Evidence for Progenitors of Chronic Lymphocytic Leukemia B Cells That Undergo Intraclonal Differentiation and Diversification

By Mariella Dono, Shiiori Hashimoto, Franco Fais, Velia Trejo, Steven L. Allen, Stuart M. Lichtman, Philip Schulman, Vincent P. Vinciguerra, Brian Sellers, Peter K. Gregersen, Manlio Ferrariini, and Nicholas Chiorazzi

Peripheral blood mononuclear cells from five patients with IgG* B-type chronic lymphocytic leukemia (B-CLL) were analyzed for the presence of clone-specific Ig H chain variable region gene mRNA transcripts linked to Cµ and/or Ca. This was assessed by (1) comparing the lengths of portions of the Vµ, Dµ, and Jµ of the IgG* CLL clones with those of the µ and α isotype-expressing B cells, (2) performing clone-specific endonuclease digestion studies, and (3) determining the DNA sequences of the µ and α isotype-expressing cDNA. Thus, when B-cell mRNA from these five patients were reverse transcribed with Cµ-specific primers and then amplified by polymerase chain reaction, dominant cDNA were found with lengths corresponding to those of the IgG* CLL B cell. In addition, in four cases, cDNA of lengths identical to those of the CLL B cell were detected when mRNA was reverse transcribed and amplified using Cµ- and/or Ca-specific primers, strongly suggesting clonal relatedness. These CLL-related µ- and α-expressing cDNA were present in greater amounts than unrelated (non-CLL) µ- and α-expressing cDNA from normal B cells that used genes of the same Vh family. When the sequences of these CLL-related Cµ- and Ca-expressing cDNA were compared with those of the IgG* CLL clones, it was clear that they were derived from the same ancestral gene as the IgG-expressing CLL B cell, thus documenting their common origin. Finally, nucleotide point mutations were observed in the µ- and α-expressing cDNA of certain patients, indicating divergence with the CLL. These data suggest that IgG* B cells, which are precursors of the leukemic B cells, exist in increased numbers in the blood of most patients with IgG* B-CLL and that these cells may differentiate, accumulate V gene mutations, and undergo isotype switching in vivo. In addition, the data are consistent with a sequential-hit model for the evolution of CLL.

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B^TYPE CHRONIC lymphocytic leukemia (B-CLL) is characterized by the expansion of a clone of mature B lymphocytes, most of whose members are blocked at the membrane Ig-expressing stage of differentiation. However, several studies have shown that a subset of these B-CLL cells may be induced to undergo terminal differentiation. For instance, in vitro, B-CLL B cells from many patients can be induced to differentiate to Ig-secreting cells when exposed to activated T cells or polyclonal B-cell activators. In addition, in vivo, a variable number of plasmacytoid cells producing the monoclonal Ig characteristic of the B-CLL can be found in the blood of certain patients, and this monoclonal Ig can be detected in the serum of a substantial number of cases when sensitive techniques are used. However, in B-CLL, the range of this maturation process appears limited, inasmuch as serologic studies have shown that isotype class switched (non-IgM) B-CLL cells are infrequent. However, these observations are at variance with those in other lymphoproliferative disorders such as lymphoma or myeloma, in which expansions of isotype-switched, non-IgM B cells are much more common or the rule. In certain instances, analyses of isotype-switched myeloma cells have shown the existence of abnormal cells at an earlier maturational stage than the bulk of the neoplastic cells.

Antigen specificity in B cells is accomplished through the assembly of distinct V, D, and J gene segments of heavy (H) and light (L) chain variable region genes. At the time of these rearrangements, the V gene sequences can be modified further by the addition of nucetides at the D-J and V-DJ junctions as well as by the loss of portions of these segments due to distinct enzymatic activities. Finally, after attainment of an intact B-cell antigen receptor complex, these rearranged genes can undergo further change due to mutation. The results of these three sets of changes (rearrangement, coding end processing, and somatic mutation) yield nucleotide sequences and lengths that are unique to an individual B-cell clone. These signature sequences identify members of a specific B-cell clone at various stages of differentiation.

In this study, we have analyzed the blood B cells from five patients with IgG*, CD5+ B-CLL with the aim of elucidating whether mRNA transcripts coding for patient-specific H chain variable region genes, but linked to Cµ and Ca H chain genes, were expressed. Indeed, such transcripts have been identified in four of the five cases, indicating that IgG-producing precursors of the IgG* CLL B cells existed in the blood and that these cells underwent isotype class switching to IgG and IgA. Collectively, the data suggest that the leukemic process may involve several steps that begin at the level of an IgM-bearing progenitor cell and include differentiation to IgA- and IgG-producing progeny, one of which accumulates a new transforming event(s) that leads to the manifest B-CLL clone.

MATERIALS AND METHODS

Patients. The B-CLL cases studied here were drawn from a cohort of patients whose expanded clone of CD5-expressing B cells produced IgG. These patients exhibited typical clinical and laboratory features of B-CLL that spanned all the Rai stages. The Ig Vh gene family coding end processing, and somatic mutation) yield nucleotide sequences and lengths that are unique to an individual B-cell clone. These signature sequences identify members of a specific B-cell clone at various stages of differentiation.

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and Vγ genes used by these IgG+ CLL cells have been analyzed and reported previously.16

Monoclonal cell suspensions were obtained from heparinized peripheral blood by centrifugation through Ficoll-Isoaque density gradients (GIBCO BRL, Gaithersburg, MD) and the surface membrane phenotypes were determined.16 Aliquots of these cells were frozen using a programmable cell freezer and stored in liquid nitrogen. The studies reported here were performed with these frozen cells and/or fresh cells, if available.

**RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) conditions.** Total RNA was extracted with guanidium isothiocyanate-cesium chloride and RT-PCR, specific for the rearranged H chain variable region genes, was performed as follows. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (GIBCO BRL) from 1 μg of total RNA using 20 pmol of oligonucleotide primer specific for the constant (C) region of the human μ, γ, and α H chains (Table 1). Ig cDNA then was amplified by a two-step PCR technique using two sets of primer pairs (60 pmol each; Table 1). The first round of amplification used an upstream leader-specific Vγ primer and a downstream Cγ-specific primer. The second round used a FR2 Vγ family-specific primer with a nested antisense primer upstream of the first Cγ primer (Table 1). The primer pairs used in individual experiments varied, depending on the gene segments to be amplified (either FR2 → Jγ or FR2 → Cγ; vide infra). PCR was performed for 30 to 35 cycles with a Perkin Elmer Cetus 9600 apparatus (Emeryville, CA) as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. After the last cycle, the synthesis was extended for 10 minutes.

**DNA cloning and sequencing.** The products of the second, nested amplifications were purified with MagicPrep DNA purification columns (Promega, Madison, WI), ligated into TA vectors, and then transformed into InvαF competent bacteria (Invitrogen, San Diego, CA). Multiple colonies were chosen randomly and sequenced in both directions by the dideoxy-chain termination method, either manually using 3′-S-dATP (Du Pont-NEN, Boston, MA) and the Sequenase Sequencing kit (US Biochemical, Cleveland, OH) or automatically using fluorescent-labeled ddNTP and T4 polymerase (1 System; Applied Biosystems, Foster City, CA).

**Ig Vγ gene fingerprinting analyses.** To assess the potential relatedness of specific rearranged VγDJH segments of individual B-CLL clones, the lengths of their Ig Vγ genes were approximated by the two-step PCR described above, except that the downstream Jγ primer was end-labeled with γ2-3′P (NEN) using T4 polynucleotide kinase (Promega). In these reactions, the primers were reduced to 3 pmol/reaction. After amplification, the radiolabeled products were electrophoresed through 4% acrylamide sequencing gel. The gel then was dried and exposed to x-ray film (Kodak, Rochester, NY) for 1 to 3 days at −80°C to visualize the radiolabeled products.

In selected experiments, the nested Vγ family-specific primer was end-labeled and the amplifications were performed as described. These radiolabeled products then were digested with specific restriction endonucleases (Pvu II and Sac I; GIBCO BRL) as recommended by the manufacturer and the fragments were electrophoresed and visualized as above. The relative amounts of these products were analyzed by comparing their incorporated radioactivity using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Identification of B-CLL patient-specific mRNA transcripts by comparing VγDIH segment lengths.** Peripheral blood mononuclear cells from five patients were selected for the following studies based on sample availability. Four of these cases (no. 001, 033, 039, and 057) had rearranged Vγ4 family genes and one (no. 030) had a Vγ3 gene (Table 2). Because of recombination and/or mutation, portions of the rearranged Ig H chain variable region genes of each patient were unique and distinct from their germline counterparts (Table 2).18,20 For patients no. 001, 030, and 033, these unique DNA sequences spanned all three gene segments (Vγ, D, and Jγ), whereas for patients no. 039 and 057 they were restricted to CDR3 and FR4.18 Nevertheless, for each patient, these gene sequences provided unequivocal markers of the individual patient’s CLL B-cell clone.

To determine whether these patient-specific VγDIH genes were associated with Ig H chain isotypes other than IgG, we used the Ig V gene fingerprinting approach.31,32 This technique allowed comparisons of VγDIH cDNA lengths generated from μ and α isotype-expressing mRNA transcripts with those generated from the γ-expressing B-CLL cell. This was accomplished with primers (Table 1) designed to specifically amplify the FR2 to JH regions of B cells using genes of the same VH family as the B-CLL cell but associated with either Cμ or Cα. The nucleotide lengths of these products then were compared with those of the corresponding IgG+ B-CLL clone as radiolabeled bands on a polyacrylamide sequencing gel (Fig 1).

In four of the five patient samples tested (no. 030, 033, 039, and 057), dominant bands with lengths identical to those of the IgG+ B-CLL, but associated with μ and/or α H chains, were detected. Because this technique takes advantage of the fact that B cells are heterogeneous, and relatively unique, in their rearranged VγDIH lengths, the demonstration that cDNA products amplified from the γ-expressing B-CLL cell mRNA transcripts and from μ- and α-expressing transcripts are of identical length is highly suggestive of a common clonal origin.

As controls, Ig cDNA were amplified from the mRNA of blood B cells from normal individuals using the same sets of primer pairs. These PCR products ran in the gel as a ladder of approximately equal intensities, indicating the heterogeneity and relatively uniform distribution of VγDIH lengths in normal circulating B cells (Fig 1; far right lane). Note that this pattern of bands was detectable in some of the B-CLL lanes (eg, patients no. 057 and 033; Fig 1), indicating the presence of normal, nonleukemic B cells in the blood of these patients. These normal B cell bands were more frequent in those lanes containing products initially amplified with the Cα primer (Fig 1). In some cases, the CLL-related band was almost the only band visible, sug-

**Table 1. PCR Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vγ4 (leader)*</td>
<td>5′ GGAATTCATGGAACATCCCTGGTTGTCTCTCC</td>
</tr>
<tr>
<td>Vγ3 (leader)*</td>
<td>5′ GGAATTCATGGAGCTGATTCAGCTGGTTG</td>
</tr>
<tr>
<td>Vγ4–FR2</td>
<td>5′ AAGGGCGTGGAGTGCCAGTGGG</td>
</tr>
<tr>
<td>S, consensus</td>
<td>5′ CTGAGGACCGCTGAGCCGGGCC       (rc)</td>
</tr>
<tr>
<td>IgG Cγ1</td>
<td>5′ CCAAGCTTGAACGCGCGCGCTG       (rc)</td>
</tr>
<tr>
<td>IgM Cδ2</td>
<td>5′ CAAAGCTTGAGGCTGATTCAGCTGGTTG</td>
</tr>
<tr>
<td>IgA Cε1</td>
<td>5′ CCAAGCTTGAACGCGCGCGCTG       (rc)</td>
</tr>
<tr>
<td>IgA Cε2</td>
<td>5′ GACCTTGAGGCGCTGTCGAGATGGC     (rc)</td>
</tr>
</tbody>
</table>

Abbreviation: rc, reverse complement.
*Underline denotes HindIII site.
1 Underline denotes HindIII site.

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suggesting that B cells of this clone were dominant and that normal B cells expressing these isotypes were scarce. This was most readily appreciated in the IgM cDNA lanes (eg, no. 039), although it is also apparent in certain IgA lanes.

It should be noted that bands of the same size as the IgG⁺ B-CLL cell could not be consistently amplified from the μ- and α-expressing transcripts of patient no. 001, indicating that this phenomenon was not universal.

Confirmation of clonal relatedness by restriction endonuclease sensitivity. As mentioned, the finding of similar $V_{H}D_{J_{H}}$ lengths among the γ-, μ-, and α-containing transcripts strongly suggests, but does not prove, that they arise from the same B-cell clone. To further confirm the clonal relatedness of the PCR products, we took advantage of the fact that the diversification events that altered the germline $D_{J_{H}}$ segments used by the B-CLL clone had created clone-specific endonuclease restriction sites in CDR3 that were not present in the germline DNA. These somatically generated sequences would not be shared by other B cells, even if they used the same germline gene segments. For example, in patient no. 039 a number of nucleotide changes in the DM1 D segment resulted in the acquisition of a site recognized by the enzyme Pvu II (underlined in Fig 3) that we used as a target for clone-specific endonuclease digestion.

Therefore, the radiolabeled cDNA generated with the Cy, Cμ, and Cα primers from patient no. 039 were either digested with Pvu II or were left untreated. When these two sets of products were electrophoresed through a 6% acrylamide gel (Fig 2A), the undigested fragments again comigrated at a position commensurate with their calculated size (252 bp). The lanes containing the enzyme-digested products of all three H chain transcripts showed a marked diminution in the amount of material migrating at 252 bp (15%, 5%, and 23% of the original counts in the γ, μ, and α undigested lanes, respectively, as determined by a phosphorimager; Fig 2B). In addition, a new set of dominant bands appeared at 192 bp, consistent with the predicted size of the enzyme digested products (Fig 2A). Note that the additional cDNA bands generated from the normal α-expressing blood B-cell transcripts were not altered by the enzyme treatment, indicating that the enzymatic digestion was clone-specific. A similar experiment was performed for patient no. 030 using Sac I with comparable results (data not shown).

DNA sequence analyses of $V_{H}D_{J_{H}}$ gene segments amplified with Cμ and Cα primers. Because the V gene sequences of the IgG-producing B-CLL clones were known, we cloned and sequenced the cDNA amplified with the Cμ and Cα primers to definitively establish their clonal relatedness with the IgG⁺ B-CLL cells of the various patients.

Three classes of sequences were identified (summarized in Table 3). The first was identical to those of the IgG⁺ CLL; the second was very similar and clearly derived from the same ancestral gene but with occasional single nucleotide differences. In contrast, the third class was completely different from those of the IgG⁺ CLL B cell (albeit derived from genes of the same $V_{H}$ family). Thus, the B cells expressing genes of the first two

<table>
<thead>
<tr>
<th>Patient</th>
<th>$V_{H}$ Family</th>
<th>Most Homologous Germline $V_{H}$ Gene</th>
<th>Percentage of $V_{H}$ Gene Difference</th>
<th>Likely Germline D Segment</th>
<th>$J_{H}$</th>
<th>Lengths* (CDR3; FR2 → $J_{H}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL 001</td>
<td>4</td>
<td>4.21</td>
<td>5%</td>
<td>D21/9</td>
<td>6</td>
<td>66bp; 255bp</td>
</tr>
<tr>
<td>CLL 033</td>
<td>4</td>
<td>4.21</td>
<td>8%</td>
<td>DLR2</td>
<td>6</td>
<td>75bp; 267bp</td>
</tr>
<tr>
<td>CLL 039</td>
<td>4</td>
<td>4.18</td>
<td>1%</td>
<td>DM11</td>
<td>2</td>
<td>57bp; 252bp</td>
</tr>
<tr>
<td>CLL 057</td>
<td>4</td>
<td>4.18</td>
<td>1%</td>
<td>DN1</td>
<td>5</td>
<td>60bp; 255bp</td>
</tr>
<tr>
<td>CLL 030</td>
<td>3</td>
<td>H11</td>
<td>6%</td>
<td>Indeterminant</td>
<td>6</td>
<td>42bp; 251bp</td>
</tr>
</tbody>
</table>

Data from Hashimoto et al.16,18

* Lengths of CDR3 calculated from the cysteine residue near the 3’ end of FR3 to the tryptophan at the beginning of FR4; lengths of FR2 → $J_{H}$ calculated from the beginning of the respective FR2 primer to the end of the consensus $J_{H}$ primer. The absolute lengths of FR2 → $J_{H}$ vary because of differences in both gene segment lengths and primer locations.

![Fig 1](https://example.com/Fig1.png)

Fig 1. $V_{H}$ gene fingerprinting analyses. A 6% acrylamide gel showing the presence of $V_{H}D_{J_{H}}$ cDNA with lengths identical to those of the leukemic cells associated with multiple H chain isotypes. In each patient sample, the dominant bands in the first lane (G; noted by arrow) correspond to the products generated from the total cellular RNA with the downstream $C_{Y}$ primer (CLL population). The major bands in the second (M) and third (A) lanes correspond to the products generated with the $C_{\mu}$ and $C_{\alpha}$ primers, respectively. The less prominent, yet distinct bands seen in some of the lanes represent products generated from the nonleukemic B cells. These are similar to those generated from mRNA of normal B cells using the $V_{H}$-FR2 and $C_{\mu}$ primers (M; far right lane).

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Table 3. cDNA Clones Detected

<table>
<thead>
<tr>
<th></th>
<th>IgM-Bearing</th>
<th>IgA-Bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clonally Related</td>
<td>Not Related</td>
</tr>
<tr>
<td></td>
<td>Identical</td>
<td>Very Similar</td>
</tr>
<tr>
<td>CLL001</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CLL030</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CLL033</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CLL039</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>CLL057</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Fig 2. Demonstration of clonal relatedness by restriction endonuclease sensitivity. (A) The cDNA generated with the Cy, Cp, and Ca primers from patient no. 039 were either digested or not with Pvu II (restriction site underlined in Fig 3) and then electrophoresed through a 6% acrylamide gel. Note that the CLL-related bands were specifically digested, whereas the additional cDNA bands generated from the normal a-expressing blood B-cell transcripts were not altered by the enzyme treatment. (B) The bands corresponding to the untreated (252 bp) and enzyme-digested (192 bp) products of the three H chain transcripts were quantified by a PhosphorImager. A similar experiment was performed for patient no. 030 using SAC1 with comparable results (data not shown).

classes were derived from the same B-cell clone as the B-CLL, whereas those of the third class were most likely derived from the residual normal B cells in the blood and were not related to the CLL clone. Figures 3 and 4 list examples of those B-CLL-related sequences derived from patients no. 039 and 057 that showed point differences from the B-CLL; identical sequences are not presented. The nucleotide sequences for patients no. 001 and 033 are not shown because, in the former case, no cDNA clones expressing the $\mu$ or $\alpha$ H chain gene could be isolated, whereas in the latter all the related clones (4 IgM and 4 IgA) exhibited complete identity with those of the IgG$^+$ CLL B cells.

The frequencies of these three classes of clones varied from patient to patient, as did the degrees of similarity with the corresponding IgG$^+$ B-CLL cell (Table 3). For example, in patient no. 039, 10 IgM-expressing cDNA were analyzed, and 9 of these were members of the IgG-expressing B-CLL clone (8 identical and 1 clonally related; Table 3 and Fig 3). However, of the 10 IgA cDNA studied, only 5 were related to the B-CLL clone (4 identical and 1 clonally related). These data suggested that, in the blood of this patient, an expanded population of IgM-producing B cells existed that were clonally related to the leukemic cell. This observation is in agreement with the PCR data shown in Fig 1 that fail to show bands, other than the dominant B-CLL band, amplified with the C$\mu$ primer.

In contrast, the IgM- and IgA-expressing cDNA from patient no. 057 were more heterogeneous (Table 3 and Fig 4). In fact, although significant numbers of $\mu$- and $\alpha$-expressing clones were analyzed, none was identical to that of the IgG-expressing B-CLL. However, 4 of 10 IgM and 4 of 10 IgA DNA clones displayed significant similarity in the FR2-JH sequence with the IgG$^+$ CLL B-cell, indicating clonal relatedness.

Finally, in patient no. 030, only cDNA expressing the CLL-specific V$\mu$DJ$\beta_2$ in association with a $\mu$ gene segment could be identified (Table 3), even though the PCR data showed the presence of an IgA-related species. The reason for this discrepancy is unclear. Of the eight IgM$^+$ cDNA sequenced, two were clonally related to the IgG-expressing B-CLL cell; one of these (M208) showed complete sequence identity with the CLL and the other (M133) was slightly different (data not shown).

Demonstration of CLL-specific V$\mu$DJ$\mu$ sequences attached to C$\mu$ and C$\alpha$. Because the DNA sequencing data mentioned above were derived from PCR products spanning FR2-JH, formal proof of the physical association of the IgG$^+$ B-CLL V$\mu$DJ$\mu$ with C$\mu$ and C$\alpha$ was not obtained. Therefore, new cDNA were generated from the same mRNA of patients no. 039 and 057 using the same set of upstream nested primers (V$\mu$ leader followed by FR2) but with nested pairs of C$\mu$-specific or C$\alpha$-specific primers (IgM Cy1,1 and 2 and IgA C$\alpha_1$1 and 2; Table 1). In each patient, sequence analyses showed clones expressing the B-CLL V$\mu$DJ$\mu$ in direct association with C$\mu$ and C$\alpha$ gene segments (data not shown), thus eliminating the possibility that the data presented above were generated artificially by cross-priming from mRNA coding for B-CLL V$\mu$DJ$\mu$-C$\gamma$.

Sequence diversity between the IgG$^+$ CLL B cells and their clonal members. As mentioned above, the nucleotide sequences of the IgM- and IgA-expressing cDNA showed varying degrees of intraclonal diversity among the patients. For example, no diversity was found among the cDNA of patient no. 033 as all of the B-CLL-related IgM- and IgA-
expressers analyzed were identical to the known IgG+ B-CLL sequence (data not shown).

However, the cDNA from patient no. 039 did exhibit some nucleotide differences (M5 and A32; Fig 3). In each of these, there is a single nucleotide difference at codon 70 of FR3 (TCC → TCA) that distinguished the V\_H genes of these cells from the germline V\_H 4.18 sequence and from that carried by the IgG+ CLL B cell. This suggests that the C → A mutation occurred in the IgM-expressing precursor and was passed on to the IgA-expressing descendants. In addition, because this mutation is present in the M5 and A32 clones but absent from the ancestral gene and from the B-CLL, it is likely that the B cell that evolved into the B-CLL clone developed an initial molecular abnormality at a maturational stage earlier than that reflected by the IgM clone. Subsequently, the IgA-expressing cDNA A32 developed three additional nucleotide differences, two involving codon 53 of FR3 (TAT → CAG) and one involving codon 81 of FR3 (AAG → AAA). The difference at codon 53 resulted in an amino acid replacement of Tyr with Gln.

In contrast, the \(\mu\)- and \(\alpha\)-expressing cDNA of patient no. 057 exhibited much more diversity involving areas of CDR2, FR3, and CDR3 (Fig 4). Thus, clones M182 and A192 showed differences in CDR2 at codons 50 (AGT → AAT) and 61 (CCG → TCG); the former resulted in a Ser → Asn amino acid replacement and the latter a Pro → Ser change. In addition, clone A192 showed substitutions at codons 92 (GTC → CTG; replacement of Gly with Leu) of FR3 and at 100C (TAT → TAC) of CDR3. This latter difference from the IgG+ B-CLL cell corresponds to the germline 4.18 sequence and may therefore represent either a nonmutated residue or a new mutation that resulted in a reversion to the original germline sequence.

As noted, several of these nucleotide substitutions resulted in amino acid replacement changes that might impact on the antigen-binding capabilities of the \(\mu\)- and \(\alpha\)-expressing B cells as compared with their B-CLL relative. These amino acid changes in patients no. 039 and 057 may be especially significant because both patients' CLL B cell use the V\_H 4.18 gene, which has been shown to rarely accumulate replacement mutations when found in B-CLL cells.18,23-25

**DISCUSSION**

Our data suggest that patients with IgG+ B-CLL frequently harbor IgM-expressing B cells that are precursors of the CLL B cell and that may switch to IgA or IgG production. The presence of clonally related, isotype class switched members of a malignant clone has been detected in other lymphoproliferative disorders, including follicular26 and Burkitt's27 lymphoma, Richter's transformation,28-30 acute lymphocytic leukemia,31,32 and multiple myeloma (MM).11-15 However, this is the first demonstration of this phenomenon in B-CLL.

A key issue in these studies is whether the B-CLL-related
This page discusses the presence of Ig variable region gene sequence diversity among precursor B cells in the bone marrow and blood of MM patients. The text mentions that several lines of evidence suggest that this is not the case in our patients. The most convincing of these are the facts (1) that within the same patient the \( \mu \), \( \alpha \), and \( \gamma \) expressing transcripts can differ from each other by mutations in the V\( \gamma \)DJ\( \gamma \) segments, a finding incompatible with a common cellular derivation (Figs 3 and 4 and Table 3); and (2) that hybridomas made from these cells produce only one isotype. In further support of this contention, others have documented the existence of precursor B cells in the bone marrow and blood of MM patients that reside in the CD45\( ^{-} \) B-cell subset and that are distinct from the mature MM cells that display a CD45\( ^{+} \), CD38\( ^{+} \) phenotype. We are presently attempting, using a three-color immunofluorescence approach, to identify the clonally related B cells in the blood of our B-CLL patients. Preliminary data suggest the presence of an expanded population of IgM\( ^{+} \), CD5\( ^{+} \) B cells in patient no. 039, although this population is much less abundant than the bona fide IgG\( ^{+} \) B-CLL population detected by routine clinical techniques.

The presence of Ig variable region gene sequence diversity among the C\( \mu \)- and C\( \alpha \)-expressing clones of B-CLL patients indicates that these B cells were still able to undergo the mutation process. This observation is in variance with those reported in MM and may indicate an innate difference between the B cells that become transformed into B-CLL versus MM, or a difference in the ability of the clonally related members to respond to antigen and/or T-cell help. In this regard, our previous studies suggested that antigen drive and selection may have been operative in the life span of the B cells overexpanded in certain of these B-CLL cases. It is possible that technical issues limited the likelihood of detecting somatic variants in MM, because in that study only the MM patient-specific regions of CDR3 were compared for the accumulation of somatic mutations. This issue requires more extensive evaluation of more patients with these diseases.

The preceding data may have implications for our understanding of the development of B-CLL and its complications. For instance, it will be important to determine whether switching of the IgM-expressing precursors to IgG\( ^{+} \) B-CLL cells is an ongoing event that continuously expands and/or replenishes the B-CLL pool. Similarly, it will be of interest to determine whether the B-CLL-related IgM- and IgA-producing B cells secrete their Ig and whether the sera of these patients contain multiple monoclonal bands corresponding not only to the B-CLL IgG monoclonal antibody (MoAb) but also to the clonally related IgM and IgA MoAb.

In this regard, it should be noted that others have reported the presence of two or more monoclonal paraproteins in various lymphoproliferative disorders, some of which have been shown to be derived from the same clone. In addition, it will be important to determine whether patients with typical IgM-producing B-CLL also develop in vivo switched cells that produce IgG and IgA antibodies. If so, this phe-
nomenon might have clinical relevance to those patients with B-CLL who develop autoimmune complications that are mediated by IgG antibodies, eg, autoimmune hemolytic anemia. We are in the process of addressing these issues. Preliminary data suggest that ≈50% of IgM+ B-CLL patients exhibit mRNA transcripts encoding patient-specific \( V_{\mu}D_{\mu}J_{\mu} \) segments in association with \( C_{\gamma} \) and/or \( C_{\alpha} \).

Finally, our data suggest that the B cell that eventually becomes the CLL clone proceeds through a multistep (at least 2) process that alters certain normal functions and not others. A proposed model of these events is shown in Fig 5: First, a normal IgM-producing B cell experiences event no. 1, which leads to an appreciable (Fig 1 and Table 3), but clinically in evident, clonal expansion. Thus, even at this pre-CLL stage, the clone appears to have a proliferative and/or survival advantage. However, despite this, the pre-CLL clone is/was able to accept appropriate signals and undergo isotype class switching and to develop Ig variable region gene mutations. Eventually, from among this pool of IgG-expressers, one member then experiences a second event that results in a much more significant clonal expansion that is recognized clinically as manifest CLL (event no. 2; Fig 5). This multistep process is more easily appreciated in IgG+ B-CLL patients because the B cells that experience events no. 1 and 2 can be distinguished by their different membrane Ig isotypes. However, we presume that a similar process occurs in IgM+ B-CLL clones, although its demonstration will require different strategies.

Based on our present and prior \(^{18} \) studies, it appears that a difference exists in the ability of the CLL B cell versus its clonal relatives to develop new Ig variable gene mutations. Although several examples of diversification were seen in the clonally related members, only rare mutations were detected within the CLL B cells, even after several years of in vivo maturation.\(^ {18} \) This difference suggests that the mutation machinery is operative in the pre-CLL B cells, but is significantly downregulated in overt CLL.

These observations also may be relevant to the different numbers of Ig V gene mutations detected in overt CLL B cells of the IgM versus IgG isotypes. Indeed, in the absence of a more profound molecular insult of the type seen in Richter’s transformation,\(^ {44} \) mutations in IgM-expressing B-CLL cells are clearly much less frequent\(^ {35-47} \) than in IgG-expressers.\(^ {18,20} \) This suggests that the likelihood of mutations being observed in a B-CLL clone increases with the duration and quality of antigenic and accessory signals delivered and the maturation attained by the B cell before its complete transformation by event no. 2.

The nature of the events postulated above that provide the increased proliferative and/or survival capacity and the decreased mutation capability are unknown. However, one might speculate that these represent molecular defects that have occurred as a result of chronic in vivo (auto)antigenic stimulation, possibly as a consequence of their known frequent autoreactive potential.\(^ {46-50} \)

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