Lack of Extensive Mutations in the V\textsubscript{H}5 Genes Used in Common B Cell Chronic Lymphocytic Leukemia

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Summary

B cell chronic lymphocytic leukemia (CLL) is a malignancy of the CD5\textsuperscript{+} B cells. Prior studies indicated that CLL B cells generally express immunoglobulin (Ig) V\textsubscript{H} and V\textsubscript{L} genes with little or no somatic mutations. However, a recent report indicated that V\textsubscript{H}251, one of three V\textsubscript{H} genes belonging to the V\textsubscript{H}5 subgroup (e.g., V\textsubscript{H}251, V\textsubscript{H}32, and V\textsubscript{H}15), not only is frequently rearranged in this disease, but also has extensive and selective mutations when expressed by CLL B cells. The extent and nature of these mutations contrasts markedly from the low level of mutations noted in V\textsubscript{H}5 genes used by normal B cells or other Ig V genes found expressed in CLL. To determine whether this difference reflects a unique property of V\textsubscript{H}251 or a previously unrecognized subgroup of CLL, we examined for V\textsubscript{H}5 Ig gene rearrangements in leukemia cells from 68 patients that satisfied clinical and diagnostic criteria for CD5\textsuperscript{+} B cell CLL. Southern blot hybridization studies with probes for V\textsubscript{H}251 and the J\textsubscript{H} locus revealed that only 7 (10\%) of the 68 monoclonal CLL cell populations had undergone Ig gene rearrangement involving V\textsubscript{H}5 genes. Two (3\%) were found to have functionally rearranged V\textsubscript{H}5 genes that shared \(\geq 98\%\) sequence homology with 5-2R1, a V\textsubscript{H}251 gene isolated from a pre-B cell acute lymphocytic leukemia. The other five CLL (7\%) had functionally rearranged V\textsubscript{H}5 genes that each shared \(\geq 99\%\) nucleic acid sequence homology with a germline V\textsubscript{H}32 isolated from human sperm DNA. These data indicate that V\textsubscript{H}251 or V\textsubscript{H}32 also may be expressed by CD5\textsuperscript{+} CLL B cells with little or no somatic mutation. These findings contrast with a recently published study on V\textsubscript{H}5 gene expression in B CLL and contest the hypothesis that extensive somatic mutation is a common property of the V\textsubscript{H}5 genes used in this disease. Further work to define the clinical and/or phenotypic characteristics of patients with leukemia cells that express mutated versus nonmutated Ig V genes may reveal subsets of CLL that possibly differ in their cytogenesis, etiopathogenesis, and/or clinical behavior.

B cell chronic lymphocytic leukemia (CLL)\textsuperscript{1}, the most common adult leukemia in Western societies, may be considered a malignancy of “B-1 B cells”, formerly referred to as CD5 (or Ly1) B cells (for reviews see references 1 and 2). Generally, the leukemia cells in this disease coexpress CD5, pan-B cell surface antigens and surface Ig. In addition, common B cell CLL may share additional characteristics with those of normal human B-1 B cells, such as the frequent production of polyclonal autoantibodies (3–5), expression of myelomonocytic surface antigens (6–10), low-level expression of CD20 (11, 12), or the ability to form rosettes with mouse erythrocytes (13, 14).

We found that the leukemia cells from many CLL patients express Ig that bear one or more autoantibody-associated cross-reactive idiotypes (CRIs) (15–18). Evaluation of the molecular basis for such CRIs revealed that each may be a serologic marker for expression of a conserved Ig V gene with limited or no somatic mutation (19–21). In addition, we and others find that CLL B cells not selected for expression of a CRI also may express Ig V genes that share extensive homology (>97\%) with other known germline Ig V genes (18, 22–26). Finally, normal or malignant murine B-1 B cells generally also express restricted, if not unique, repertoires of Ig V genes that display little or no somatic mutation (27–30). Collectively, these studies imply that B-1 B cell malignancies use restricted repertoires of Ig V genes that have not diversified substantially from the germline DNA.

However, there is an apparent exception to this generalization. Humphries et al. (31) noted that the leukemia cells from related CLL patients expressed extensively mutated Ig

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\textsuperscript{1} Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; CRI, cross-reactive idiotype; FR, framework region.
Vn genes belonging to the Vn,5 subgroup. In addition, they noted that 30% of patients with B cell CLL have rearranged Vn genes that belong to this subgroup. These same investigators examined 11 additional CLL B cell populations that also expressed Ig encoded by Vn,5 genes (32, 33). Since prior studies indicated that Ig Vn genes of this small subgroup are highly conserved and nonpolymorphic (34), these investigators compared the primary nucleic acid sequences of the expressed Vn genes with that of known Vn,5 genes. In 10 of 11 CLL, they found the expressed Vn,5 genes to differ substantially from those of known germline sequences. Importantly, nonconservative nucleic acid base differences were found clustered primarily in sequences encoding the Ig CDRs which form the pocket of the presumed Ig antigen-combining site. Such substitutions often are noted in Ig selected in an antigen-driven secondary immune response. Accordingly, it appears that these CLL express somatically mutated Ig Vn genes that have been selected for their ability to bind some unknown antigen(s).

It is not certain whether these leukemias represent a distinct subset of CLL or whether they express mutated Ig Vn gene because they use Ig Vn genes of the Vn,5 subgroup. The Vn,5 gene subgroup is relatively small, consisting of only two functional genes (Vn,251 and Vn,32) and one pseudogene (Vn,15) (31, 35, 36). The Vn,251 gene is distinctive in that it is transcribed at relatively high levels in the germline nonrearranged configuration (36). Conceivably, such transcriptional activity could affect higher rates of Ig gene rearrangement and/or somatic mutation of this Vn gene in CLL B cells or leukemia B cell precursors. Alternatively, despite also being CD5+ (32, 33), the CLL cells examined by these investigators may constitute a discrete subset of chronic leukemia that differs from that of other B cell CLL in its physiology of Ig gene expression. For these reasons, we decided to examine for Vn,5 gene rearrangements in the leukemia cells from 68 patients that satisfied diagnostic and clinical criteria for B cell CLL.

Materials and Methods

Patients. Peripheral blood samples were obtained from 68 patients fulfilling diagnostic and immunophenotypic criteria for common B cell CLL at the UCSD Medical Center, the Veteran's Administration Hospital, or the Scripps Clinic and Research Foundation (all in La Jolla, CA; 37-41). The median age of the patients was 63, ranging from 41 to 79 years. The patients were heterogeneous with respect to clinical stage (ranging from Rai stage 0 to stage IV) and prior therapy for CLL. 52 (76%) of the patients were male, reflecting the known 5:2 male/female bias in the prevalence of common B cell CLL (42). Each patient is assigned a unique three-letter identifier (listed in capital letters).

Flow Cytometry. Direct immunofluorescence analyses of leukemia cells were performed using a flow cytometer (FACSscan™; Becton Dickinson & Co., San Jose, CA) as described (38). PE- or fluorescein-conjugated mAbs specific for CD20, CD19, or CD5, and their respective isotype control mouse IgG, were purchased from Becton Dickinson & Co. Other mAbs were as described (43).

DNA Isolation and Southern Blotting. Genomic DNA was isolated from the leukemia cells as described (44). A total of 10 μg of DNA was digested with fivefold excess of restriction enzyme in appropriate buffers and then loaded onto a 0.8% agarose gel (GIBCO BRL, Gaithersburg, MD). After electrophoresis for 650 volt-hours, the size-separated DNA was transferred onto nylon for Southern blot hybridization with radiolabeled probes as described (35, 44). Final washing conditions consisted of two 20-min washes at 65°C in 0.2 x SSC in 0.1% NaDodSO4 (Vn,251 probe), or 0.1 x SSC in 0.1% NaDodSO4 (Jn probe) (1 x SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

Probes and Oligonucleotides. The probe for Vn,251 consisted of a 1.0-kb XbaI/PstI fragment of WSI (35) that included most of the Vn,251 coding region along with a few hundred bp of the 5' flanking region. The Jn probe consisted of a 2.1-kb Sau3A fragment spanning the genomic DNA of Jn,2 through Jn,6. DNA probes were radiolabeled to a sp act of 10^4 cpm/μg with α-[32P]CTP (New England Nuclear, Boston, MA) via random hexamer priming (45). Oligonucleotides and probes such as the Vn,5 third framework region (FR) probe (5' GCA GAC TTC TCC TCA GG 3') and the β-globin internal probe (5' GCA GAC TTC TCC TCA GG 3') were synthesized as described (15). These probes were 5' end-labeled using γ-[32P]ATP and polynucleotide kinase to a sp act of 2 x 10^7 cpm/pmol, as described (46).

PCR Amplification of Genomic DNA. 1 μg of genomic DNA was amplified using the PCR technique as described (44). Primers corresponding to the sense strand of the leader sequence of the Vn,251 gene (dGCGAATTCAGCTTCAACGGAAGTCTCTGGCC) and the antisense strand of a Jn, consensus sequence (dGGCGAATTCACGGAAGTCTCTGGCC) were used to amplify Vn,5 genes that were juxtaposed to Jn through Ig gene rearrangement. To control for template fidelity, PCR using oligonucleotides dACACAACTGTGTTCACTAGC and dCAACTTCCTAGGCGTGACC (R, G/A) were used to amplify Vn,5 genes that were juxtaposed to Jn through Ig gene rearrangement. To control for template fidelity, PCR using oligonucleotides dACACAACTGTGTTCACTAGC and dCAACTTCCATCCAGTTCCCTAGC was performed to amplify the β-globin gene in the genomic DNA of all samples, as described (20).

Cloning and DNA Sequencing. The amplified DNA segments were digested with HindIII and EcoRI to cut the linkers incorporated into the oligonucleotide primers, purified and ligated into a HindIII/EcoRI-cut pUC19 vector (Bluescribe; Stratagene, La Jolla, CA) for transformation of competent Escherichia coli, as described (44). Plasmids containing the insert were isolated for double-strand (ds)DNA sequencing as described (47, 48).

Results

Flow Cytometric Analyses. Direct immunofluorescence analyses of the mononuclear cells from each subject substantiated the diagnosis of common B cell CLL, indicating that >70% (mean 92%, range 71-99%) of the lymphocytes in each blood sample coexpressed pan-B cell surface antigens, CD5 and κ or λ L chains (data not shown). Of the 68 leukemia cell populations studied, 39 (57%) expressed Ig κ L chains and 29 (43%) expressed λ L chains.

Southern Blot Analyses. Genomic DNA isolated from each of the 68 samples was digested with BamHI and HindIII for Southern blot hybridization analysis. Hybridization of filter-immobilized DNA with the radiolabeled Jn probe revealed each to have monoclonal Ig gene rearrangements (Fig. 1, and data not shown). 45 (65%) of the samples had two detectable Jn gene rearrangements, whereas the remaining 23 samples each had only one nongermline Jn restriction fragment.

Using the Vn,251 probe, we found that most DNA
samples had the germline $V_{\delta}5$ gene pattern of placental DNA (Fig. 1, lane A, and data not shown) (31). The $V_{\delta}251$, $V_{\delta}32$, and $V_{\delta}15$ genes are contained in the 7.5-, 3.2-, and 1.4/1.5-kb fragments, respectively (e.g., Fig. 1, lane A [NOM]). These four bands were identified in 37 (54%) of the 68 samples tested. In 26 (39%) of the samples, the 3.2-kb $V_{\delta}32$ gene fragment was not detected, suggesting that the germline $V_{\delta}32$ genes of both alleles were lost, possibly through Ig gene rearrangement. However, previous studies demonstrated that the germline DNA of many individuals actually lack this 3.2-kb BamHI/HindIII $V_{\delta}32$ restriction fragment (31). As such, the absence of this band in these CLL DNA samples instead may be due to genetic polymorphism.

In 7 (10%) of the 68 CLL DNA samples, we identified nongermline bands with the $V_{\delta}251$ probe. In all cases, these nongermline bands matched those detected using the human $J_{\delta}$ probe, arguing that $V_{\delta}5$ genes within these CLL had undergone Ig gene rearrangement (Fig. 1). Two (3% of the total) had rearranged BamHI/HindIII fragments of 8.5 kb, suggesting that these CLL samples each had a rearranged $V_{\delta}251$ juxtaposed to $J_{\delta}4$ (e.g., Fig. 1, lane 2A [HAN]) (31). In the other five samples, we detected nongermline BamHI/HindIII fragments of 3.0–4.0 kb that matched rearranged bands that hybridized with the human $J_{\delta}$ probe (Fig. 1, samples 3 [CAV], 4 [HOW], 5 [PET], 6 [ANG], and 7 [KER]). However, such BamHI/HindIII restriction fragments should be too small to contain a rearranged $V_{\delta}251$ gene juxtaposed with any $J_{\delta}$ gene segment, unless alternative BamHI or HindIII restriction sites were introduced into or around this gene through Ig gene rearrangement, somatic mutation, and/or genetic polymorphism. More likely, these relatively small restriction fragments contain $V_{\delta}32$ rearranged with either $J_{\delta}4$ (HOW, Fig. 1, lane 4A), $J_{\delta}5$ (ANG and KER, Fig. 1, lanes 6A and 7A) or $J_{\delta}6$ (CAV and PET, Fig. 1, lanes 3A and 5A), respectively.

**Sequence Analyses of Rearranged $V_{\delta}5$ Genes.** To examine the $V_{\delta}5$ genes juxtaposed to $J_{\delta}$ through Ig gene rearrangement, we performed the PCR on the genomic DNA from each of the 68 samples using primers corresponding to the sense strand of the $V_{\delta}251$ leader and the antisense strand of a $J_{\delta}$ consensus sequence. We found that only the CLL samples noted to have $V_{\delta}5$ gene rearrangements by Southern analyses yielded PCR products that hybridized with either an oligonucleotide corresponding to the third FR of $V_{\delta}251$ or the genomic $V_{\delta}251$ probe (data not shown). On the other hand, each of the 68 DNA samples yielded 112-bp gene fragments in control PCR using $\beta$-globin specific primers (data not shown). Therefore, the inability to amplify $V_{\delta}5$ Ig gene rearrangements in the other DNA samples could not be ascribed to inadequate template quality or nonspecific PCR inhibitors.

Nucleic acid sequence analyses confirmed that each of these seven CLL DNA samples contained rearranged $V_{\delta}5$ genes (Figs. 2 and 3). The two CLL DNA samples reasoned to have possible $V_{\delta}251$ gene rearrangements by Southern (DOB and HAN) were found to have rearranged $V_{\delta}5$ genes that shared \textasciitilde98% sequence homology with 5-2R1, a $V_{\delta}251$ gene isolated from an acute pre-B cell leukemia (49) (Fig. 2). The $V_{\delta}$ gene of DOB is identical to that of 5-2R1. The $V_{\delta}$ of HAN, on the other hand, differs from 5-2R1 by four nucleotides in the first Ig FR and two in the third Ig FR (Fig. 2). However, only two of these base differences are non-conservative, resulting in a Gly $\rightarrow$ Arg substitution at position 26 and a Ser $\rightarrow$ Arg change at position 82B (numbering according to Kabat et al. [50]). The rearranged $V_{\delta}5$ genes of the other five CLL (e.g., CAV, HOW, PET, ANG, and KER) were found to share \textasciitilde99% nucleic acid sequence homology to a germline $V_{\delta}32$ gene isolated from human sperm DNA (Fig. 2) (31, 51). Except for a conservative base change in the codon for the amino acid at position 7 in the first Ig FR, CAV and HOW are identical to $V_{\delta}32$. The other three $V_{\delta}$ genes also differed from $V_{\delta}32$ by one (PET).
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or two (ANG or KER) base changes in the intron (marked
intervening sequence, Fig. 2).

Each of the rearranged V₅ Ig genes detected in our
survey had a unique sequence encoding the third CDR
(CDR3) (Fig. 3). The CDR3 sequence of each had stretches
with high homology to known germline Dₖ and Jₖ gene
segments (50, 52). The sequences encoding the CDR3 of
PET, CAW, or HOW each had highest homology with D₅₁.
Those of DOB or KER had the highest homology with Dₓ₅₁,
whereas the CDR3 sequence of HAN or ANG had the highest
homology with DA₄ or DK₁, respectively. Of the seven dis-
parate CDRs, we deduce that three (DOB, HOW, and KER)
were encoded by J₅, two (ANG and KER) by J₆, and two (CAV and PET) by J₆ (Fig. 3).

Discussion

We examined the leukemia cells from 68 randomly selected patients that satisfied clinical and diagnostic criteria for CD5⁺ B cell CLL. This group of CLL patients was noted to have a mean age and sex distribution comparable with that noted in earlier surveys of patients with this disease (39, 42). Furthermore, FACS analyses demonstrated that the leukemia cells from each patient coexpressed pan-B cell surface antigens, CD5 and K or k Ig L chains. Of these CLL samples, we found that 7 (10%) had Ig gene rearrangements involving the V₅ gene family. Nucleic acid sequence analyses of these Ig V₅ genes did not reveal evidence for intraclonal diversity or Ig somatic mutations. Instead, genetic polymorphism apparently accounts for some of the differences noted in V₅ genes detected in this study.

In any case, it is unlikely that we failed to detect rearranged V₅ genes found expressed in this disease (18–26).

In contrast with previous reports on the V₅ genes rearranged in CLL (31), V₅,32 and not V₅,251 was the most frequently rearranged V₅ gene detected in our survey. Initially, V₅,32 was assumed to be a pseudogene due to a termination codon at amino acid position 28 (31, 35). However, Sanz et al. (34) reexamined the original V₅,32 clones and found errors in the reported sequence, allowing these investigators to conclude that V₅,32 was in fact functional. The rearranged V₅,32 genes observed in the current survey also lack any termination codons and appear functional. In view of the finding that many individuals apparently lack the V₅ germline band, the relatively frequent use of V₅,32 in CLL appears remarkable. Indeed, the absence of the V₅,32 germline band may indicate that a relatively high proportion of individuals do not possess this Ig V gene in their germline DNA. However, other explanations are possible, including polymorphisms affecting the restriction sites in and around V₅,32 and, at least in the current survey, Ig gene rearrangements and/or deletions by the leukemia cells studied.

In any case, it is unlikely that we failed to detect rearranged V₅ genes in our survey that incurred extensive and selected somatic mutations. We performed Southern blot analyses using washing conditions that allow for detection of rearranged V₅ genes that share >80% homology with V₅,251.
In addition, over half of the V\(_\alpha251\) probe used in these analyses corresponded to the 5' flanking region and intron of the V\(_\alpha251\) gene, regions not subject to selected Ig somatic mutation. Furthermore, using the V\(_\alpha251\) probe we detected both V\(_\alpha32\) and V\(_\alpha15\), V\(_\alpha5\) genes having 95 and 86% coding region nucleic acid sequence homology with V\(_\alpha251\), respectively. Finally, PCR on all leukemia samples using oligonucleotides to amplify V\(_\alpha5\) genes juxtaposed with J\(_\alpha\) demonstrated that only those DNA samples that had V\(_\alpha5\) gene rearrangement by Southern also generated PCR products that hybridized with oligonucleotides or full-length DNA probes for V\(_\alpha251\) and related V\(_\alpha5\) genes.

These results contrast markedly with those recently reported by Cai et al. (32, 33), indicating that highly mutated V\(_\alpha251\) genes frequently are expressed in human B cell CLL. Earlier reports indicated that V\(_\alpha251\) gene may be rearranged in the leukemia cells of 30% of the patients with this disease (31). More importantly, nucleic acid sequence analyses revealed extensive numbers of base substitutions from the putative germ-line V\(_\alpha251\) sequence. Moreover, the noted pattern of non-conservative base substitutions in the V\(_\alpha251\) gene used by these CLL B cells was typical of Ig V genes expressed in secondary immune responses to antigen (32, 33). In contrast, we found that only 2 of 68 CLL cell samples use V\(_\alpha251\), and that these two apparently express this gene with little or no somatic mutation.

Such differences may reflect heterogeneity within what currently is considered B cell CLL. Subgroups of CLL may exist that arise from different B cell lineages and/or stages of B cell differentiation. Such differences may be reflected in the Ig V genes used by each subgroup. Already, we have noted that CD5\(^-\) B cell CLL, in contrast with CD5\(^+\) B cell CLL, may display intraclonal diversity in their expressed Ig V genes (25, 53). Although both the current survey and that of Cai et al. (32, 33) examined CD5\(^+\) B cell CLL, there also may exist subgroups within this category of CLL that express Ig similar to that found in the primary (e.g., nonmutated) versus secondary (e.g., mutated) humoral immune response. Arguably, the leukemia cells in the former category may be those that express "natural" or polyreactive autoantibodies that frequently are detected in CD5\(^-\) B cell CLL (3-5). On the other hand, those CLL in the latter category may originate from B cells previously selected to express Ig with affinity for some unknown environmental or self-antigen(s). Antigen selection may not be unique to this subgroup, however. Recent Ig chain mixing studies employing murine transfectedomas have indicated that the polyreactive binding activity of Ig encoded by nonmutated Ig V genes also may be a selected specificity (54). In addition, as demonstrated in this survey, it is not likely that the observed differences in the extent of observed Ig mutation depends upon expression of Ig V genes of the V\(_\alpha5\) subgroup, in particular V\(_\alpha251\). Rather, there may exist subsets of CD5\(^+\) B cell CLL that have differences in their cytogenesis and/or etiopathogenesis. Comparison of B cell CLL that express nonmutated versus highly mutated Ig V genes may reveal features that can be used to discriminate between these two types of leukemias, possibly allowing us to define subsets of CLL that have different clinical features and/or therapeutic requirements.

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