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Lack of Intraclonal Diversification in Ig Heavy and Light Chain V Region Genes Expressed by CD5⁺ IgM⁺ Chronic Lymphocytic Leukemia B Cells: A Multiple Time Point Analysis

Edward W. Schettino,* Andrea Cerutti,* Nicholas Chiorazzi, † and Paolo Casali2*

To analyze the modalities of clonal expansion of chronic lymphocytic leukemia (CLL) cells, we sequenced at multiple time points the V(D)J genes expressed by CD5⁺ IgM⁺ CLL B cells in three patients. All three V(D)J gene sequences were found to be point mutated. The mutation frequency in the Ig VH (3.96 × 10⁻² and 2.41 × 10⁻² change/bp) and VH and VL (6.67 × 10⁻² and 1.74 × 10⁻² change/bp) genes of two CLLs (1.19 and 1.32, respectively) was similar, and higher than that in the corresponding gene segments of the third CLL (1.69; 3.4 × 10⁻³ and 6.67 × 10⁻³ change/bp). In all three CLLs, there was no preferential representation of nucleotide changes yielding amino acid replacement (R mutations), nor was there any preferential segregation of R mutations within the Ig V gene complementarity-determining regions. In all three CLLs, the somatic mutations were all identical in multiple Ig VH and VH transcripts at any given time point, and were all conserved at multiple time points throughout a 2-yr period. The lack of concentration of R mutations in the complementarity-determining regions and the lack of intraclonal heterogeneity suggest that Ag may no longer be able to play a significant role in the clonal expansion of these cells. This conclusion would be strengthened further by the germine configuration of the bcl-1 and bcl-2 proto-oncogenes that are translocated in neoplastic B cells that display significant traces of intraclonal diversification and Ag-dependent selection, such as B-prolymphocytic leukemia and low grade follicular non-Hodgkin lymphoma. The Journal of Immunology, 1998, 160: 820–830.

Chronic lymphocytic leukemia (CLL) is the most frequent form of adult leukemia in Western societies, accounting for 30% of all leukemias. In the recently proposed definition of the disease, the malignant B cells are surface CD5⁺ (1, 2), express mainly IgM, and are thought to represent the neoplastic expansion of a single clone that replaces the normal polyclonal B cell population in the peripheral blood (3–8). CLL B cells putatively arise as a transformant of B-1a lymphocytes. In healthy humans, B-1a cells are committed mainly to the production of IgM, and account for 5 to 30% of the normal circulating, tonsillar, and splenic B lymphocytes (9–11). They also account for the majority of B cells in the fetus and neonate (10, 12, 13). B-1a cells have long been thought to be primordial elements that rearrange only a restricted selection of V(D)J genes, and that lack the machinery for Ig gene hypermutation. Consistent with this view,

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3 Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; CDR, complementarity-determining region; FR, framework region; H chain, heavy chain; L chain, light chain; PMN, polymorphonuclear cell; R, replacement (mutation); S, silent (mutation).
CD5- IgM- CLL cells suggests that Ag may no longer be capable of inducing clonal diversification in these leukemic cells.

Materials and Methods

PBMCs and immunophenotyping

B lymphocytes were enriched from PBMCs by depletion of T cells and monocytes (21). Enriched B cells were reacted with FITC- or phycoerythrin-labeled mouse mAbs to human CD5, CD19, CD3, CD25, CD23, CD40, HLA-DR, Igk, IgA, IgG, IgM, IgD, or IgG (Coulter Immunology, Hialeah, FL, and Becton Dickinson Labware, Bedford, MA), in ice-cold sterile PBS, pH 7.4, containing 1% BSA and 1% human AB serum (Life Technologies, Gaithersburg, MD). After washing with the same buffer, the cells were applied to a Becton Dickinson FACSscan fluorescence flow cytometer (Becton Dickinson, San Jose, CA) for analysis (21).

PCR amplification, cloning, and sequencing of expressed Ig V(D)J gene cDNA

mRNA was extracted from CLL B cells using the Mini RiboSep Ultra mRNA Isolation Kit (Becton Dickinson). First strand cDNA, synthesized using the SuperScript First Strand cDNA Synthesis Kit (Life Technologies) (16, 17, 22–24), was used as a template (100 ng) for PCR in a volume of 50 μl containing 200 nM of each dNTP, 2.5 U of Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), and 10 pmol of each oligonucleotide primer. Six individual PCR amplifications were performed for the H chain. Each reaction included a sense leader VH primer, specific for the members of the six VH families, in conjunction with an antisense Cκ oligonucleotide primer. Each sense oligonucleotide primer consisted of a degenerate sequence encompassing an area of Ig gene leader region plus an EcoRI site, as follows: VH 1, 5′-GGAATTTCAAGCTTGGGCTGCACT(GC)TCT(GT)C-3′; VH 2, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; VH 3, 5′-GGGAATCACATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; VH 4, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; VH 5, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; and VH 6, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′. Due to the relatedness of the VH1 to the VH3 family (25), the VH1 family members can be amplified by the VH1 family primer. The H chain antisense oligonucleotide primer consisted of the reverse complement (5′-CCGAATTCGAGCTGGGTGAGCGCTGCTCCCGTCTCC-3′) of a 21-nucleotide Cκ sequence plus an EcoRI site. The L chains were amplified in five individual PCRs. Each PCR included a sense leader Vκ or Vλ primer specific for the members of the L chain gene families, in conjunction with an antisense Cλ oligonucleotide primer. Each sense oligonucleotide primer consisted of a degenerate sequence encompassing an area of Ig gene leader region plus an EcoRI site, as follows: Vκ1, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; Vκ2, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; Vκ3, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; Vκ4, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; Vκ5, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′; and Vκ6, 5′-GGGAATCTAGGAACTATGACATCACA(TG)CT(TG)CGCT(GT)C-3′. The number of expected R mutations in the Ig V segment CDRs and FRs

Clonality of the CLL B cells

In addition to the single PCR amplification product, the clonality of the CD5- B cells was assessed by Ig gene rearrangement analysis using a genomic Igκ gene probe on HindIII, EcoRI, and BamHI DNA digests (28). Briefly, B cell genomic DNA (5 μg) was digested with a fivefold excess of EcoRI, BamHI, or HindIII (Boehringer Mannheim Corp., Indianapolis, IN) in appropriate buffer, loaded onto a 0.8% agarose gel (Life Technologies), and electrophoresed at 22 V for 24 h. Size-fractionated DNA was transferred overnight onto Hybond-N nylon membranes (Amerham Life Sciences, Arlington Heights, IL) and prehybridized at 37°C for 4 h. Membranes were incubated overnight at 37°C in hybridization solution containing a γ-32P-labeled 2.2-kb genomic Igκ gene DNA probe, and then washed four times before being autoradiographed at −70°C for 16 to 48 h with Kodak XAR film (Eastman Kodak Co., Rochester, NY).

bcl-1 and bcl-2 proto-oncogene configuration in the CLL B cells

To analyze the configuration of the bcl-1 and bcl-2 proto-oncogenes, the nylon membranes blotted with DNA from the CLL B cells, and previously hybridized with the γ-32P-labeled Igκ DNA, were stripped of the Igκ probe, according to the manufacturer’s protocol, and then reacted with the following probes: MTC on the HindIII digest for the major translocation cluster of bcl-1 (29); p94PS on both EcoRI and BamHI digests for the second break point on bcl-1 (29); and pFl-1 and pFL-2 on HindIII and BamHI digests for the major and minor break points of bcl-2 (30, 31). After four washings, the membranes were autoradiographed at −70°C for 16 to 48 h with Kodak XAR film.

Results

Phenotypic analysis and clonality of the CLL B cells

PBMCs were obtained from three male patients (69 to 75 yr of age: one African-American, 1.19; and two Caucasians, 1.32 and 1.69), who fulfilled the established diagnostic criteria for CLL (1, 2, 30, 32), ranging from Rai stage I to stage II (Table 1). Direct immunofluorescence analysis of the T cell-depleted PBMCs from each CLL patient showed that virtually all of these cells were surface IgM+, κ+ or λ+, CD19+, HLA-DR+, and CD23+ (Table 1), with less than 1% being CD3+ and/or CD10+ (data not shown). More than 99% of these CD19+ cells expressed surface CD5 at high density (Fig. 1), consistent with a virtually complete replacement of the PBL by the clonally expanded neoplastic B cells.

The monoclonality of these cell populations was verified by isolating genomic DNA from each of the CLL B cells and separately digesting it with BamHI, HindIII, and EcoRI for Southern blot hybridization analysis. The reaction of the filter-immobilized DNA with the radiolabeled genomic Igκ probe yielded, in each CLL, a hybridization pattern consistent with a monoclonal Igκ gene rearrangement (Fig. 2). Consistent with a monoclonal B cell composition, only one of the six VH gene recognition sequence. PCR amplification of the germline VH6 gene consisted of 30 cycles of denaturation (95°C, 1 min), annealing (65°C, 1 min), and extension (72°C, 2 min).

Analysis of Ig V gene mutations

The number of expected R mutations in the Ig V segment CDRs and FRs was calculated using the formula R = n × CDR Ri or FR Ri × CDRrel or FRrel, in which n is the total number of observed mutations, Ri is the ratio of CDR Ri or FR Ri inherent to CDR or FR sequences, and CDRrel or FRrel are the relative size of the CDRs or FRs (26, 27). The CDR Ri or FR Ri inherent to the respective progenitor germline genes are as follows: V6-1, CDR = 0.7935, FR = 0.7404; V4-59, CDR = 0.8218, FR = 0.7217; DP-88, CDR = 0.7801, FR = 0.7991, FR = 0.7533; lv318, CDR = 0.8128, FR = 0.7319; and A23, CDR = 0.7901, FR = 0.7463. A binomial probability model was used to evaluate whether the excess of R mutations in CDRs or FRs (q = CDRrel or FRrel Ri or FRrel × FR Ri), and k is the number of observed R mutations in the CDRs or FRs (26).
family primers, \( V_{\mu} \), \( V_{\lambda} \), or \( V_{H} \), yielded a product of appropriate size (approximately 500 bp) when used in conjunction with the antisense \( C_{\kappa} \) primer to amplify cDNA reverse transcribed from mRNA isolated from each CLL sample, 1.19, 1.32, and 1.69, respectively (Fig. 3). Accordingly, only one of the five L chain primers, \( V_{x} \), yielded an amplification product of appropriate size (approximately 350 bp) in each CLL sample when used in conjunction with the antisense \( C_{\kappa} \) or \( C_{\lambda} \) primer (data not shown).

**Sequences of the CLL B cell \( V_{H}D_{JH} \) genes**

The PCR-amplified Ig \( V_{H}D_{JH} \) gene DNAs were cloned and sequenced. Each sequence was derived from the analysis of four to
VH gene sequence was found to be unique to the CLL B cell clone; thus, the likelihood that these differences were due to allelic polymorphism is highly improbable. Finally, the contention that the differences detected in the VH gene segment sequences expressed by 1.19, 1.32, and 1.69 CLL cells represented somatic mutations, as compared with their respective germline VH gene sequences, was supported further by the presence of point mutations in the sequences of the JH gene segments of CLLS 1.19 and 1.32, and in the sequences of the Jk and Ja gene segments of each CLL B cell clones.

The nucleotide and deduced amino acid sequences of the expressed junctional VHDJH gene segments from the CLL B cell clones were compared with those of the reported germline D, DIR, and Jk genes (Fig. 4) (45–49). The best-fit D and/or DIR genes were identified according to the two following criteria: first, priority was given to the VH or JH gene sequence when VH and D, or D and JH gene sequences overlapped; and second, if more than one candidate D and/or DIR gene was identified, the germline D gene sequence, displaying the longest stretch of identity (with a minimum match of five nucleotides in a stretch of 7 bp, or with a minimum match of five nucleotides in row) to that of the expressed D gene segment, was assigned. One of the three clones, 1.69, expressed a D segment that resulted from the utilization of a single germline D gene; the second, 1.19, resulted from the utilization of two different germline D genes, possibly as result of a D-D fusion, one of which was inverted; and the third, 1.32, from the utilization of three different D genes (Fig. 4 and Table II). Thus, the D gene segments expressed by the CLL B cells represented the rearrangement of a heterogeneous assortment of germline D genes in conventional, fused, or inverted configurations. Two of the three CLL-expressed D genes (1.19 and 1.32) were 5’ flanked by nontemplated residues (A additions); and all but that encoding D1.32 were also 3’ flanked by nontemplated nucleotides. CLL 1.19 and 1.32 utilized Jk6b and Jk4b genes, respectively, that were both truncated and mutated (Fig. 4 and Table II). CLL 1.69 utilized a Jk6b gene that was both intact and unmutated. The deduced amino acid sequences of the DJH segments of the three CLLS were divided into CDR3 and FR4 stretches (Fig. 4), according to Kabat et al. (50). The CDR3 sequences were highly divergent in composition and ranged in length from 15 to 22 amino acids; the FR4 sequences were invariable in length and displayed little diversity.

**Sequences of the CLL B cell VkJk and VaJk genes**

The nucleotide and deduced amino acid sequences of the VkJk gene segments expressed by the three CLL B cells are depicted in Figure 5, A and B, respectively, and summarized in Table II. Like the VH gene locus, the human Ig κ- and λ-chain loci have been
characterized recently in detail, and virtually all germline Vκ and Vλ gene segments have been sequenced (50–53), thus providing a representative assortment of unmutated germline templates to which the expressed Vκ and Vλ genes can be compared.

The Ig Vκ gene expressed by CLL 1.19 B cells was of the Vκ1 subgroup, and its sequence contained 19 somatic point mutations as compared with that of the germline gene 02/012. The CLL 1.69 Ig Vκ gene sequence contained two somatic point mutations as

FIGURE 3. Nucleotide and deduced amino acid sequences of the VH gene segments expressed by the three CLL B cell clones (1.19, 1.32, and 1.69). Dashes indicate identities. The top sequence in each cluster is that of the germline gene to which the remaining sequences of the cluster are compared. Solid lines on top of each cluster depict CDRs. Lower case letters denote untranslated sequences. Sequences encompassed by the VH leader and VH6 23-bp spacer primers are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers U31936, U31937, and U31962.
Table II. Structure of the expressed CLL B cell Ig V(D)J genes

<table>
<thead>
<tr>
<th>V(D)J Gene</th>
<th>CDR</th>
<th>FR</th>
<th>R</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.19 V6-1</td>
<td>96.0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>1.22 V4-59</td>
<td>97.2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1.69 Vl318</td>
<td>99.7</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Analysis of the somatic point mutations in the CLL B cell Ig V(D)J genes

In the absence of negative or positive selective pressure on a gene product, R and S mutations distribute randomly throughout the coding sequence. If a DNA segment displays a number of R mutations higher than that expected by chance alone, it is likely that a positive pressure was exerted on the gene product to select for those mutations, as it occurs in the V segment CDRs of mature Abs in which nucleotide changes yield a high R:S mutation ratio. Conversely, if a DNA segment displays a number of R mutations lower than that expected by chance, it is likely that a negative pressure was exerted on the gene product to select against mutations such that the protein structure is preserved, as is in the FRs of Ab, in which nucleotide changes yield a low R:S mutation ratio. We calculated the number of expected R mutations in the CLL 1.19 and V6 genes, as proposed by Chang and Casali (26). In our calculations, the general replacement frequency (Rf) value of 0.75, originally calculated by Jukes and King (54) for random hypermutation of a gene product that need not be conserved in structure, was substituted with the Rf values derived from the analysis of each individual germline VH, VK, or VL gene sequence, as listed in Materials and Methods. The introduced correction yields a more accurate estimate of the frequency of R mutations expected by chance, only, given the demonstration by Chang and Casali (26) that a significant number of human Ig V gene segments display a CDR codon composition that is inherently more prone to R mutations.

The observed distribution of the R and S mutations in the VH and VL gene segments and FRs is summarized in Table II, which also reports the expressed mutations in the CDRs and FRs. In the CDRs and FRs, the V6-1 gene contained five R mutations, two of which were located in the CDR2, and seven S mutations, of which five were located in the FRs, yielding an R:S ratio of 2:0 in the CDRs and 3:7 in the FRs; the VL gene contained 11 R mutations, six of which were located in the CDRs, and five S mutations, of which two were located in the FRs, yielding an R:S mutation ratio of 6:3 in the CDRs and 5:2 in the FRs. In the CDRs and FRs, the VL gene contained four R mutations, three of which were located in the CDRs, and one S mutation located in the FR1, yielding an R:S mutation ratio of 3:0 in the CDRs and 0:1 in the FRs. In the CDRs and FRs, the VH gene contained only one R mutation located in the FR1, yielding an R:S mutation ratio of 0:1 in the CDRs and 1:0 in the FRs; the VK gene contained one R mutation, located in the FR1, and one S mutation, located in the CDR3, yielding an R:S mutation ratio of 0:1 in the CDRs and 1:0 in the FRs. In all three VH genes, the number of R mutations in the CDRs was lower than expected (Table II). In one VL gene segment (1.69), it was compared with that of the VL2 germline gene A23. Finally, the CDR 1.32 Ig VL gene sequence contained five somatic point mutations as compared with that of the VL3 germline Jv318 gene.

Comparison of the expressed Ig VH gene nucleotide and deduced amino acid sequences with those of the germline Jκ and Jλ genes (Fig. 5B) showed that CLL 1.19 utilized a mutated Jκ5 gene; CCL 1.69 utilized a mutated Jκ2 gene; and CCL 1.32 utilized a mutated Jκ2/Jλ3 gene that was 5' flanked by a CCT triplet coding for a Pro residue. This additional CCT triplet could have resulted from the direct juxtaposition of the two deoxycytosine nucleotides (CC) 5' of the heptamer/nonamer sequences of the donor VL gene segment and the deoxynthymidin residue (T) 3' of the heptamer/nonamer sequences of the donor Jλ gene segment (53).
FIGURE 4. Nucleotide and deduced amino acid sequences of the D and J_H gene segments expressed by the three CLL B cell clones (1.19, 1.32, and 1.69). Each expressed sequence represents a composite sequence of the multiple time point and multiple transcript analysis. Dashes indicate identities. The nucleotide sequences of relevant portions of the germline D and J_H genes are given for comparison, and appear above or below the expressed Ig D and J_H gene segments as underlined strings. Unencoded nucleotides (N) are segregated 5' and/or 3' of the D gene segment. The deduced amino acid sequences are divided in CDR3 and FR4.

FIGURE 5. Nucleotide and deduced amino acid sequences of the V_LJ_L gene segments expressed by the three CLL B cell clones (1.19, 1.32, and 1.69). Dashes indicate identities. A, V gene segments; the top sequence in each cluster is that of the germline gene to which the remaining sequences of the cluster were compared. Solid lines on top of each cluster depict CDRs. Sequences encompassed by the V_L leader primers are underlined. B, J gene segments; the nucleotide sequences of relevant portions of the germline J genes are given for comparison, and appear above the expressed J gene segments as underlined strings. The deduced amino acid sequences are divided in CDR3 and FR4. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers U31963, U31964, and U31965.
lower than expected; in the two others (1.19 and 1.32), it was higher than expected (Table II); however, this excess can be due to chance only. Thus, these results demonstrate that in each of the CLLs analyzed, both the mutated Ig VH and VL gene segments did not show any preferential enrichment for R mutations in the CDRs. In addition, in all CLL VH and VL gene segments but one (1.19 VH), the number of R in the FRs was not different from that expected on the basis of chance only. In the 1.19 VH gene segment, the scarcity of R mutations in the FRs reached a level of significance, demonstrating that a negative pressure was probably exerted on the gene product so that the protein structure is preserved.

Lack of intraclonal diversification in the CLL B cell clones

To determine whether selective forces were influencing the clonal expansion of the three CLL B cells in vivo at times different from that initially considered, we analyzed the intraclonal diversity of each case by selecting multiple time points during a 1.5- or 2-yr time period. The expressed Ig VHDJH gene from CLL 1.19 was analyzed over a 1–1/2-yr period at four separate time points (Figs. 3 and 4, and Table I). Each of the VH gene segments as well as the sequences of the CDR3 and FR4 were absolutely identical at each of the four time points, and no additional point mutations were present. The expressed Ig VHDJH genes from CLLs 1.32 and 1.69 were both analyzed over a 2-yr period by selecting three separate time points (Figs. 3 and 4, and Tables I and II). Like CLL 1.19, all of the somatic point mutations in the VH gene segment (1.32 and 1.69, respectively), as well as the sequences encoding the CDR3 and FR4, were absolutely identical at each of the three time points, and no additional point mutations were observed.

bcl-1 and bcl-2 proto-oncogene configuration

Translocation of the bcl-1 and bcl-2 proto-oncogenes has been associated with other B cell neoplasia that display significant traces of intraclonal diversification and Ag-dependent selection of somatic point mutations. bcl-1 is translocated in mantle zone lymphoma and B-prolymphocytic leukemia (2), and bcl-2 is translocated in low grade follicular non-Hodgkin lymphoma (55). In both cases, translocation of these proto-oncogenes has been associated with the high degree of clonal expansion and cellular division characteristic of these B cell disorders. To analyze whether these proto-oncogenes were translocated in the three CLLs, B cell genomic DNA from each of the CLL B cells was subjected to Southern blot analysis using four different probes for either the bcl-1 or bcl-2 loci, as detailed in Materials and Methods. The hybridization pattern was consistent with that of both proto-oncogenes being in germline configuration (Fig. 6).

Discussion

These studies show that IgM⁺ CLL B cells can express Ig V(D)J gene segments containing variable numbers of somatic point mutations. They also show that these mutations do not display any
preferential enrichment of R changes within the CDRs, and, in each CLL patient, they are absolutely conserved not only in multiple Ig cDNAs from the same time point, but also throughout multiple time points over a 2-yr period. The scarcity of R mutations in the CDRs, and absolute conservation of the sequences of the Ig V(D)J gene cDNA transcripts among different B cells of the same clonotype, are consistent with an absence of intraclonal diversification. These findings were derived from the analysis of three patients diagnosed with CD5⁺ IgM⁺ CLL, each of whom fulfilled the established clinical and morphologic diagnostic criteria for the disease, including B cell surface expression of both CD5 and CD23 at high density. The CLL nature and the Ag-independent emergence of these neoplastic clones were strengthened further by the germline configuration of the bcl-1 and bcl-2 proto-oncogenes, which translocate (to chromosome 14) in neoplastic B cells undergoing a high degree of intraclonal somatic diversification and selection by Ag, such as in mantle cell lymphoma and follicular lymphoma (56, 57).

The first issue addressed by these studies was whether (IgM⁺) CLL B cells can express somatically mutated Ig V genes. In each of the three B cell clones, the expressed V_H gene segment sequences displayed a number of differences as compared with those of the closest reported germline genes V6-1, V4-59, and DP-88. The probability that these differences stemmed from the utilization of heretofore unreported germline gene was estimated to be negligible in each of the three CLL V_H gene segments due to the following considerations. V6-1 has been shown to be among the most conserved Ig V_H genes throughout the human population. V4-59 has been reported to be polymorphic, but the allelic variants in this gene have been shown to consist of only few nucleotide differences (38, 39). DP-88 is one of the 13 related variants of the V1-69 gene. Each of these variants has been sequenced, thereby producing a complete profile of this gene’s polymorphism (41, 42).

The contention that the expressed Ig V_H genes constitute somatically mutated forms of V6-1, V4-59, and DP-88 templates is supported further by the presence of somatic mutations in the juxtaposed D and J_genes, and by the isolation of a V_H gene template identical to the germline V6-1 gene from the genomic PMN DNA of patient 1.19. In each B cell clone, the load of somatic mutations in the expressed Ig V_P,D,J_gene segment was accompanied by a comparable load of putative somatic mutations in the paired L chain variable segments. For instance, the CLL 1.19 B cell V_k gene sequence displayed 19 nucleotide differences when compared with that of the closest reported κ-chain germline gene, 02/012; the CLL 1.32 B cell V_l gene sequence displayed five nucleotide differences when compared with that of the closest reported λ-chain germline gene, λv318; and the CLL 1.69 B cell V_k gene sequence displayed two nucleotide differences when compared with that of the closest reported κ-chain germline gene, A23. Like their H chain counterparts, the J_k and J_a genes were also mutated.

The next question asked by our experiments was whether the nature and the distribution of the R mutations in the CLLs Ig V segment were consistent with a selection by Ag. Ag-selected Abs have been shown to include a higher frequency of R mutations in the Ig V gene CDRs than in the FRs, in which the proportion of S mutations may be greater. The recent findings by Chang and Casali (26) suggest that when assessing the Ag-selected nature of somatic point mutations in an expressed Ig V gene, the inherent susceptibility to amino acid replacement or replacement frequency, R_p, needs to be calculated for the progenitor germline gene sequence. The R_p is then used to calculate the theoretically expected number of R mutations in the CDRs or FRs of that particular gene given a random distribution of R mutations. This principle was applied to the analysis of the Ig V genes of the three CLL subjects. For each expressed H or L chain V gene and in each CLL patient, the number of expected CDR and FR R mutations was calculated, and used to determine, on the basis of a binomial distribution model, the probability that any excess and scarcity of R mutations in the CDRs and FRs were due to chance only. The results of these calculations clearly indicated that in none of the three CLL B cell H and L chain V segments was there evidence for positive selection of R mutations in the CDRs, a result, in general, of a process of Ag-driven clonal selection. In some cases, it could be hypothesized that a negative, rather than a positive, selective pressure applies to R mutations, that is, the unmutated product rather than a somatically mutated form of the germline gene is selected by Ag. This is a distinct possibility (58), but has never been experimentally substantiated in vivo. Even in such a case, however, negative pressure on R mutations in CDRs should be accompanied by a similarly negative pressure in the FRs to preserve a structurally sound Ab molecule. Thus, a significant scarcity of FR R mutations should be an important feature of Ag-selected Ab-producing cell clones. The present findings show, with the exception of the CLL 1.19 V_H gene segment, no significant negative selection of R mutations in the FRs of the Ig H and L chain V segments expressed by the three CLL B cell clones.

As shown by the study of a variety of specific experimental Ab responses, Ag-driven selection and expansion of a B cell clone not only result in positive selection of R mutations in the Ig V segment CDRs, but also lead to a significantly high degree of intraclonal diversification, revealed at the DNA transcription level by the appearance of colinear Ig V(D)J DNA sequences sharing and differing in various numbers of somatic point mutations. Intraclonal diversification has been shown in neoplastic equivalent of GC B cells, such as follicular lymphoma (57, 59, 60), and one patient with CD5⁺ CLL (61). In these cells, as in normal cells undergoing specific expansion and selection by Ag, somatic diversification has been found to be associated with positive selection of CDR R mutations and negative selection of FR R mutations. To better verify whether selective forces were influencing the biologic behavior of the CLL clones in vivo, we addressed the issue of intraclonal diversification by not only analyzing multiple independent bacterial isolates from a given time point, but by also examining multiple time points of interest during a 1½- to 2-yr period. By following these clones individually through their natural history in vivo, we failed to detect any nucleotide variation throughout our time point analysis study, therefore concluding that these CLL B cells lacked traces of intraclonal diversification. Lack of intraclonal diversification in CLL B cells is supported further by Fais et al. (62, 63), who demonstrated that clonally related IgG- and IgA-switched progeny of IgM⁺ CLL B cells fail to accumulate appreciable numbers of new somatic mutations in their Ig V genes. However, IgM⁺ progenitors of IgG⁺ CLL B cells retain the ability to accumulate somatic mutations (64), presumably because they have yet to receive the final “hit” in the transformation process. The occurrence of switching without the accumulation of V gene mutations suggests that the processes of differentiation and diversification are not necessarily linked, and that in CLL B cells, clonal differentiation can occur in the absence of V gene mutation.

Taken together, our findings suggest that antigenic stimulation is unlikely involved in the clonal diversification of our panel of CD5⁺ IgM⁺ CLL B cells. They differ from those by Hashimoto and coworkers (65), who detected a high number of somatic mutations in the expressed Ig H chain genes in two (CLLS 055 and 030) of seven IgG⁺CD5⁺ CLLSs, as compared with their respective germline genes (V4-34 and H11, respectively). The distribution of R mutations in both of these CLL IgG⁺ clones was consistent with
selection by Ag. Thus, different selective forces are present in IgG-producing CLL cell, as discussed by Hashimoto et al. (65), as compared with IgM-producing CLL cells, as shown by this study.

The different selective pressures that are applied to IgM + and IgG + CLLs need to be further investigated. The majority of studies on CLL suggest that the B cells are clonally expanded, and use a biased set of Ig V genes, which are generally unmaturated, to encode for low affinity, polyreactive autoantibodies (8). These findings are consistent with the general unmaturated nature of the putative non-neoeplastic CLL progenitors, B-1a cells. Recent findings by us and others (16–20), however, have demonstrated that normal human B-1a lymphocytes can produce somatically mutated and Ag-selected Abs, indicating that these cells do indeed possess the machinery for somatic hypermutation, and can undergo an affinity maturation process, and therefore suggesting that CLL B-1a cells may also be able to mutate the expressed Ig V(D)J genes. By indicating that mutations can occur in the V genes of both H and L chains, and that the numbers and locations of these mutations are closely paralleled in individual patients, our findings in IgM + CLLs extend those by Hashimoto et al. in IgG + CLLs (65).

However, unlike these previous studies in IgG + CLL, they did not show evidence of a pattern of Ag selection of R mutations, nor did they show any evidence of intraclonal diversification over a significant period of the disease. It is thus unlikely that the reported somatic mutations are inherent to the clonal evolution of CLL, but rather represent a pre-existing feature of the normal B-1a cell clones “hit” by the transforming event(s).

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References


