Immunoglobulin Phenotype on B Cells Correlates With Clinical Stage of Chronic Lymphocytic Leukemia

By Frances S. Ligler, John R. Kettman, R. Graham Smith, and Eugene P. Frenkel

The present study examines the relative amounts of surface immunoglobulin (Ig) on lymphocytes obtained from 64 patients with chronic lymphocytic leukemia (CLL) and correlates these findings with the clinical stage of disease. Since the maturing B cell first expresses surface IgM, followed by IgD, and subsequently by IgG, IgA, or IgE, the surface Ig phenotype can be used as a marker of differentiation. Surface Ig was analyzed using the fluorescence-activated cell sorter under carefully controlled conditions.

Although the cells involved in chronic lymphocytic leukemia (CLL) have relatively uniform morphological features, survival of the patient does not reflect that uniformity. The dominant tumor cell resembles the classic mature small lymphocyte and, unlike malignant lymphomas of the B-cell type, subdivision of cases of CLL on histologic criteria has failed to provide information of value in predicting the natural history of the disease. Cell size, mitogen reactivity, and the degree of bone marrow involvement are among the measurements that have been suggested as indicators of survival.1,10 Dubner et al.2 correlated the presence of large cells resembling prolymphocytes or lymphoblasts with a poor prognosis. Others, however, have not been able to correlate size with survival.3,4 Another approach to predicting survival was based on murine studies that showed that the response pattern to different mitogens reflected the maturation stage of B lymphocytes.5 Robert et al. subjected cells from patients with CLL to a variety of mitogens6 and found that a low response to phytohemagglutinin and high response to dextran sulfate and lipopolysaccharide or to a low concentration of anti-β2-microglobulin was associated with more aggressive disease. Robert and Nilsson7 suggested that the mitogen-responsiveness of cells from CLL also reflected the state of maturation of the cells and that less mature cells were associated with more aggressive disease. Finally, Gray et al.2 proposed that the percent of bone marrow space occupied by tumor was related to the survival of the patient. However, this parameter by itself was insufficient to determine prognosis.

Rai et al.8 developed a method of staging CLL based on easily measurable clinical parameters that correlated directly with patient survival. These studies were confirmed by Binet et al.9 and Peterson et al.,10 who showed that patients with less clinical evidence of disease (i.e., stages 0 and I) have significantly longer survivals than patients with more evidence of mass disease. Recent attempts to further refine this staging scheme11,12 have provided even more evidence of its simplicity and relative reliability.

Observations based on the study of carcinomas and other nonlymphoid neoplasias suggest that tumor aggressiveness is related to maturity in the tumor cells as assessed by morphology, with differentiation favoring more benign disease. However, histology of lymphocytes is not always useful in distinguishing mature or more differentiated lymphocytes from less mature. For example, blast morphology may be found in pre-B-cells or in activated mature cells. On the other hand, evaluation of surface immunoglobulin (Ig) aids in the discrimination of the many differentiation stages of normal B cells.13-15 Assuming that the relative amount of IgM, IgD, and IgG on the surface of lymphocytes from patients with CLL could be used to identify the relative degree of differentiation of the tumor cells, the purpose of this study was to see if the relative degree of differentiation would determine the extent of dissemination and aggressiveness of the tumor as measured by the clinical stage.

Materials and Methods

Patients

Peripheral blood was obtained from 64 patients with CLL. All patients exhibited increased absolute lymphocyte counts at the time of analysis (>4,000 lymphocytes/μl). None of the patients exhibited
paraproteinaemia, and none of the leukemic cells formed rosettes with sheep erythrocytes.6 The clinical data on all the patients was reviewed, and the patients were staged at the time of analysis according to the Rai classificationa as follows: 12, stage 0; 10, stage I; 15, stage II; 9, stage III; 18, stage IV. In all but one case, the Rai stage was unchanged from the stage at diagnosis.

**Immunofluorescence Staining**

Viable lymphocytes were isolated from heparinized peripheral blood by centrifugation over 9% Ficoll (Sigma Chemical Co., St. Louis, MO). 33% Hypaque (Winthrop Laboratories, New York, NY). Cells were washed with phosphate-buffered saline, pH 7.2, resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 20% fetal calf serum (GIBCO), and incubated at 37°C for 16–20 hr to remove cytophilic immunoglobulin. All anti-heavy-chain reagents prepared as described elsewhere. Anti-heavy-chain reagents were carefully checked for specificity by staining of monoclonal populations of cells and radioimmunoassay, and then were used at optimal concentrations ranging from 2 to 20 μg/ml. Cells stained with normal rabbit immunoglobulin, no primary antibody, or the irrelevant anti-light-chain reagent were used as controls. After 20 min, cells were washed and resuspended with fluoresceinated goat anti-rabbit Ig (0.1 mg/ml).2 Ten minutes later, the cells were washed and fixed in 4% paraformaldehyde. Before analysis, they were resuspended with phosphate-buffered saline and resuspended in Isoton (Coulter Electronics, Hialeah, FL).

**Cytocfluorimetry**

Cells were examined on the Becton-Dickinson FACS III by exposure to a laser light of 488 nm at an intensity of 500 mW. The fluorescence signal was determined with a photomultiplier (Type 9524, EMI GEMCO, Inc.) potential of 600 V. Fluorescence and scatter signals were standardized daily using both fluorescent glutaraldehyde-fixed chicken erythrocytes22 and nonfluorescent osmium-fixed chicken erythrocytes and from a normal individual.

**RESULTS**

Specialized Conditions for Analysis of CLL

Under conditions appropriate for the analysis of surface Ig at the density found on normal B lymphocytes (500 V, 300 mW), the fluorescence intensity from the low density of surface Ig on CLL cells is difficult to distinguish from autofluorescence. By increasing the output of laser light to 500 mW, adjusting the voltages applied to the photomultiplier tube to 600 V, and optimizing the signal-to-noise ratio with fluorescent and nonfluorescent particles, the fluorescence of the weakly stained CLL cells can be accurately measured. Under these conditions, the number of stained B cells in normal peripheral blood does not change, but their brightness exceeds the signal range accommodated in the scale where the CLL cells are scored and their fluorescence is registered in the last channel.

**Scatter Analysis**

In comparison to the scatter distribution of cells from normal peripheral blood, CLL cells were homogeneous with regard to scatter and always had a clearly defined peak (Fig. 1). However, three types of variation among CLL scatter profiles were evident. The first and most obvious variable in the CLL scatter histograms was the peak channel, which reflects the predominant cell size. The second variable, the width of the peak, reflected the size heterogeneity of cells within the CLL. The third difference among scatter profiles was the degree to which the curves were skewed by the presence of large cells. The two CLL profiles in Fig. 1 differ predominantly in terms of the peak channel and the width of the peak.

In order to measure these three scatter parameters, the following evaluations were made of scatter profiles from the lymphocytes from each of the 64 cases of CLL (Fig. 2). The peak channel was considered to represent the predominant cell size. The distance, expressed in terms of number of channels, between the half-peak heights measured the degree of heterogeneity. Finally, the ratio of the number of channels between the half-peak heights and the peak channel...
Fig. 2. Measurements of scatter. Scatter profiles for different CLL cells were compared after determining the channel of the scatter peak (Y) and the channels of half maximum peak height (X,Z). "Y" was designated as peak scatter and reflected the predominant cell size. "Z - X" was a measure of the width of the peak, reflecting the degree of size heterogeneity. "(Y - Z)/(Y - X)" was considered a measure of skewness of the size distribution.

was used as the parameter of the degree of skewness of the size distribution. None of these three parameters correlated with stage of disease.

Fluorescence Analysis

Fluorescence analysis of 62 of 64 cases of CLL showed monoclonality, with respect to Ig light chain type. Staining with anti-light-chain reagents was not detected on the cells in the remaining 2 cases of CLL.

Table 1. Correlation of Rai Classification and Light Chain Type

<table>
<thead>
<tr>
<th>Stage</th>
<th>Light Chain Type</th>
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<tr>
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<tr>
<td>0</td>
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<tr>
<td>I</td>
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<tr>
<td>II</td>
<td>1</td>
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<td>IV</td>
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<td>Total</td>
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Cells were collected from patients with CLL and analyzed as described in Materials and Methods. Values are expressed as number of CLL patients.

(Table 1). Figure 3 displays histograms of cells from three representative cases of CLL; the cells were stained with anti-κ and anti-λ reagents. In all three cases, the amount of fluorescence due to autofluorescence showed up in the profile from cells stained using the irrelevant anti-light-chain reagent. The amount and distribution of fluorescence of cells stained using the appropriate anti-light-chain reagent is clearly variable. Cells in CLL-A stain weakly and are relatively homogeneous in terms of the amount of surface Ig, generating an almost Gaussian curve at low fluorescence channels. In contrast, CLL-B cells are quite heterogeneous in terms of the amount of Ig on their surface, and cells from CLL-C are stained more brightly, though their fluorescence intensity is still less than that of most normal B cells. In some CLL, the fluorescence intensity increased in proportion to cell size. This proportionality has been discussed elsewhere.25

Unlike the scatter curves, the fluorescence curves often had no clear-cut peak (see Fig. 3C). For this reason, cumulative distribution curves were generated.

Fig. 3. Fluorescence of CLL. Cells from three patients with CLL were isolated, stained using anti-κ (——) or anti-λ (-----), and analyzed as described in Materials and Methods. These representative profiles show a CLL with relatively homogeneous, weak fluorescence (A); a CLL with heterogeneous staining producing double peaks in the fluorescence histogram (B); and a CLL with relatively bright fluorescence, though the level of intensity is still below that of most normal B cells (C).
to better define the fluorescence of the CLL (Fig. 4). Each point on the cumulative distribution curve represents the percentage of cells in a given sample with a fluorescence intensity less than or equal to the channel number. Portions of the original histogram that represented residual normal populations, often Ig-negative, were not included in the calculation of the cumulative distribution curve if the original histogram showed separate peaks for CLL cells and residual normal populations. The channel number at the 50th percentile was considered to be the median fluorescence or the "fluorescence of the CLL." 

CLL cells were considered positive for a given heavy chain if the 50th percentile (median fluorescence) of the cumulative distribution curve obtained using that anti-heavy-chain reagent was at least five channels above the 50th percentile obtained from the histogram of cells stained only with the fluoresceinated goat anti-rabbit Ig. Five channels were selected as the minimum increase, after careful inspection of the original data (dot plots) substantiated that this displacement of the cumulative distribution curve reflected a visible difference between stained and unstained populations.

Heterogeneity was calculated as the distance between the channel at the 75th percentile and the channel at the 25th percentile. Skewness of the distribution of fluorescence intensities was calculated as the ratio of the distances between the 75th and 50th percentile and the 50th and 25th percentile.

In order to make certain that variations in quantities of fluorescence at low levels were reproducible, two experiments were performed. First, aliquots of the same sample were analyzed repeatedly on a daily basis for 2 wk. The profiles showed no variation. Secondly, samples were collected from one patient for 4 consecutive months. The median fluorescence (50th percentile) shifted by less than 20 channels during the entire period, and the shape of the cumulative distribution curves remained relatively constant. These data reassured us that there was neither significant day-to-day variation in analysis procedures nor month-to-month variation between samples collected from a single patient. Serial samples drawn on patients with stable disease over a longer period also showed little change. Only one patient showed a significant change in staining pattern. The original sample stained only with anti-μ and anti-δ heavy chain reagents, but a second sample, drawn 6 mo later, showed that half of the CLL cells had also acquired γ.

Six of the 64 patients had no detectable heavy chain on their cell surface. These leukemias were not segregated by stages. They are probably a combination of alpha-bearing tumors and leukemias with amounts of μ, δ, or γ below the level of detection. Only one of the leukemias without μ, δ, or γ was also light-chain-negative. Forty-one cases of CLL bore all 3 heavy chain types. These "triples" were found in all stages.

Only one observation from these studies showed complete correlation with a single Rai classification. Of the 64 cases of CLL, there were 4 κ+μ+δ+γ+ tumors for which the light chain fluorescence was much brighter than the totaled fluorescence from the heavy chains, suggesting the presence of a fourth heavy chain class or free light chain (Table 2). All of these patients were stage IV. Twenty-one of the 64 cases of CLL had μ as the brightest heavy chain; of these, 16 were stage II–IV. Eight had γ as the brightest heavy chain: 7 of these 8 were in stages 0–II.

**DISCUSSION**

The low intensity of the fluorescence of cells from patients with CLL has been documented both by
fluorescence microscopy26-29 and by cytofluorimetry.30 By very careful manipulation of both reagents and sorter conditions, differences in the amounts of heavy and light chains can be quantitated even at the low density characteristic of CLL cells. Changes in the cell sorter conditions compared to those used for the analysis of normal peripheral blood include an increase in the intensity of the laser light, an increase in the voltage applied to the photomultiplier tubes, and daily optimization of the signal-to-noise ratio using both fluorescent and nonfluorescent particles.

The analysis of CLL cells under these conditions is reproducible. Repeated analysis of a single sample shows that the sensitivity of the measurement is constant from day to day, and repeated blood samples from a single CLL patient show that the Ig phenotype and distribution of fluorescence are stable and characteristic for each individual CLL.

The sensitivity of the instrumentation reveals a new degree of heterogeneity in CLL. While CLL cells appear morphologically uniform, both the amount of light scatter and the low amount of Ig on the surface of cells within a single CLL may be relatively homogeneous or variable. Where the amount of intracranial surface Ig varies, the density may or may not increase in proportion to cell size.25 The cause and significance of these different types of intracranial heterogeneity is unknown. There is no obvious relationship between the cellular heterogeneity and the stage of the CLL according to the Rai classification.

The quantitative information on the relative amounts of each heavy chain class on the surface of CLL cells, as defined by the present methodology, was used to try to characterize differences in “aggressiveness” of CLL. Though some normal B cells may remain only IgM+, the major differentiation path is marked by a shift in cell surface Ig phenotype from IgM+ to IgM+, IgD+. This mature B cell may acquire IgG, IgA, or IgE and/or lose one or both of its existing heavy chain classes as it becomes a recirculating memory cell or an antibody-producing cell.13-15 This sequence of heavy chain expression correlates with the sequence of heavy chain genes described by Honjo and others.31,32 The hypothesis underlying the present study is that the relative amounts and the classes of surface heavy chains on the cells in CLL reflect their relative degree of differentiation which, in turn, determines the “aggressiveness” of the tumor.

As shown in Table 2, CLL patients whose tumor cells exhibited IgM as the dominant surface Ig had a more advanced stage (and thereby poorer prognosis), while patients whose tumor cells exhibited IgG as the dominant surface Ig had a lesser stage (and thereby better prognosis). Tumors with a dominance of surface IgD are found in all stages, suggesting that IgD-predominant CLL, and indeed IgM+, IgD+, IgG+ cells, are at a pivot point along their differentiation pathway.

The finding that cell size, as judged by scatter analysis,24 is unrelated to prognosis is inconsistent with some earlier reports,1 but can be explained by several considerations. The models of B-cell differentiation using heavy chain markers include large cells at both ends of the pathway, so that the differentiation status of a cell cannot be identified on the basis of its size alone. In addition, right angle scatter is affected by internal structure as well as by the size of a cell, though to a much lesser degree. While the difference in internal structure between lymphocytes from different CLL patients would seem too small to cause a shift in the light scatter, such a possibility cannot be ruled out. The measurement of skewness of the scatter profile based on half-peak heights only reflects the presence of larger CLL cells if they constitute major subpopulations, and thus does not necessarily contradict data correlating the presence of small numbers of large cells with a poor prognosis.27-29 Heterogeneity within both the scatter and fluorescence profiles might be expected to reflect instability of the tumor population, but no correlation with stage of the leukemia was evident using the present methodology.

Several papers have suggested that CLL with a κ light-chain type are less aggressive than those with a λ light-chain type.33,34 We did not find any segregation of λ isotype with more advanced clinical stage of disease (Table 2).

The suggestion that free light chain35,36 exists on the surface of some of the stage IV tumors is interesting in the light of the work of Gordon, Smith, and associates.37,38 They showed that free light chain is the major immunoglobulin secreted by CLL cells with low amounts of surface IgM or no surface Ig detectable by fluorescence microscopy, and that variable amounts of free light chain are secreted by cells with easily visible IgM or IgG. On this basis, they suggest that B cells

<table>
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<th>Stage</th>
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<td>IV</td>
<td>4</td>
<td>7</td>
<td>9</td>
<td>1</td>
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Total | 4 | 21 | 22 | 8 |

Patients with CLL were classified using the Rai system. Cells were collected and analyzed as described. Values are expressed as number of CLL patients. Tumors with no predominant heavy chain are not included in the table.
secrete free light chains at an early stage of differentiation. That the cell sorter analysis showed low densities of IgM, IgD, and IgG on the 4 CLLs that probably bear a lot of free light chain suggests that the synthesis of free light chain by neoplastic cells is not restricted to those with the IgM' or IgM', IgD' phenotype characteristic of early B cells. Additionally, it is unclear how free light chains might continue to associate with the cell surface, even after overnight incubation at 37°C.

Our data are somewhat at variance with those of Hamblin et al., who found that CLL patients with surface IgM pursued a more benign course than groups with surface IgM and IgD or no surface Ig detectable using the fluorescence microscope. However, due to the greater sensitivity of cytofluorimetry, many more tumors staining for two or three heavy chain classes were detected in this study than in their studies. When patients from this study with bright surface IgM and little or no Ig of other classes were examined as a group, they tended to have a more, rather than less, advanced stage of CLL. Since the study group of Hamblin et al. was small and limited to an elderly population of women, they may have identified a special subpopulation not prevalent in our study group.

While the percentages of tumors bearing all three heavy-chain classes is higher in this study (41 of 64) than is usually reported, the identification of such "triples" among human lymphocytes is not without precedence. Ault reported that the majority of IgG-bearing spleen cells also had surface IgM and IgD, and Stevenson found IgG on five of nine IgM', IgD' CLL patients who had no paraproteinemia. The simultaneous expression of three heavy chains is not easy to justify in the light of current knowledge of the sequential activation of heavy chain genes. Coexpression of μ and δ is explained by RNA splicing, but the gene for δ is assumed to be deleted upon activation of the gene coding for γ. According to this model, maintenance of δ expression after deleting the δ gene would require very long-lived messenger RNA. Despite the above model, it may be possible that, in these tumors, the genes coding for all three classes are expressed simultaneously.

The possibility that triples (IgM', IgD', IgG' cells) in reality bear only two heavy-chain classes and bind the third through Fc receptors cannot be entirely ruled out. CLL cells are able to express receptors for μ as well as γ Fc. The overnight incubation of the cells to remove cytophilic Ig and the monoclonality of the subsequent light chain staining argue against residual Ig bound to Fc receptors. However, a low degree of cytophilic light chain of the type not expressed by the tumor might go undetected in the presence of significantly higher amounts of monoclonal surface light chain.

The data presented here show that patients with CLL whose cells phenotype by surface Ig as comparatively immature cells (IgM-predominant) have a more advanced stage (i.e., "more aggressive") leukemia than those with a phenotype of more mature cells (IgG-predominant). Patients with CLL do not necessarily progress through successive Rai stages, and it is not known if a patient that does progress to a more advanced stage has a tumor whose surface Ig phenotype changes. Sequential studies on the present population are in progress. In addition, different CLL patients with the same stage vary both in terms of survival, as reported by users of the Rai classification, and by surface phenotype. Overlapping survival curves have been reported for CLL patients in stages 0 and I, I and II, II and III, and III and IV. Whether or not surface phenotype provides more specific information about prognosis will be determined only after longitudinal study.

ACKNOWLEDGMENT

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