenotype by having moderate or bright expression of CD20, CD79b, and/or FMC7 (Table 1). Moderate or bright expression was found for CD20 in 36% (25/70), for CD79b in 18% (11/60), and for FMC7 in 7% (5/70) of the samples. In only 7% (4/60) of specimens was moderate or bright staining for all 3 B-cell antigens seen in the same specimen. Representative examples of dim, moderate and bright staining for these three markers are shown in Figure 4. No correlation was found between the qualitative staining intensity of CD20, FMC7 and CD79b and the morphologic classification as typical CLL or CLL/PL since 38% (20/53) and 40% (4/10) of these cases, respectively, showed moderate or bright staining of one, or more, of the pan B-cell markers studied. Similarly, no correlation was found between these morphologic categories and staining intensity of the B cell markers individually. The small number of cases of transformation to large cell lymphoma precluded meaningful evaluation of the relationship of this morphologic category to intensity of expression of the pan B-cell markers studied.

The frequency was determined with which a particular pan B-cell marker offered support for a diagnosis of CLL when another pan B-cell marker expressed uncharacteristic moderate or bright staining intensity. Thus, the percentages of cases with negative or dim expression of the other two pan B-cell markers was calculated when moderate or bright staining was seen in one marker. When FMC7 was moderate or bright in 5 cases, CD20 was never negative or dim and CD79b was negative or dim in 1 of the 5 cases (20%). Of the cases that had moderate or bright expression of CD20, FMC7 was negative or dim in 20 of 25 cases (80%), whereas CD79b was negative or dim in 13 of 21 cases (62%). Negative or dim staining of either FMC7 or CD79b was seen in 17 of 21 cases (81%) with moderate or bright CD20 staining. Therefore, in cases with moderate or bright CD20 staining intensity, assessment of staining intensity of FMC7 alone offers the best support for the diagnosis of CLL. Assessment of staining intensity of both FMC7 and CD79b in these cases did not substantially add further support of a diagnosis of CLL than did assessment of FMC7 alone. When CD79b was uncharacteristically moderate or bright, FMC7 was negative or dim in 7 of 11 cases (64%) and CD20 was negative or dim in only 3 of the 11 cases (27%). Again, use of both FMC7 and CD20 did not add further support for CLL than did FMC7 alone in these qualitative assessments of staining intensity. In this context, it should be noted that FMC7 demonstrated a typical pattern of dim or negative staining intensity in 93% of the specimens. Performing similar comparisons within mor-

phologic subtypes was precluded due to the small number of total cases with moderate or bright staining within the subset designated as CLL/PL (4 cases) and as transformation to large cell lymphoma (2 cases).

Qualitative differences in intensity of expression separated by more than 1 grade (i.e., dim versus bright, negative versus moderate, or negative versus bright) was found for 21% (15/70) of the comparisons between CD20 and FMC7. Similar discrepant staining intensity was found in 15% (9/60) of the comparisons between CD20 and CD79b and 10% (6/60) between FMC7 and CD79b. Linear least squares comparisons of geometric mean fluorescence intensities demonstrated a moderate degree of correlation for staining intensity between CD20 and FMC7 ($r^2 = 0.5$) and limited or no correlation between CD20 and CD79b ($r^2 \leq 0.1$) and between FMC7 and CD79b ($r^2 \leq 0.1$; Fig. 5).

Comparison of Cases With Two Samples From the Same Patient

Six of the patients had 2 different samples evaluated by flow cytometry. It was possible to morphologically categorize both samples in five patients. The morphologic classification remained the same in all of the 5 patients when comparing the 2 different samples. Three patients had typical morphology for CLL, 1 had CLL/PL, and 1 had transformation to large cell lymphoma. A qualitative difference in intensity of expression separated by more than 1 grade was found in 2 patients when comparing the 2 samples. One of these cases was morphologically categorized as typical CLL. Initially, a bone marrow sample showed clonal kappa light chain restricted B cells that were CD79b⁻, but a peripheral blood sample two months later showed kappa light chain restricted B cells with moderate expression of CD79b. In that case, CD20 was initially dim with moderate intensity detected in the later sample while FMC7 remained negative. The second case showed a transformation to large cell lymphoma in bone marrow and peripheral blood. The clonal lambda light chain restricted CD19⁺, CD5⁺, CD23⁺ cells were moderate CD20⁺, dim FMC7⁺, and CD79b⁻ in the bone marrow aspirate. An essentially concurrent peripheral blood sample tested 4 days later showed lambda light chain restricted B-cells that were bright CD20⁺, bright FMC7⁺, and bright CD79b⁺.

DISCUSSION

A substantial percentage (40%) of cases of CLL in this study demonstrated moderate or bright staining of one or more of the 3 pan B-cell markers, CD20, CD79b, and FMC7. Moderate or bright staining for CD20 was so frequently seen, including in 36% of CLL cases with typical morphology, that in the classic paradigm of dim CD20 staining being associated with CLL, one could question the relevance of using CD20 for the immunophenotypic evaluation of a peripheral blood lymphocytosis. However, it should be noted that in light of the moderate correlation in staining intensity between CD20 and FMC7, which only rarely was not dim or negative, the frequent "atypical" staining intensity for CD20 may predominantly reflect a

Table 1
Morphologic Category and Intensity of Expression of Pan B-Cell Markers
by Flow Cytometric Analysis in CLL

Patient	Specimen ^a	Morphologic classification	Intensity of expression by flow cytometric analysis		
			CD20 (n = 70)	FMC7 (n = 70)	CD79b (n = 60)
1	PB	Typical	Moderate (9.5)	Negative (1.8)	Not done
2	PB	Typical	Dim (2.3)	Negative (1.4)	Negative (1.9)
3	PB	Typical	Dim (4.8)	Negative (1.9)	Negative (1.5)
4	PB	Typical	Bright (20.6)	Moderate (10.0)	Dim (3.0)
5	PB	Typical	Moderate (7.6)	Negative (1.4)	Dim (4.4)
6	PB	Typical	Dim (2.5)	Negative (1.9)	Dim (3.1)
7	PB	Typical	Dim (3.3)	Negative (1.4)	Dim (4.7)
8	PB	Typical	Dim (4.9)	Negative (1.7)	Negative (1.9)
9	PB	Typical	Moderate (7.7)	Negative (1.5)	Moderate (9.9)
10	PB	Typical	Dim (3.8)	Negative (1.8)	Negative (1.7)
11	PB	Typical	Dim (2.9)	Negative (1.1)	Dim (2.1)
12	PB	Typical	Moderate (8.7)	Dim (3.0)	Dim (2.4)
13	PB	Typical	Negative (1.9)	Negative (1.2)	Dim (3.6)
14	PB	Typical	Dim (4.5)	Negative (1.9)	Dim (1.3)
15	PB	Typical	Dim (4.9)	Dim (2.4)	Negative (1.1)
16	PB	Typical	Dim (4.6)	Negative (1.9)	Negative (1.7)
17	PB	Typical	Dim (2.9)	Negative (1.4)	Negative (1.9)
18	PB	Typical	Dim (4.4)	Negative (1.8)	Dim (3.7)
19	PB	Typical	Dim (4.2)	Negative (1.8)	Dim (3.7)
20	PB	Typical	Moderate (10.0)	Moderate (9.8)	Moderate (9.4)
21	PB	Typical	Dim (3.9)	Negative (1.2)	Dim (3.5)
22	PB	Typical	Moderate (6.2)	Negative (1.9)	Dim (3.3)
23	PB	Typical	Dim (4.3)	Negative (1.4)	Dim (2.3)
24	PB	Typical	Moderate (7.5)	Negative (1.7)	Negative (1.4)
25	PB	Typical	Moderate (11.2)	Negative (1.4)	Moderate (7.8)
26	PB	Typical	Dim (4.6)	Negative (1.2)	Dim (2.4)
27	PB	Typical	Moderate (8.9)	Negative (1.2)	Dim (4.9)
28	PB	Typical	Dim (4.5)	Negative (1.0)	Negative (1.5)
29	PB	Typical	Negative (1.9)	Negative (1.0)	Dim (2.8)
30	BM	Typical	Dim (2.7)	Negative (1.9)	Not done
31	BM	Typical	Moderate (9.9)	Negative (1.9)	Not done
32	BM	Typical	Dim (4.8)	Negative (1.4)	Negative (1.4)
33	BM	Typical	Moderate (8.5)	Dim (3.2)	Moderate (10.0)
34	BM	Typical	Dim (3.4)	Negative (1.0)	Negative (1.7)
35	BM	Typical	Dim (2.5)	Negative (1.5)	Negative (1.9)
36	BM	Typical	Moderate (7.8)	Negative (1.8)	Dim (5.0)
37	BM	Typical	Moderate (7.3)	Negative (1.6)	Negative (1.9)
38	BM	Typical	Bright (23.9)	Bright (13.9)	Moderate (8.4)
39	BM	Typical	Negative (1.3)	Negative (1.2)	Negative (1.4)
40	BM	Typical	Negative (1.4)	Negative (1.1)	Not done
41	BM	Typical	Moderate (8.1)	Dim (3.6)	Negative (1.7)
42	BM	Typical	Dim (2.7)	Negative (1.4)	Dim (2.7)
43	BM	Typical	Dim (3.3)	Negative (1.8)	Dim (2.5)
44	LN	Typical	Dim (4.0)	Negative (1.3)	Negative (1.9)
45	LN	Typical	Dim (3.9)	Negative (1.9)	Moderate (9.3)
46	LN	Typical	Dim (4.9)	Negative (1.7)	Dim (4.9)
47	PB	Typical	Moderate (7.5)	Negative (1.9)	Not done
	PF	Not categorized	Moderate (9.6)	Dim (3.0)	Not done
48	PB	Typical	Dim (4.5)	Dim (3.5)	Dim (2.9)
	BM	Typical	Negative (1.3)	Negative (1.4)	Not done
49	BM	Typical	Dim (4.5)	Negative (1.2)	Dim (3.6)
	PB	Typical	Moderate (6.3)	Negative (1.5)	Moderate (6.1)
50	BM	Typical	Dim (3.1)	Negative (1.6)	Negative (1.2)
	BM	Typical	Moderate (7.8)	Negative (1.1)	Negative (1.9)
51	PB	CLL/PL	Dim (3.6)	Negative (1.0)	Dim (2.9)
52	PB	CLL/PL	Dim (2.1)	Negative (1.3)	Not done
53	PB	CLL/PL	Moderate (8.3)	Negative (1.2)	Negative (1.6)
54	BM	CLL/PL	Dim (3.8)	Negative (1.1)	Not done
55	BM	CLL/PL	Negative (1.1)	Negative (1.3)	Negative (1.9)
56	BM	CLL/PL	Dim (4.0)	Negative (1.5)	Moderate (8.1)
57	BM	CLL/PL	Dim (4.2)	Negative (1.4)	Dim (4.5)
58	BM	CLL/PL	Bright (37.2)	Bright (54.2)	Moderate (6.7)
59	BM	CLL/PL	Dim (3.7)	Negative (1.4)	Not done
	LN	CLL/PL	Moderate (8.7)	Negative (1.7)	Negative (1.1)
60	LN	LCL	Dim (3.7)	Negative (1.4)	Dim (2.3)
61	BM	LCL	Moderate (6.6)	Dim (2.3)	Negative (1.3)
	PB	LCL	Bright (36.3)	Bright (49.3)	Bright (22.6)
62	PB	Not categorized	Negative (1.0)	Negative (1.2)	Negative (1.2)
63	LN	Not categorized	Dim (3.7)	Negative (1.9)	Negative (1.0)
64	LN	Not categorized	Negative (1.9)	Negative (1.1)	Moderate (6.4)

^aBM, bone marrow aspirate; CLL, chronic lymphocytic leukemia; LCL, large cell lymphoma; LN, lymphnode; PB, peripheral blood; PF, pleural fluid; PL, prolymphocytic leukemia.

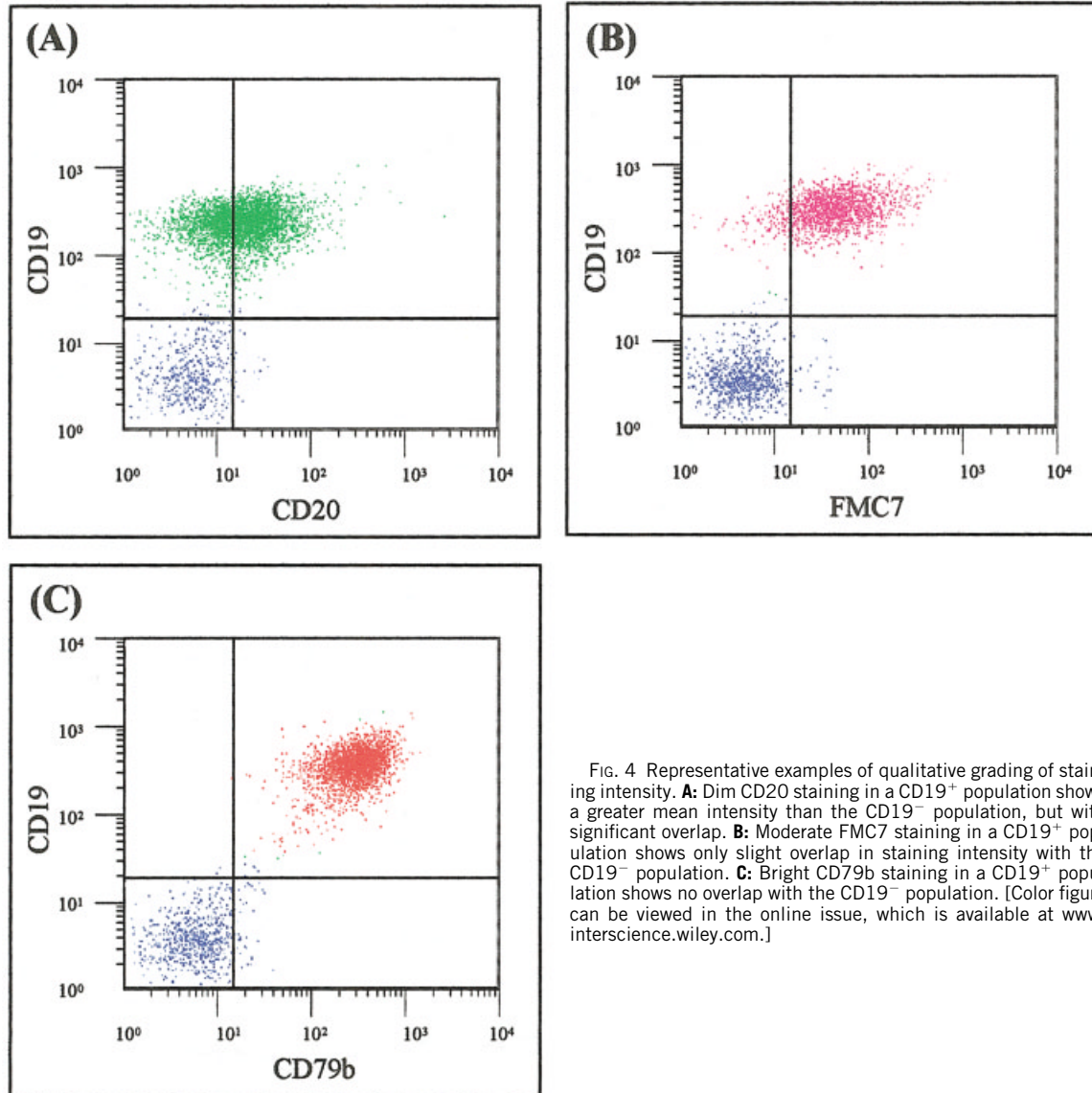


FIG. 4 Representative examples of qualitative grading of staining intensity. **A:** Dim CD20 staining in a CD19⁺ population shows a greater mean intensity than the CD19⁻ population, but with significant overlap. **B:** Moderate FMC7 staining in a CD19⁺ population shows only slight overlap in staining intensity with the CD19⁻ population. **C:** Bright CD79b staining in a CD19⁺ population shows no overlap with the CD19⁻ population. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shift in relative staining intensity of CD20 versus FMC7. Moderate or bright staining of CD79b and FMC7 was also seen in occasional to rare cases of CLL, 18% and 7%, respectively. Appreciation of these phenomena is relevant because the distinction between CLL and mantle cell lymphoma (MCL), an often problematic differential diagnosis, has important consequences. Not only can MCL be difficult to distinguish from CLL morphologically, but the neoplastic cells similarly express CD5. However, in contrast to CLL, MCL is usually CD23⁻, FMC7⁺, and CD79b⁺ (1,7,17,18), although dim CD23⁺ staining may be seen (18). MCL is more often aggressive than CLL and patients may benefit from earlier and different therapeutic approaches (19–22). In morphologically difficult cases of CD19⁺CD5⁺ B cell lymphomas, particularly if dim CD23⁺ coexpression is seen, the findings in this study would indicate that the diagnosis of CLL may not be precluded

based on moderate or bright staining for FMC7 or CD79b. In these difficult cases, fluorescence in situ hybridization or cytogenetics for the t(11:14) or immunohistochemistry for cyclin D1 would likely be more effective in clarifying CLL versus MCL (23–26). In addition, other B-cell lymphomas that may rarely express CD5 [marginal zone lymphoma (27–29), lymphoplasmacytic lymphoma (1,30–33), de novo large B-cell lymphomas (34–42) and de novo polymphocytic leukemia (43,44)] should also be considered in the setting of a CD5⁺ B-cell lymphoma with unusual immunophenotypic features.

Appreciation of the immunophenotypic heterogeneity of CLL may also be important for identifying and understanding the variable entities that likely exist within the spectrum of CLL. The most recent classification of hematopoietic and lymphoid neoplasms (45) emphasizes the evolving understanding that neoplasms with distinct clin-

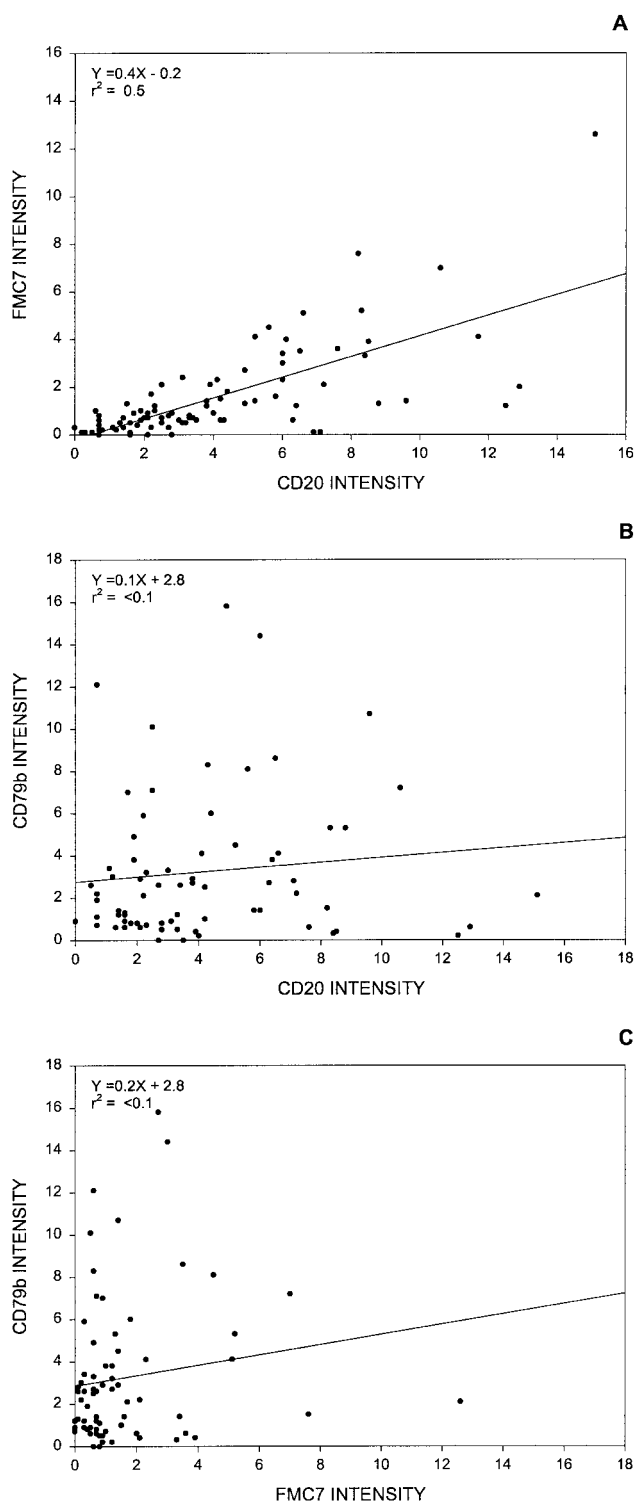


FIG. 5 **A**: Linear least squares comparison of geometric mean fluorescence intensities shows limited correlation for staining intensity between CD20 and FMC7. Even less correlation is demonstrated between CD20 and CD79b (**B**) and between FMC7 and CD79b (**C**).

ical and pathologic features are defined by unique genetic abnormalities. In CLL, various cytogenetic aberrancies have long been recognized (46–49). Continued advances in genetic analysis have recently shown that the most common lesions in CLL are del11q and del13q14 (50,51). Furthermore, molecular analysis has shown that the status of immunoglobulin heavy chain gene rearrangement and somatic hypermutation is variable in CLL and carries independent prognostic information (52–54). However, definitive relationships have not been completely determined between morphology, immunophenotype, clinical behavior and genetic abnormalities in CLL. Elucidation of these relationships requires further appreciation of subtle variations including immunophenotypic variations among cases of CLL. Further understanding of the heterogeneity of CLL may also lead to development of more targeted therapeutic approaches. The results of this study show that staining for the pan B-cell markers studied, particularly CD79b and FMC7, were not redundant. In the context of studying heterogeneity within CLL, assessment of both of these markers may be useful.

Compared to the two other pan B-cell markers studied, FMC7 most often showed characteristic negative or dim expression which was seen in 93% of the CLL specimens in this series. Negative or dim expression of FMC7 has been shown to be characteristic for CLL by other investigators. In one study, absence of FMC7 expression and weak expression of slg were the most reliable markers in discriminating CLL from other B-cell malignancies, including MCL (1). In that study, markers commonly positive in CLL (CD5, CD23, and CD22) were expressed in a substantial number of other B-cell neoplasms, whereas lack of FMC7 expression was much less frequent in other B-cell neoplasms. In another study (55), significant differences in the intensity of FMC7 were demonstrated between CLL and MCL, but these differences could not be demonstrated with CD20. Our findings are also in concordance with a study that showed CD79b is more consistently negative or dim than CD20 in CLL (7). In addition, quantitative studies found that CD79b was bound to less antigen binding sites on CLL cells than those of MCL (17). This latter finding had greater statistical significance than the difference seen in CD20 antigen binding sites per cell between CLL and MCL (17).

In the context of the expected dim or negative staining for each of the pan B-cell markers studied, these findings show that if FMC7 had been the only marker used, 5 of 70 cases would have had an indeterminate, or “atypical,” immunophenotype for CLL. It should be noted, however, that all cases studied were CD19⁺, CD5⁺, and CD23⁺. Further, in all 5 of these cases, assessing the intensity of CD20 expression did not lend further support for a diagnosis of CLL because CD20 staining intensity was also either moderate or bright. Assessment of CD79b staining would have been helpful in 1 of the 5 cases where it showed dim staining intensity. Conversely, when CD79b was “atypical” (moderate or bright), CD20 was also not helpful, having moderate or bright staining intensity in all 11 cases. However, FMC7 was “typical” in 6 of these 11

cases. Thus, using antibody panels such as FMC7/CD19/CD23, FMC7/CD19/CD5, or FMC7/CD19/CD5/CD23 without using CD20 may be justified and help reduce the cost of immunophenotypic analysis. Whether addition of CD79b is warranted, or cost effective, is not as clear because little correlation between CD79b and FMC7 staining intensity was seen, and in 1 of the 5 cases demonstrating moderate or bright FMC7 staining, CD79b maintained a typical dim expression pattern. Study of a larger number of cases of CLL with moderate or bright expression of FMC7 is likely to be required to make a more conclusive statement regarding the value of adding CD79b in the analysis of CLL. However, at present, these data would point to some benefit from the addition of CD79b. These conclusions contradict those of Hubl et al. (6) who emphasized the value of assessing CD20 staining intensity for mature B-cell neoplasms and that the use of FMC7 added little information in the evaluation of CLL. Our study would support, at least in the context of the paradigm of dim or negative staining for these markers in B-CLL, that FMC7 is a better correlate than CD20. However, considering the relative degree of correlation in staining intensity for these two markers, and that FMC7 may be an epitope on the CD20 antigen (12), this may more reflect a relatively consistent shift in the staining intensity for these 2 markers, with CD20 being brighter, than an actual discrepancy in results. Quantitative assessment of staining intensity (56-62), and comparison to normal, might better resolve which, if either, is a better choice. Lastly, direct comparison of the results in this study to the scoring systems proposed by Matutes et al. and others (1,14) is not possible because different methods were used in analysis of the data. In contrast to the use of fixed integration regions established based on an isotypic control to calculate a percent positive cells in the Matutes et al. report, in this study, the non-parametric distribution of antigen expression in a specific cell population was examined and is supported by others (63). Nonetheless, this study would support using a similar panel of antibodies as those used in the proposed scoring systems with the exception of the potential redundancy of CD20 and FMC7. However, it should probably be stressed again that in cases of "atypical" pan B-cell antigen staining, particularly when coupled with dim CD23 staining, that the use of other information such as clinical course, cytogenetic studies, fluorescence in situ hybridization for the t(11;14) and/or immunohistochemistry for cyclin D1 may be more effective in ruling in, or out, the diagnosis of MCL.

The highest degree of correlation between the pan B-cell antigens studied in B-CLL, as assessed by linear least squares analysis of the geometric mean fluorescent intensities, was found between CD20 and FMC7. However, this correlation could be considered only moderate ($r^2 \approx 0.5$) when compared to that seen in a small series of other B cell lymphomas ($r^2 \approx 0.86$; data not shown) and it is not surprising that discrepancies between the qualitative assessment (negative versus moderate or bright, and dim versus bright) of these two markers were frequent in B-CLL. Better correlation between intensity of expression

for CD20 and FMC7 might be expected since there is evidence that the FMC7 antigen is an epitope of the surface protein encoded by the CD20 gene (12). As mentioned above, the findings reported here appear to be to some degree in contrast to those of Hubl et al. (6) who concluded that CD20 and FMC7 were highly correlated. However, patients with CLL represented only a subset of the patients in that study. Furthermore, diminished correlation of CD20 and FMC7 was observed in those cases where CD20 was weakly expressed. The authors attributed this latter finding to a possible inability to resolve dim staining of FMC7 from nonspecific staining. Thus, the high degree of correlation between CD20 and FMC7 found in that study (6) may have been driven by the cases with brighter staining of CD20 and FMC7. We also found excellent correlation between CD20 and FMC7 staining intensities in normal B-cells and in other mature B-cell neoplasms as noted above (data not shown). It is possible that the use of additional blocking agents (30% newborn calf serum) to decrease nonspecific binding in this study may have contributed to an increased ability to reproducibly stain and interpret the intensity of expression of CD20 and FMC7 in cases of CLL with dim levels of expression. Thus, even if the FMC7 antigen is an epitope on the CD20 antigen, we believe our data suggest that it may be differentially expressed, or have differential accessibility for detection, in CLL as compared to normal B cells and other lymphoproliferative disorders.

Interestingly, qualitative discrepancies were observed less frequently between CD20 and CD79b and between FMC7 and CD79b than the qualitative differences between CD20 and FMC7. However, the correlation coefficients were even less for CD20 versus CD79b ($r^2 \leq 0.1$) and FMC7 versus CD79b ($r^2 \leq 0.1$) than between CD20 versus FMC7. This difference is partially reflective of the broad range of staining intensities within any one qualitative intensity category (negative, dim, moderate or bright) in that each encompassed approximately 5-fold differences in geometric mean intensity, and further, only changes of two categories or more were considered relevant. Thus, the staining intensity of two markers might have been qualitatively interpreted as concordant, while in fact the geometric mean intensities were very different. In this regard, it is worth noting another study which, although using different criteria for assessment of antigen expression and statistical methods for comparison of data, also found no correlation between expression of CD79b and FMC7 for CLL (13).

Heterogeneity of the intensity of expression of the pan B-cell markers studied within B-CLL was also highlighted by the discrepant staining found in two different samples from two patients. In one of these patients, a bone marrow aspirate and peripheral blood sample were separated by 2 months. In the second patient, a bone marrow and peripheral blood sample were essentially concurrent. In the first patient, it is possible that the variation in intensity of expression of immunophenotypic markers within the same patient could be related to clonal evolution that may occur over time. It is more difficult to explain the differ-

ences found in the second patient with concurrent samples showing transformation to large cell lymphoma in both the bone marrow and peripheral blood. However, demonstration of differential expression of CD20 in CLL according to site of sample has been reported (64) with the highest mean number of CD20 antibodies bound per cell in peripheral blood lymphocytes and lower mean numbers of CD20 antibodies bound per cell found in bone marrow aspirate and lymph node aspirate samples. Interestingly, the patient in our study with concurrent immunophenotyping of peripheral blood and a bone marrow aspirate was found to have increased staining intensity of all 3 pan B-cell markers on peripheral blood lymphocytes as compared to cells of the bone marrow aspirate.

No correlation was found between qualitative staining intensity and the morphologic categories of typical CLL and CLL/PL. This finding was somewhat unexpected in light of several studies that have shown an apparent correlation between morphology and immunophenotype (65-71). In addition, CLL with trisomy 12 has been associated with atypical morphology (65-71). Furthermore, cases of CLL with trisomy 12 have been shown to be associated with an atypical immunophenotype including increased CD20 expression (66,70) and expression of FMC7 (65,68,70,71). The majority of these studies concentrated on the differences between cases of CLL with or without trisomy 12. The correlation found between morphology and expression of CD20 and FMC7 in these studies was thus largely drawn from indirect comparisons. Additionally, two other studies found that cases morphologically categorized as atypical CLL had higher CD79b expression than typical CLL (12,72). Because cytogenetic studies were not performed on the majority of the cases in our study, the cases were not divided between those with or without trisomy 12. This difference may explain part of the reason why we did not find a correlation between intensity of expression of the pan B-cell markers and the morphologic categories of CLL. The correlation between atypical morphology and immunophenotype may not be strong in cases of CLL without trisomy 12. Indeed, a subset of CLL patients without trisomy 12 that had identical morphologic features as cases with trisomy 12, including cases of CLL/PL, showed decreased intensity of expression of CD20 when compared to cases with trisomy 12 (70). Matutes et al. (68) also found a lack of association between the morphologic category of CLL/PL and increased FMC7 expression in cases without trisomy 12 when compared to cases with trisomy 12. Moreover, CLL with transformation to large cell lymphoma has been shown to commonly retain the major immunophenotypic features of CLL, including negative or dim expression of FMC7 (73).

In summary, the findings in this study of a cohort of B-CLL patient samples calls attention to the variability of staining intensity for CD20, FMC7 and CD79b. With the exception of moderate correlation between geometric mean fluorescence intensity between CD20 and FMC7, little or no correlation was seen between these markers. FMC7 was the most consistently dim or negative among

the 3 pan B-cell markers in cases of CLL, whereas CD20 was the least. The latter finding lends some support for the elimination of CD20 in immunophenotypic panels designed for specifically evaluating peripheral blood lymphocytosis, at least within the paradigm that staining for these three antigens is dim or negative in CLL. However, it should be noted again that this primarily may reflect a shift in relative staining intensity of CD20 versus FMC7. Unexpectedly, increased intensity of expression of CD20, FMC7, and CD79b was not correlated with increased numbers of prolymphocytes.

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