Biclonal Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is well characterized clinically and immunophenotypically. Demonstration of a monotypic CD19+, CD5+ B-cell population is central to the diagnosis. We report 2 cases of biclonal CLL. Two elderly men were encountered with an absolute lymphocytosis consisting of the typical CD5+, CD19+, CD23+ B-cell population seen in CLL; however, immunoglobulin light chain restriction by flow cytometry was not apparent as B cells expressed kappa or lambda light chains without a clear monotypic population. Molecular genetic analysis of flow cytometry–sorted cells (kappa and lambda populations) revealed in both cases 2 monoclonal B-cell populations. The characterization of these cases and a review of the issues surrounding biclonal CLL are presented.

Chronic lymphocytic leukemia (CLL) is a well-characterized, mature, B-cell lymphoid leukemia and is the most common type of leukemia in older adults. Criteria for the diagnosis of CLL are well established and include lymphocytosis of 5,000/µL (5 × 10⁹/L), fewer than 55% prolymphocytes, 30% or more lymphocytes in the bone marrow, and a CD5+ monotypic B-lymphocyte immunophenotype.¹ Immunophenotyping has become an integral part of the classification of the chronic B-cell leukemias and often is considered essential in the initial diagnosis. CLL has a characteristic immunophenotype, expressing CD5, CD19, CD23, and dim monotypic surface immunoglobulin.¹ Scoring systems have been devised to help in the immunophenotypic diagnosis of CLL that include these and other antigens such as FMC7 and CD79b.²,³

We have seen 2 cases of chronic lymphocytic leukemia that on flow cytometric immunophenotyping showed a polytypic pattern by immunoglobulin light chain analysis. However, further study demonstrated the biclonal nature of these leukemias, each with a kappa and lambda monoclonal. Theoretic and diagnostic considerations of biclonality are discussed.

Materials and Methods

Flow Cytometric Immunophenotyping and Cell Sorting

Four-color flow cytometric immunophenotyping was performed in the clinical laboratories of the Cleveland Clinic, Cleveland, OH, using monoclonal antibodies Table I. Cells were stained according to standard methods and subjected to analysis using a FACScalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA). Data were collected and analyzed using a CD45–side scatter lymphocyte gate with CellQuest software (Becton Dickinson).
Flow cytometric cell sorting was performed on Ficoll-separated mononuclear cells in the Flow Cytometry Core laboratory at the Cleveland Clinic with a FACSVantage (Becton Dickinson) using kappa-FITC/lambda-PE (Becton Dickinson) dual staining to obtain a purity of more than 95% kappa-positive and lambda-positive B-cells.

**Southern Blot and Polymerase Chain Reaction**

DNA was isolated from leukocyte cell suspensions according to manufacturer’s protocol (DNAzol, Gibco, Bethesda, MD) followed by ethanol precipitation. Southern blotting with phosphorus 32–radiolabeled probes was performed according to published methods4 using the probe and enzyme combinations given in Table 2.

**Polymerase Chain Reaction Analysis**

Polymerase chain reaction (PCR) for immunoglobulin heavy chain gene rearrangement was performed using the Taq master mix kit (Qiagen, Valencia, CA) and framework III consensus primers as previously described.5,6 PCR products were analyzed using 6% polyacrylamide gel electrophoresis and visualized with ethidium bromide staining under UV light.

**Cytogenetic Analysis**

Standard karyotyping of G-banded metaphases was performed in the Cytogenetics Laboratory of the Cleveland Clinic using 3-day cultures of 12-o-tetradecanoylphorbol-13-acetate stimulated cells (unsorted). Twenty metaphases were analyzed per case.

**Patients**

**Case 1**

In a 73-year-old man, lymphocytosis was found incidentally on routine examination in 1986, and CLL was diagnosed. Because of a change in physician and lack of previous records, flow cytometric immunophenotyping was performed in May 1998. Results of the CBC count at diagnosis were as follows: hemoglobin, 13.5 g/dL (135 g/L); WBC count, 45.600/µL (45.6 × 10^9/L); and platelet count, 128 × 10^3/µL (128 × 10^9/L). Results of the differential count were as follows: neutrophils, 10% (0.10); lymphocytes, 83% (0.83); monocytes, 6% (0.06); and eosinophils, 1% (0.01). The physical examination revealed a palpable spleen tip 1 to 2 cm below the costal margin but no adenopathy. The clinical impression was Rai stage II. The last clinical follow-up in January 1999 showed no change.

**Case 2**

In a 77-year-old man with a history of coronary artery disease, a lymphocytosis was found incidentally during a routine visit in May 1998. The CBC results at diagnosis were as follows: hemoglobin, 16.5 g/dL (165 g/L); WBC count, 20,000/µL (20.0 × 10^9/L); and platelet count, 355 × 10^3/µL (355 × 10^9/L). Results of the differential count were as follows: neutrophils, 26% (0.26); lymphocytes, 69% (0.69); monocytes, 2% (0.02); eosinophils, 2% (0.02); and basophils, 1% (0.01). The physical examination revealed no lymphadenopathy or organomegaly. The clinical impression was Rai stage 0. The last clinical follow-up in June 1999 showed no change.

**Results**

**Morphologic Analysis**

Review of the Wright-stained peripheral blood smear from both patients showed lymphocytosis with similar morphologic features. As is typical in B-cell CLL, the lymphocytes were small with round nuclear contours and scant cytoplasm. The chromatin was condensed, and nucleoli were not evident. Cleaved cells and cells with irregular nuclei were absent. Prolymphocytes were rare.

**Immunophenotyping and Molecular Genetic Analysis**

Flow cytometric immunophenotyping in case 1 demonstrated a dominant B-cell population expressing CD5, CD19, low-intensity CD20, CD23, CD45, and low-
Intensity CD79b Figure 1A. Two separate populations could be seen from examining the CD19 expression intensity. The percentages of kappa and lambda expressing B cells were 55% and 45%, respectively (CD19 gated). Further analysis demonstrated that the brighter CD19+ cells expressed lambda light chains, and the dimmer CD19+ cells expressed kappa light chains Figure 1B. Case 2 showed a similar phenotype, although CD19 expression levels were uniform without evidence of separate populations. Kappa and lambda percentages were 71% and 21%, respectively (CD19-gated). Thus, although there was an abnormal B-cell phenotype in both cases, kappa and lambda expression appeared polytypic.

Cells from case 1 were sorted into separate kappa- and lambda-expressing populations by flow cytometry. Southern blot analysis in case 1 using an immunoglobulin heavy chain gene probe yielded monoclonal populations that were distinct by using a combined BamHI/HindIII DNA digest. Two bands were seen in each digest indicating biallelic rearrangement. A BamHI DNA digest using a kappa light chain probe showed a single band in the kappa-expressing cells and failed to show any bands in the lambda-expressing cells. The latter is consistent with deletion of the kappa gene loci Figure 2A. Southern blot analysis of the DNA extract from peripheral blood (unsorted cells) in case 2 showed evidence of bicalonality with 4 nongermline bands in a BamHI/HindIII digest using an immunoglobulin heavy chain probe and 2 nongermline bands in a BamHI digest using a kappa light chain probe Figure 2B. Because of low cell yield after cell sorting in case 2, insufficient DNA was obtained for Southern blot analysis of the individual kappa and lambda populations.

PCR for immunoglobulin heavy chain gene rearrangement was performed in DNA extracts of these sorted cells. Framework III primers yielded a PCR product with a single band in the kappa population, while the lambda population showed 2 bands, 1 of a size similar to that seen in the kappa sorted cells and 1 of a different size Figure 3.

Cytogenetic Analysis

Karyotyping of a 3-day culture of phorbol ester–stimulated cells (unsorted) in case 1 showed 15 cells with trisomy 12 (47,XY,+12) and 5 normal cells (46,XY). Case 2 showed a normal male karyotype.

Discussion

These cases represent 2 unusual examples of bicalonary PLL. Both showed a significant absolute lymphocytosis with a dominant abnormal B-cell population coexpressing CD5, CD19, and CD23. In addition, the cells were dimly positive for CD20 and had decreased expression of CD79b. All these immunophenotypic feature are typical for B-PLL.1,7,8 However, immunoglobulin light chain analysis showed a polytypic pattern. Further study using molecular genetic techniques demonstrated the presence of 2 distinct monoclonal populations.

In case 1, the kappa- and lambda-sorted cells were subjected to Southern blot analysis for immunoglobulin heavy and light chain analysis and yielded different gene rearrangement patterns. Although sorted cells were not available for Southern blot analysis in case 2, the heavy chain probe demonstrated 4 nongermline bands consistent...
Figure 1 A, Flow cytometric dot plots demonstrating CD5+ (fluorescein isothiocyanate [FITC]), CD19+ (allophycocyanin [APC]), CD23+ (phycoerythrin [PE]) B cells in each case without clear monotypic light chain staining. CD19-gated kappa/lamba percentages were 55%/45% and 71%/21% for cases 1 and 2, respectively. The triple CD5+/CD19+/CD23+ B cells are shown in black in the first 2 columns. In the third column, the CD19+/lambda-positive (PE) cells are shown in magenta, and the CD19+/kappa-positive (FITC) cells are shown in blue. B, Flow cytometric dot plots from case 1 demonstrating that the dimmer and brighter subpopulations of CD19+ cells are the separate monoclonal (bright, lambda [red]; dim, kappa [green]). PerCP, peridinin chlorophyll protein.
with the presence of more than 1 clone. The pattern would be consistent with 2 populations each with biallelic rearrangement. PCR for immunoglobulin heavy chain $\text{J}_\text{H}$ probe shows different patterns of biallelic rearrangement in the BamHI/HindIII digest. The kappa probe shows a rearrangement in the kappa population. No bands are seen in the lambda lane consistent with deletion of the kappa locus in this population.

Biclonal CLL is not well characterized. Only a handful of cases have been reported. Gonzalez-Campos et al.\(^9\) reported a single case of biclonal CLL that demonstrated 2 CD5+ lambda B-cell clones that were distinguishable by a different intensity of light chain expression. Wong and colleagues\(^10\) reported a biclonal CLL case documented with 2 independent cytogenetic abnormalities. Again, monotypic light chain restriction was present. Crossen and colleagues\(^11\) reported an oligoclonal CLL that was monotypic but contained at least 3 separate cytogenetic clones. More recently, Fernhout et al.\(^12\) reported a rare occurrence of CLL in 4 elderly siblings; for 2, Southern blot analysis revealed bicalonality, and cytogenetic analysis showed independent clones. One case was bitropic, and the other was surface immunoglobulin–negative (undetectable).

In contrast with the present cases, 1 case reported by Fernhout et al.\(^12\) coexpressed CD8 on the CLL cells. CD8 has been reported to be an indicator of a poor prognosis.\(^13\)
In an unusual report, Rechavi and colleagues\textsuperscript{14} studied 38 cases of CLL by Southern blot analysis and found multibandng patterns of IgH in 58% of samples, suggesting that biclonality may be more common than previously thought. Bitypical cases were not reported. Many of these cases with a multibandng pattern were assumed to be due to clonal evolution, occurred in patients with advanced stage disease, and may not be true independent clones.\textsuperscript{14} Clonal evolution at the karyotypic level has been shown to be common in a prospective study.\textsuperscript{15} To our knowledge, only 1 other well-documented case of bitypic clonal CLL similar to the 2 present cases has been described.

While the 2 present cases can be considered bitypic and biclonal, it is uncertain whether either represents a truly independent clonal process or whether the 1 clone represents a subclone of the other. Since independent cytogenetic clones were not found, definitive evidence for independent clones is not present.\textsuperscript{16}

Recent studies of the mutational status of the \textit{IgH} genes in CLL suggest that the cells may derive from either naïve B cells or post-germinal center memory cells.\textsuperscript{17,18} However, ongoing somatic mutations (which might in some cases lead to different gene rearrangement patterns) are believed not to occur, thus favoring the supposition that the 2 present cases are not biclonal as a result of clonal evolution.\textsuperscript{19-21} Furthermore, the 2 patients, particularly the patient described in case 2, presented with relatively low-stage disease, making clonal evolution as postulated by Rechavi et al\textsuperscript{14} less likely as a cause of second clone. This, of course, presupposes that the CLL developed at a mature stage and, thus, did not undergo further rearrangement.\textsuperscript{11,16} One cannot formally exclude a common immature B-cell precursor that subsequently rearranged \textit{IgH} genes to form 2 mature subclones.

The main diagnostic issue highlighted by the present report is that rare cases of CLL can seem polytypic by immunophenotyping. The fact that the patients we describe had significant absolute lymphocytosis with the appropriate morphologic features of CLL prompted further study to document clonal B-cell populations by molecular genetic techniques.

A differential diagnostic consideration is so-called persistent polyclonal B-cell lymphocytosis. Patients generally are asymptomatic young women who smoke and have the HLA-DR7 allele.\textsuperscript{22} These cases are CD5– B-cell proliferations, and occasional binucleated cells are evident on review of the peripheral blood smear (lacking in the present cases). As the name implies, the cases are polyclonal. Recently, polyclonal rearrangement of the \textit{bcl}-2 and immunoglobulin genes was reported.\textsuperscript{23} Interestingly, a case of a CD5+ polyclonal B-cell lymphocytosis mimicking CLL was reported.\textsuperscript{24} This case was polytypic by flow cytometric immunophenotyping. Flow sorting was not performed. Southern blot analysis of the mixed population was interpreted as polyclonal; however, examination of the illustrated autoradiograph suggested a possible biclonal pattern. One additional case of CD5+ persistent polyclonal B-cell lymphocytosis was reported that had a germline pattern shown by Southern blot analysis for B- and T-cell receptors.\textsuperscript{25}

The clinical significance of biclonal CLL is not certain since follow-up in the present cases is relatively short. In case 1, a typical long indolent course is apparent. However, it is unknown whether the biclonal nature of the disease is a new occurrence or was present at original diagnosis. The biclonal nature of case 2 seems to be an early occurrence. The stability of these clones over time and the clinical course, which to date seems indolent, will be of interest. We have had the opportunity to restudy case 2 nine months after the original flow cytometric analysis. The relative percentage of each clone remained the same (data not shown).

The occurrence of biclonal CLL is unusual and raises questions about whether these patients are particularly susceptible to developing CLL. There are reports of familial CLL and increased risk of CLL in relatives of patients with the disease.\textsuperscript{12,26-28} However, the factors involved (environmental or genetic) in this phenomenon are uncertain. One report suggests that HLA-B35 may be operative.\textsuperscript{26} HLA-typing was not performed in the present cases.

We report 2 cases of bitypic biclonal CLL that had the typical morphologic and immunophenotypic features of CLL except for the apparent polytypic pattern of light chain expression. Molecular genetic analysis confirmed the clonal nature of the 2 populations. This report calls attention to this uncommon entity and highlights an example in which light chain ratios can be deceiving. Careful attention to the morphologic features of the blood smear and the full immunophenotype will aid in the diagnosis.

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\textbf{References}


