Phenotypic Heterogeneity of B Cells in Patients With Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

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Key Words: Aberrant marker; Chronic lymphocytic leukemia; Small lymphocytic lymphoma; CD2; CD7; CD10; CD13; CD33; CD34

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Abstract

Although some studies have examined the expression of aberrant markers such as CD2, CD7, CD10, CD13, CD33, and CD34 on B cells in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), a uniform multiparametric analysis of the frequency of expression of these markers using stringent criteria is lacking. By using 3-color flow cytometry, we analyzed 117 cases (bone marrow, 71; blood, 31; lymph nodes, 15) for coexpression of aberrant markers with CD19. Marker expression was considered positive when present on at least 20% of CD19+ cells. Of 117 cases, 40 (34.2%) showed expression of 1 or more aberrant markers. Expression of 4 aberrant markers was seen in 1 case, 3 in 4 cases, 2 in 15 cases, and 1 in 20 cases. Kaplan-Meier survival curves and the log-rank test revealed that the group with aberrant markers showed significantly shortened overall survival compared with the group without aberrant markers (P < .001). There is considerable phenotypic heterogeneity in CLL/SLL, and expression of aberrant markers indicates aggressiveness.

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), although a relatively indolent neoplasm, is known to show considerable heterogeneity with respect to clinical course and life expectancy among patients. For a biologic correlate to this diversity, there has been considerable interest in the phenotypic heterogeneity of B cells in CLL/SLL. Expression of nonlineage markers in acute leukemias,1-6 high-grade lymphomas,7-9 and multiple myeloma10-12 has been associated with an adverse prognosis. This finding encouraged study of the frequency of expression of aberrant markers in CLL/SLL.

As a result, several studies have evaluated the expression of T-cell (CD2 and CD7), myelomonocytic (CD13 and CD33), progenitor cell (CD34), and immature B-cell (CD10) markers in CLL/SLL.13-24 However, a good number of these studies were case reports.14-18,25 Among the studies in which a number of cases of CLL were analyzed,19-24 there was considerable variation in the frequency of cases with CD2, CD13, and CD33 expression. The wide variation in the frequency of expression of these markers in CLL/SLL may be due to difference in the methods used. In some studies, the marker was considered positive in comparison with negative control samples,16,22-24,26 and in others, single-color flow cytometry was used.21,24 By using these methods, the coexpression of markers in B cells cannot be assessed accurately, and contaminating nonlineage cells in the gate cannot be excluded. Marker coexpression with CD20 was analyzed by some authors25,27; however, CD20 is not a sensitive marker: it can be absent (B.K. and C.S., unpublished data), weak, or expressed heterogeneously in CLL/SLL.28 In some studies, the marker expression was considered positive when present on at least 10% of CD19+ cells.29 By using 10% as a cutoff,
there can be false-positive cases, especially when marker expression is heterogeneous.

The clinical significance of CD2 expression in CLL/SLL is controversial. Expression of CD2 has been associated with an aggressive clinical course or advanced stage of disease at diagnosis. However, Kaleem et al. found no clinical significance for CD2 expression in CLL at diagnosis. The expression of CD13 and CD33 in B-cell CLL has been associated with unfavorable clinical and prognostic factors. In these studies, the prognostic effect of the expression of aberrant markers was assessed indirectly based on stage of the disease or pattern of bone marrow involvement at diagnosis, but not by a direct measure such as the analysis of long-term survival.

We used 3-color flow cytometry to evaluate coexpression of CD2, CD7, CD10, CD13, CD33, and CD34 with CD19 in 117 cases of CLL/SLL involving bone marrow, peripheral blood, and lymph nodes. We used CD19 to assess coexpression because it is well known to express uniformly and brightly on B cells in CLL/SLL. Marker expression was considered positive when present on at least 20% of CD19+ cells. The percentage of cases positive for each marker was determined. Survival analysis was performed using the Kaplan-Meier survival curve, and survival in patients with expression of aberrant markers was compared with a group without expression of aberrant markers by using the log-rank test.

Materials and Methods

Cases

Specimens from 117 cases of CLL/SLL involving bone marrow (71), peripheral blood (31), and lymph nodes (15), collected in the pathology and flow cytometry departments, Roswell Park Cancer Institute, Buffalo, NY, were studied retrospectively. In patients with multiple specimens (bone marrow, peripheral blood, lymph nodes), only the earliest specimen was included in the study to avoid duplication. The median interval between the date of initial diagnosis and the date of specimen analysis was 3 months (range, 0-295 months). The flow cytometric data from the initial diagnostic workups were available for the majority of cases. Some follow-up cases, however, also were included because sequential flow cytometric analyses in many cases during a period of time did not show alteration of expression of aberrant markers as a result of treatment or disease evolution. All specimens were studied by morphologic examination; when relevant, cases were studied by immunohistochemical analysis for CD3, CD5, CD10, CD20, CD23, bcl-1, bcl-2, bcl-6, and myeloperoxidase. Specimens that were equivocal owing to overlapping feature of follicular lymphoma, mantle cell lymphoma, or marginal zone lymphoma based on combined morphologic, immunohistochemical, and flow cytometric criteria were not included. B-cell and T-cell gene rearrangement data were available for 28 cases.

There were 76 men and 41 women (M/F ratio, 1.9:1) with an average age of 60.4 years and a median age of 60.8 years at diagnosis (age range, 31-85 years). The survival data for these patients were retrieved from the tumor registry. Eighteen cases (15.4%) were excluded from the survival analysis because of lack of pertinent information for the survival study. Laboratory (lymphocyte count, hemoglobin value, and platelet count) and clinical (lymphadenopathy and splenomegaly) data at the time of initial diagnosis were available for 86 cases for clinical staging according to a modified Rai system. Monoclonal Antibodies

Monoclonal antibodies, their clones, the fluorochromes used in the study, and the manufacturers’ names are given in Table I. Each antibody was titrated before its use in the panel to ensure that each reproducibly stained its target cell to the same degree in samples from healthy donors as previously reported. Panels were prepared in 100-test batches for consistency. The 3 antibody combinations used were as follows: (1) CD3/CD4/CD8; (2) CD2/CD7/CD19; (3) CD5/CD10/CD19; (4) CD5/CD23/CD19; (5) CD5/CD19/CD34; (6) CD11c/CD20/CD22; (7) CD14/CD45/HLA-DR; (8) CD13/CD19/CD33; (9) CD38/CD45/CD56; (10) CD19/κ/λ.

Cell Staining

The bone marrow and peripheral blood samples, anticoagulated with EDTA, were counted and filtered through 75-μm mesh and pipetted into a 15-mL conical tube (Falcon, Lincoln Park, NY). The details of our immunophenotyping procedure have been reported previously. Briefly, the tube was filled with phosphate-buffered saline (PBS)-heparin (10 U/mL) and centrifuged at 1,500 g for 3 minutes. The PBS-heparin was aspirated off, and the cells were washed again in 15 mL of PBS without heparin.

To approximately 1 mL of pellet that had been suspended in residual PBS buffer, 67 μL of mouse IgG (3 mg/mL) was added to block Fc receptor and nonspecific antibody binding. After a 10-minute incubation on ice and without washing, 50 μL of the cell suspension (0.5-1.0 × 10⁶ cells) was distributed into 12 × 75-mm Falcon plastic tubes containing the appropriate amount of monoclonal antibody panels. The cells were lysed by adding 3 mL of lysing reagent (Sigma, St Louis, MO) after 15 minutes of incubation on ice. Cells were centrifuged at 1,500g for 3 minutes, washed in PBS, and resuspended in 200 μL of 2% ultrapure formaldehyde (Polysciences, Warrington, PA.).

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Lymph node specimens were collected in RPMI, and suspensions were prepared by dispersing through a 60-µm mesh screen into Hanks buffered saline with Hepes buffer (pH 7.2; 20-mmol/L concentration of organic buffer). After centrifugation, cells were resuspended in 1 mL of PBS, counted, and adjusted to 0.5 to 1.0 × 10^6/mL, and 67 µL of mouse IgG (3 mg/mL) was added to block Fc receptor and nonspecific antibody binding. Fifty microliters of suspension was added to tubes containing the monoclonal antibody panels and incubated for 25 minutes on ice. After washing once with 3 mL of PBS, the pellets were resuspended in residual buffer and fixed with 2% ultrapure formaldehyde.

By using isotype controls labeled with the appropriate fluorochrome and setting positive and negative quadrants, the percentage of positive cells for each set of monoclonal antibodies was determined. A second control tube to assess viability was included containing 5 µg/mL of ethidium monoazide. The ethidium monoazide–containing tube was placed 18 cm away from a 20-W fluorescent light for 10 minutes to covalently link the ethidium monoazide to DNA. The cells then were treated with lysing reagent, washed, and fixed along with the other samples.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA) configured for 3-color fluorescence measurement. All data were obtained in list mode and displayed as 2-parameter dot plots using WinList software (Verity Software, Topsham, ME). Lymphocytes were gated based on low forward and low side scatter characteristics. CD19 was present in all tubes with aberrant markers, which enabled us to gate on CD19+ cells to exclude contaminating T cells and myelomonocytic cells.

The percentage of B cells coexpressing CD19 and any other marker was determined by dividing the total number of marker-positive cells by the total number of CD19+ cells and multiplying by 100. Marker expression was considered positive when present on at least 20% of CD19+ cells. In positive cases, the median values for corresponding markers also were calculated. In this study, CD2, CD7, CD10, CD13, CD33, and CD34 were considered aberrant markers for CLL/SLL.

Survival Analysis

By using the Kaplan-Meier curve and the log-rank test, survival in the following groups was compared: (1) overall, survival of patients with and without expression of aberrant markers, (2) survival based on expression of aberrant markers among patients stratified to the Rai intermediate-risk category, and (3) survival based on expression of aberrant markers among patients with CD38 expression.

Results

As shown in Figure 1 and Figure 2, the expression of CD2 and CD7 was seen in 17.1% and 3.4% of cases, respectively. In positive cases, the percentages of CD19+ cells expressing CD2 ranged from 21.2% to 100.0% (median, 0.5 to 1.0 × 10^6/mL, and 67 µL of mouse IgG (3 mg/mL) was added to block Fc receptor and nonspecific antibody binding. Fifty microliters of suspension was added to tubes containing the monoclonal antibody panels and incubated for 25 minutes on ice. After washing once with 3 mL of PBS, the pellets were resuspended in residual buffer and fixed with 2% ultrapure formaldehyde.

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40.1%) and CD7 from 22.8% to 57.6% (median, 29.9%). For 13 of 24 cases with CD2 and CD7 expression, gene rearrangement studies had been done, and none showed T-cell gene rearrangement. CD13 and CD33 expression was seen in 17.1% and 0.9% of cases, respectively. CD13 expression ranged from 20.5% to 67.3% (median, 34.8%) of CD19+ cells. CD33 expression was present only in 1 (0.9%) of 117 cases. Of 117 cases, 12 (10.3%) showed CD10 expression with a range of 20.4% to 80.35% (median, 45.9%) of CD19+ cells. CD34 expression ranged from 21% to 55.4% (median, 31.6%) of CD19+ cells in 9 (7.7%) of 117 cases.

Histograms of representative cases of CD2, CD7, CD10, CD13, and CD34 coexpression with CD19 are shown in Figure 3. The quadrants were set based on negative isotype controls. For clarity and to avoid contaminating T cells and myelomonocytic cells, CD19+ cells were gated, and then their marker expression was evaluated. In these cases, all aberrant markers, except CD34, showed heterogeneous expression, meaning a continuum of expression from negative to positive on CD19+ neoplastic cells.

Of 117 cases, 40 (34.2%) showed expression of 1 or more aberrant markers (CD2, CD7, CD10, CD13, and CD34) coexpression with CD19 are shown in Figure 3. The quadrants were set based on negative isotype controls. For clarity and to avoid contaminating T cells and myelomonocytic cells, CD19+ cells were gated, and then their marker expression was evaluated. In these cases, all aberrant markers, except CD34, showed heterogeneous expression, meaning a continuum of expression from negative to positive on CD19+ neoplastic cells.

For 7 cases in group 1 and 11 in group 2, no pertinent clinical data were available; therefore, these cases were excluded from the survival analysis, leaving 33 cases for analysis in group 1 and 66 in group 2. In group 1, 21 patients (64%) died of progressive disease, as did 22 patients (33%) in group 2. For cases included in the survival analysis, the mean follow-up was 85.2 months (median, 74.3 months; range, 4-348 months). Figure 4 shows Kaplan-Meier survival curves comparing groups 1 and 2. The median survival periods for groups 1 and 2 were 88 and 150 months, respectively, with a statistically significant difference (P < .001; log-rank test). For the subset of patients with expression of multiple aberrant markers, no significant survival
A difference was found compared with the subset with expression of a single aberrant marker. The mean age ± SD at diagnosis for patients with multiple aberrant markers was 61.6 ± 11.4 years (range, 36-81 years) and for those with 1 aberrant marker was 60.1 ± 12.8 years (range, 31-85 years), which was not statistically different.

We analyzed CD38 expression in all cases, although it was not combined with CD5 and CD19 as reported earlier. By using 30% as the cutoff for CD38 expression as recommended previously, we found 18 cases positive for CD38 (11 in group 1 and 7 in group 2). The median survival for the group that coexposed CD38 and aberrant markers was 70 months compared with 140 months for the group that expressed CD38 without aberrant markers ($P < .02$; log-rank test) [Figure 5]. The median age for group 1 was 63.5 years (range, 36-81 years) and for group 2 was 60 years (range, 31-85 years). The male/female ratios for groups 1 and 2 were 1.2:1 and 2.3:1, respectively. Eighty-six cases (group 1, 27; group 2, 59) were classified according to the modified Rai system. In group 1, there were 4 patients in the low-risk, 13 in the intermediate-risk, and 10 in the high-risk groups. In group 2, there were 28 patients in the low-risk, 17 in the intermediate-risk, and 14 in the high-risk groups. Since patients who belong to the Rai intermediate-risk group are clinically the most heterogeneous, we analyzed the survival of the 30 patients (group 1, 13; group 2, 17) [Figure 6]. The median survival for group 1 was 87 months compared with 168 months for group 2 ($P = .0002$; log-rank test).

### Discussion

Our study differs from all others in that the coexpression of aberrant markers with CD19 was evaluated for every case. In this way, there can be no doubt about the presence of the marker on malignant B cells. We considered the expression

### Table 2

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*Data are given as percentage of cells expressing the marker. CD2, CD7, CD10, CD13, CD33, and CD34 were considered aberrant markers because of their infrequent expression in chronic lymphocytic leukemia/small lymphocytic lymphoma. The expression of 4 aberrant markers was seen in 1 case, 3 in 4 cases and 2 in 15 cases. Table 2 also shows the percentage of CD19+ cells that coexposed CD5 and some double marker combinations (CD2/CD7, CD5/CD10, and CD5/CD34). Cases with a single aberrant marker and values less than 20%, which are considered negative, are not shown in the table.
of aberrant markers to be positive when present on at least 20% of CD19+ cells. The 20% cutoff has been accepted as an optimum value for a marker to be considered positive if the expression is heterogeneous.30

In our study, CD2 and CD7 (T cell–associated markers) were seen in 17.1% and 3.4% of cases, respectively (Figures 1 and 2). Some case reports on CD2 expression in CLL/SLL14-16 do not acknowledge the frequency of expression of this marker in cases of CLL/SLL. However, Newman et al24 reported CD2 expression in fewer than 5% of the patients with CLL in their series, while Kurec et al23 observed CD2+ cells in 17 (28%) of 61 patients. Kurec et al23 and Newman et al24 assessed marker expression in comparison with negative control samples and did not evaluate coexpression with a B-cell marker. To our knowledge, CD7 expression in CLL/SLL has not been reported.

Our study showed expression of CD13 and CD33 (myelomonocytic markers) in 17.1% and 0.9% of cases, respectively (Figures 1 and 2). Several series have been published on CD13 expression in CLL/SLL13,20,21,27,29 and CD33 in cases of CLL/SLL. However, in these studies, the frequency of CD13 expression ranged from 1.9% to 50% and of CD33 expression from 0% to 66%. The discrepancy in expression of CD13 and CD33 among different studies may be due to technical factors such as the antibody clone, the sensitivity of individual methods, Fc receptor binding producing artifactual data, contamination of myelomonocytic cells in the gate, and lack of assessment for coexpression with CD19.

CD10 expression commonly is found in precursor B-cell neoplasms, Burkitt lymphoma, and follicular lymphoma. A few reports have been published on CD10 expression in B-cell CLL,17,18 but the exact frequency of CD10 expression in cases of CLL/SLL is not known. In our study, CD10 expression was observed in 12 (10.3%) of 117 cases and all cases also were CD5+, indicating that we were not dealing with follicular lymphoma, which sometimes is morphologically indistinguishable from CLL/SLL in the bone marrow. Immunohistochemical analysis for bcl-6 also was used to exclude follicular lymphoma. In 7 cases (Table 2), coexpression of CD5, CD10, and CD19 was seen.

CD34 is a progenitor cell marker commonly found in acute leukemias. Estrov et al37 were able to generate CLL colonies in vitro from CD34+ progenitor cells. Gahn et al25 observed the presence of trisomy 12 in sorted CD34+ cells in patients with B-cell CLL and suggested that the malignant transformation in B-cell CLL may involve early hematopoietic stem cells. However, the exact frequency of CD34 expression in CLL/SLL is not well documented. In the 117 cases in our study, 9 (7.7%) (Figures 1 and 2) demonstrated CD34 expression, and in 6 cases (Table 2), more than 20% CD5+/CD19+/CD34+ cells were present, indicating that CD34 was present on CD5+ cells.
In our study, 2 or more aberrant markers were expressed in 20 (50%) of 40 cases (Table 2), which suggests that these markers have a tendency to cluster in the same patient. Clustering of aberrant markers persisted in patients who underwent repeated flow cytometric analysis, which indicates that this is not a random phenomenon. Although we were unable to demonstrate unequivocally that all these markers were expressed on the same cell in each patient, we found coexpression of CD5, CD10, and CD19 in 7 (58%) of 12 CD10+ cases and coexpression of CD5, CD19, and CD34 in 6 (67%) of 9 CD34+ cases. In the 4 CD7+ cases, 2 (50%) showed coexpression of CD2. When the sum (percentage) of cells expressing 2 markers exceeds 100, some degree of coexpression is indicated for 2 markers. This clustering of aberrant markers has not been described previously. Sequential flow cytometric analysis in subsets of patients with aberrant markers and without aberrant markers for a long period showed persistence of aberrant markers in the former subset and absence of aberrant markers in the latter subset, indicating that the expression of aberrant markers is not influenced by treatment or evolution of the disease.

Survival analysis\(^3\)\(^6\) seems to be a better method for assessing clinical behavior in CLL/SLL than other parameters such as clinical stage at diagnosis or pattern of bone marrow involvement. By using Kaplan-Meier survival analysis (Figure 4), we observed a significantly shorter overall survival (\(P < .001\); log-rank test) in group 1 than in group 2. This is similar to CD38 expression in B-cell CLL, which has been reported to carry an adverse prognosis.\(^3\)\(^6\) We analyzed the combined effect of CD38 and aberrant markers on survival. By using Kaplan-Meier survival analysis (Figure 5), we demonstrated that the group with coexpression of CD38 and aberrant markers had a significantly shorter survival (\(P < .02\); log-rank test) than the group that expressed CD38 alone. Damle et al\(^9\) analyzed survival based on CD38 expression in the Rai intermediate-risk group and found that expression of CD38 carried an adverse prognosis in this group, suggesting that the influence of CD38 is independent of the Rai stage. Similarly, when we analyzed survival of those classified in the Rai intermediate-risk category in groups 1 and 2, group 1 had a significantly shorter survival than group 2 (\(P = .0002\); log-rank test; Figure 6). This study shows that the presence of aberrant markers is a prognostic marker independent of CD38 expression and Rai clinical stage.

Our finding of the expression of 1 or more aberrant markers in CLL/SLL is similar to that reported in acute leukemias,\(^1,4,8-40\) multiple myeloma,\(^10,12\) and intermediate- and high-grade lymphomas.\(^7,9\) Acute leukemias expressing nonlineage markers are thought to arise from precursor cells with potential for multilineage differentiation.\(^3,39\) This view is supported by multilineage blast transformation in chronic myeloid leukemia, which is a known stem cell disorder.\(^41-45\) In lymphoid malignant neoplasms, which are thought to arise from precursor cells that are frozen at different stages of ontogeny,\(^45-47\) the reason for expression of nonlineage markers is not well understood. This may be due to aberrant gene expression as a manifestation of malignancy or malignant transformation occurring in progenitor cells with the potential for multilineage differentiation. In a study by MacKenzie and Lewis,\(^48\) the same karyotypic abnormalities in malignant plasma cells and lymphocytes were found in a patient, and they suggested that the initiating cells in plasma cell myeloma might be early B lymphocytes. The expression of CD117 (stem cell marker) in a subset of patients with multiple myeloma\(^49\) also suggests the primitive nature of myeloma precursors.

In our study, we observed retention of the progenitor cell marker, CD34, in a subset of CLL/SLL, suggesting a primitive precursor for these neoplastic cells. Our findings are supported by the findings of Estrov et al,\(^37\) who generated CLL colonies in vitro from CD34+ progenitor cells, and Gahn et al\(^25\) who observed trisomy 12 in sorted CD34+ cells in patients with B-cell CLL. The tendency for expression of multiple nonlineage markers along with CD34 in the same case favors a primitive precursor, rather than secondary acquisition of all of these markers. Aggressive behavior in cases with aberrant markers also suggests that these neoplastic cells have a primitive precursor, because the biologic behavior of the tumor is determined principally by the differentiation state of the precursor cell, and less differentiated
precursor cells are thought to give rise to a neoplasm that has great malignant potential. If oncogenesis is considered a multistep process, the initial insult can happen in a primitive or stem cell, which still retains its capacity to differentiate until the malignant transformation occurs under the influence of tumor promoters. Probably the stage at which tumor promoters act corresponds to the precursors of lymphoid malignant neoplasms that are considered to have been frozen at different stages of ontogeny.

Our study demonstrated that the neoplastic cells in CLL/SLL are immunophenotypically heterogeneous and that a subset of CLL/SLL expresses aberrant markers such as CD2, CD7, CD10, CD13, CD33, and CD34. Patients in whom the aberrant markers are found have a significantly decreased overall survival compared with patients without aberrant markers.

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