Hematopathology / BICLONAL CLL WITH SEPARATE CLONAL ORIGINS

Molecular Characterization of Chronic Lymphocytic Leukemia With Two Distinct Cell Populations

Evidence for Separate Clonal Origins

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Key Words: Chronic lymphocytic leukemia; CLL; IgH rearrangement; Biclonal; Immunophenotyping

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Abstract

We report a case of an elderly woman with persistent lymphocytosis in whom flow cytometric immunophenotyping revealed 2 distinct clonal B-cell populations with different light chain restrictions. One clone was CD19+ (dim), CD5+ (bright), CD23+ (moderate), and κ+; the other clone was CD19+ (moderate), CD5+ (bright), CD23+ (dim), and λ+. We separated the 2 clones by flow cytometry from peripheral blood lymphocytes for further molecular and cytogenetic analysis. Polymerase chain reaction (PCR) analysis of the immunoglobulin heavy chain (IgH) genes revealed that the clones had bands of slightly different size. The PCR products of the framework 3 (FR3) region were cloned, and sequence analysis confirmed that each population had distinct, clone-specific IgH gene rearrangements. Fluorescence in situ hybridization (FISH) analysis revealed trisomy 12 in the λ-restricted B-cell clone, whereas the κ-restricted population had normal FISH patterns. Our results demonstrated that the immunophenotypically different cell populations originated from 2 separate clones.

Chronic lymphocytic leukemia (CLL) is a well-characterized B-cell lymphoproliferative disorder of mature lymphocytes identified by a combination of clinical, morphologic, immunophenotypic, and genetic abnormalities.1,2 In some cases, there may be 2 or more B-cell clonal populations in the same patients, raising the question of whether the populations are oligoclonal or of the same clonal origin. Several studies provided evidence for a biclonal B-cell lymphoproliferative disorder based on different light chain restrictions by flow cytometric analysis, different sizes of bands for framework 3 (FR3) regions of the immunoglobulin heavy chain gene (IgH) by polymerase chain reaction (PCR) amplification, IgH rearrangements by Southern blot analysis, or different karyotypic abnormalities.3-10 However, there were only a few reports that addressed the B-cell clonal relationship by sequencing the IgH rearrangements in the biclonal B-cell lymphoproliferative disorders.11,12 Because identification of clonal-specific IgH belongs among the most reliable methods to determine a clonal B-cell origin,13 we report a case of CLL with 2 immunophenotypically distinct B-cell populations that molecular and cytogenetic analyses demonstrated to originate from 2 separate clones.

Materials and Methods

Case Report

In March 2004, an 82-year-old woman was evaluated for persistent lymphocytosis (lymphocyte count, 53,000/µL [53 × 10⁹/L]). Physical examination revealed no evidence of lymphadenopathy or hepatosplenomegaly. Lymphocytes with
condensed chromatin, no nucleolus, and scant cytoplasm constituted 85% of the WBCs of the peripheral blood sample. The results of other hematologic and biochemical studies were within the normal range. The patient was diagnosed with CLL, Rai stage 0, and treated with intermittent chlorambucil, and her clinical condition was unchanged at last follow-up in January 2005.

### Flow Cytometric Immunophenotyping

For flow cytometric analysis, a 100-µL aliquot of peripheral blood was added to an appropriate monoclonal antibody combination. A whole-blood lysis method with Optilyse C Lysing Solution (Immunotech, Marseille, France) was used to eliminate RBCs, and the lymphocytes were immunophenotyped by using 3-color direct immunofluorescence on a Beckman Coulter FC-500 (Coulter, Miami, FL). The following antibodies were used in relevant combinations and conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or PC5 (PE-Cy5): CD3, CD4, CD5, CD2, CD7, CD8, CD16, CD56, CD57, CD25, T-cell receptor (TCR)-αβ, TCR-γδ, CD19, CD20, CD23, FMC7, and CD79b (Coulter) and κ and λ light chain (DAKO, Carpinteria, CA). Lymphocytes were identified and gated on a forward vs side-scatter histogram. Autofluorescence control and internal control were used for setting the cursors to interpret the surface markers.

### Cell Sorting

Ficoll-separated lymphocytes were washed and resuspended at 2 × 10⁶ cells/mL in phosphate-buffered saline supplemented with 1% human serum albumin (PBS/HAS; Bayer, Elkhart, IN). Next, 50-µL aliquots of cells were stained with 20 µL of κ-FITC and λ-PE and were incubated in the dark at 4°C for 30 minutes. Cells were washed 3 times, resuspended in 0.5 mL of ice-cold PBS/HAS, and sorted on a FACS Vantage (BD Biosystems, San Jose, CA) equipped with an Enterprise laser (Coherent, Santa Clara, CA) to obtain populations of κ+ and λ+ cells with a purity of more than 98%.

### PCR Amplification, Cloning, and Sequencing Analysis

DNA was extracted from peripheral blood and sorted clones using standard procedures. PCR analysis of B-cell clonality was performed with primers to the FR3 and joining region (J) of the IgH gene essentially as described by Aubin et al. The sequence of the FR3 primer was 5′-ACA CGG C(C/T)(G/C) TGT ATT ACT GT-3′ and for the JH primer was 5′-TGA GGA GAC GGT GAC C-3′. The amplification products were separated on a 1% agarose gel, and the predominant 100- to 120-base-pair DNA products were excised and purified using QIAquick Gel Purification columns (Qiagen, Valencia, CA). The purified fragments were ligated into the pGEM-T vector using the pGEM-T Easy Vector System I (Promega, Madison, WI) and plated onto Luria Bertani agar containing ampicillin and X-gal. Positive colonies were expanded in Luria Bertani media containing ampicillin and X-gal. The DNA was quantified by spectrophotometry and approximately 200 fmol of each plasmid sequenced using a Beckman Coulter CEQ 8000 DNA Sequencer (Beckman Coulter, Fullerton, CA) (University Health Network DNA Sequencing Facility, Toronto, Canada). The sequence data were assigned on the basis of their similarity to BLAST sequences and confirmed by homology to each germline sequence. Assignment of D and J segments was performed as defined by Ichihara et al and Yamada et al. It should be noted that a minimum of 7 homologous consecutive nucleotides generally is thought to be a prerequisite for D gene assignment.

### Fluorescence In Situ Hybridization Analysis

Standard cyt centrifuged preparations were prepared from the flow cytometry–sorted, κ+ and λ+ B-cell clones and submitted for fluorescence in situ hybridization (FISH) analysis. Each slide was fixed in methanol/glacial acetic acid (3:1), air dried, and pretreated in 2× standard saline citrate (SSC) at 37°C for 40 minutes; dehydrated in a series of 2-minute incubations with 70%, 90%, and 100% ethanol; and air dried.

The following locus-specific DNA probes were used: 13q14.3 (D13S319); 11q23(ATM); and α-satellite probes for the pericentromeric region of chromosome 12 (D12Z3), chromosome 8 (D8Z2), and 17p13.1(p53), all from Vysis (Downers Grove, IL). Interphase FISH was performed as dual-color hybridization with green (SpectrumGreen)- or red (SpectrumRed)-labeled fluorochromes. The probes

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**Image II** Peripheral blood sample showing lymphocytosis with mature lymphocytes (Wright, ×1,000).
were denatured under a coverslip using a hybridization mixture containing 50% formamide and 10% dextran sulfate.

The cells were hybridized overnight at 37°C, washed with 0.4× SSC at 72°C for 2 minutes, and transferred to 1× phosphate buffer detergent at room temperature for 1 minute. Antifade was added, and the cells were imaged on a fluorescent microscope equipped with a double bandpass filter for FITC and Texas red. Two hundred cells were scored from each slide. If the percentage of cells with abnormal signal patterns exceeded 10% of the evaluated cells, the specific genetic abnormality was scored as positive, as previously established.22

Results

Flow Cytometric Immunophenotyping

Flow cytometric immunophenotyping demonstrated a B-cell population expressing CD19, CD20, CD5, CD23, and FMC7, but examination of CD19 expression intensity revealed 2 separate populations. Gating on the CD19+ populations revealed κ- and λ-expressing B cells at 80% and 20%, respectively. Further analysis revealed that the CD19+ (moderate) cells expressed λ light chains, bright CD5, and dimmer CD23. The dimmer CD19+ cells expressed κ light chains, bright CD5, and moderate CD23 Image 2. The λ+ population had a slightly brighter CD20 expression than that from the κ+ population; both clones had similar patterns of heterogeneous expression of CD38. Both clones also dimly expressed FMC7 with similar intensity.

Molecular Analysis

PCR analysis for IgH rearrangements using FR3 primers showed a dominant band of approximately 120 base pairs of slightly different sizes in the 2 sorted cell populations Image 3. Each PCR product was purified and cloned. Three clones from each PCR product were sequenced successfully and showed identical sequences from each of the 2 cell populations. The sequences from population 1 were distinct from those of population 2 in their D and J segments Table 1. Sufficient material was not available from either sorted clone to perform Southern blot analysis to detect IgH rearrangements.

Interphase FISH Analysis

The flow cytometry–sorted cell populations were examined for the most common genetic aberrations in B-cell CLL: 13q deletions, 11q23 deletions, trisomy 12, and 17p (p53) deletions. The only abnormality detected was a trisomy 12 in 75% of the λ-restricted cells Image 4. The t(11;14), a typical translocation characteristic of mantle cell lymphoma, was not present in either clone (data not shown).
Discussion

B-cell CLL with more than 1 clone is rare and not well characterized. Sklar et al. suggested that at least 2 methods must be used to prove biclonality. There are several situations when more than 1 \( IgH \) rearrangement can be detected in patients with B-cell lymphoproliferative disorders: (1) Patients with CLL might have a cell clone expressing 2 different immunoglobulin molecules on the cell surface. The lack of allelic exclusion has been shown to be higher in CLL than in normal cells. (2) Patients have 2 \( IgH \) rearrangements detectable by PCR (one in-frame and one out-of-frame; biallelic rearrangements), but only the productive one is translated into an immunoglobulin protein. (3) Bimorphic lymphomas and biclonal leukemias represent situations in which patients have 2 malignant clones, each expressing a different immunoglobulin molecule. Common and separate clonal origins of the 2 tumor parts have been reported. It is important to identify patients with biclonal disease because they may have a poorer outcome.

In our case, FACS analysis showed 2 immunophenotypically different populations, cytogenetic analysis revealed 2 different clones with a specific genetic change in the \( \lambda^+ \) clone, and, it is important to note, molecular sequencing confirmed 2 distinct FR3 regions consistent with 2 separate and distinct B-cell populations.

In a case of biclonal or bitypic lymphoproliferative disorder, one also would ask the question whether it represents a truly biclonal process or whether one clone represents a subclone of the other. In this case, each of the clones carried distinct VDJ rearrangements. In clone 1, the JH6c segment was used, in contrast with the JH4b segment used in clone 2. Unlike in clone 1 in which the D segment (D4-11 or D4-4) sequences were identified, the actual sequence of the D segment homologous to

<table>
<thead>
<tr>
<th>Clone</th>
<th>V Segment</th>
<th>D Segment (D4-11 or D4-4)</th>
<th>JH6c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-ACACGGCTGTGATTACTGTG CGAGAGA-</td>
<td>-GCCCCGCACTAACTACGTGG -CTCA-</td>
<td>-ACTACTACTACTACTATGGACGTCACCCTCTCTCA-3'</td>
</tr>
<tr>
<td>2</td>
<td>5'-ACACGGCCGTGTATTACTGTG CGAGAGA-</td>
<td>-TGCCGTAGTTAGCCAGGGACGTTG ACTGCAAGT-</td>
<td>-ACTACTTTTACTAGGCGACCAGGAACCCC TGTCACCCTCTCTCA-3'</td>
</tr>
</tbody>
</table>

D segment, sequence corresponding to a D segment germline sequence; J segment, sequence corresponding to a J segment germline sequence; N, N nucleotides; V segment, sequence corresponding to a V segment germline sequence.

The sequence data were assigned on the basis of their similarity to BLAST sequences and confirmed by homology to each germline sequence.

![Table 1](image1.png)

![Table 1](image2.png)

**Image 1** Fluorescence in situ hybridization analysis of chronic lymphocytic leukemia cells with D13S319 (red) and CEP12 (green) probes showing normal signal patterns in the \( \kappa^+ \) clone (A) but 3 CEP12 signals indicating trisomy 12 in the \( \lambda^+ \) clone (B).
the germline sequence could not be found in clone 2, probably owing to extensive somatic mutations. There have been multiple studies showing CDRIII sequences when the D segment gene could not be assigned. This has been explained by extensive mutations or nucleotide additions or loss. Both clones had productive immunoglobulin because the sequences of both FR3 regions were in-frame. The present case showing 2 unrelated VDJ rearrangements and an independent cytogenetic lesion (trisomy 12) found exclusively in one clone but not the other strongly supports an independent biclonal process.

Recent studies of the mutational status of the IgH gene in CLL suggest that the cells may be derived from naive B cells or post–germinal center memory cells. In view of a mature CLl suggest that the cells may be derived from naive B cells or post–germinal center memory cells. This has been explained by extensive mutations or nucleotide additions or loss. Both clones had productive immunoglobulin because the sequences of both FR3 regions were in-frame. The present case showing 2 unrelated VDJ rearrangements and an independent cytogenetic lesion (trisomy 12) found exclusively in one clone but not the other strongly supports an independent biclonal process.

Recent studies of the mutational status of the IgH gene in CLL suggest that the cells may be derived from naive B cells or post–germinal center memory cells. In view of a mature B-cell phenotype of both clones, the malignant transformation may occur in 2 independent B cells during or after germinal center development. Indeed, it has been suggested that antigen receptor editing or revision, hypermutation, and other genetic instability events in the germinal center and beyond make essential contributions to many chromosomal aberrations in lymphomas. Therefore, it is possible that in our case, while each B-cell population expands its own clone, additional genetic changes accumulate, such as trisomy 12 in the + clone. However, one cannot formally exclude the possibility that the oncogenic events may occur as early as in pro-B cells or pre-B cells during VDJ recombination in the bone marrow.

Fewer than 5% of chronic B-cell lymphoproliferative disorders in leukemic phase have more than 1 clone. The disease in patients with CLL with 2 B-cell clones can be classified into CLL + CLL and CLL + non-CLL subgroups. In this case, one CD19+ (dim), CD5+ (bright), and CD23+ (moderate) and the other CD19+ (moderate), CD5+ (bright), and CD23+ (dim) B-cell populations with different light chain restrictions were identified, consistent with an immunophenotypic profile of CLL + CLL clones. It was suggested that patients with CLL and non-CLL populations require treatment within the first 18 months significantly more often than patients with only CLL or 2 CLL clones. In the present case, however, because of the clinical symptoms, anemia and significant increase of lymphocytes, our patient was treated with chlorambucil 6 months after diagnosis.

We report a case of a B-cell lymphoproliferative disorder with 2 CLL clones. Comprehensive immunophenotypic, cytogentic, and molecular analysis clearly demonstrated 2 separate clonal origins with different VDJ rearrangements, but the issue remains unresolved about which stage the oncogenic events occurred in during B-cell development. The recognition of biclonal CLL has clinical implications because patients may require earlier therapeutic interventions.

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References


