Molecular Analysis of \( V_H \) and \( V_L \) Regions Expressed in IgG-Bearing Chronic Lymphocytic Leukemia (CLL): Further Evidence That CLL Is a Heterogeneous Group of Tumors

By Saskia B. Ebeling, Mieke E.M. Schutte, and Ton Logtenberg

We report the heavy (H) and light (L) chain variable (V) region sequences of cDNAs encoding the Ig receptor of two cases of CD5+ IgG-bearing CLL P87 and P103. In both CLL cases the H chain was encoded by members of the \( V_{III} \) gene family. The L chain expressed by P87 belonged to the \( V_{IV} \) subgroup, whereas P103 used a member of the \( V_{III} \) subgroup. The \( V_{III} \)P87 gene differed by only three nucleotides from 3B1P1, a \( V_{III} \) gene previously cloned from a fetal liver cDNA library. Nucleotide sequence analysis demonstrated that the \( V_{III} \)P103 gene differed by seven nucleotides from its most homologous germline counterpart, the Humkv325 gene, a highly conserved gene frequently expressed in IgM-bearing CLL. The nucleotide sequences of \( V_{III} \)P103 and \( V_{IV} \)P87 could not be reliably matched with reported germline V genes. The analysis of multiple independently obtained \( V_H \) and \( V_L \) cDNA clones from each tumor showed a lack of intraclonal diversification. The data show that V regions expressed in isotype-switched CD5+ CLL may be either in/near germline configuration or somatically mutated. Furthermore, these tumors, like their IgM-bearing counterparts, do not seem to undergo intraclonal diversification.

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MATERIALS AND METHODS

Patient population. Heparinized blood samples were collected from two patients with documented CLL. Mononuclear cells (MNC) were prepared by Ficoll-hypaque (ICN Flow, Costa Mesa, CA) density gradient centrifugation as described.10 MNC were stained with fluorescein isothiocyanate-conjugated MoAbs to human CD5, CD19 (Becton Dickinson, Mountain View, CA) IgM, IgD, IgG, IgA, \( \kappa \), and \( \lambda \) light chains (Southern Biotechnology Associates, Birmingham, AL) and analyzed on a FACScan (Becton Dickinson). The two CLL cases P87 and P103 described in this study expressed CD5 and surface IgG, IgD isotype, respectively (Table 1 and Fig 1). Expression of membrane IgM or IgD was not detectable. Tumor loads were \( 139 \times 10^5 \) and \( 46 \times 10^6 \) MNC/mL of peripheral blood, respectively.

Preparation of RNA and first-strand cDNA synthesis. RNA was extracted from \( 10^6 \) cells using the guanidinium isothiocyanate method. First-strand cDNA was prepared from 10 \( \mu \)g of total RNA according to standard protocols.11 Briefly, an oligo-(dT) primer was hybridized to 10 \( \mu \)g RNA and extended with \( 10 \) U of avian myeloblastosis virus (AMV) reverse transcriptase.12 For each patient, two independent cDNA samples were prepared.

Cloning and sequencing of \( H \) and \( L \) chain \( V \) regions. For cloning of the \( V_H \) and \( V_L \) regions, cDNA samples were amplified in the polymerase chain reaction (PCR) using a 3' \( C_V \)-specific primer and each of a set of 5' \( V_H \) gene family-specific primers as previously described (Table 2).13 The 3' and 5' primers contained XbaI and EcoRI endonuclease restriction sites, respectively, to facilitate subcloning of the PCR products.

The \( V_H \) region expressed by P103 was amplified with 5' \( V_H \) gene family-specific and 3' \( C_V \)-specific primers containing BamHI and SalI endonuclease restriction sites respectively. The \( V_L \) region ex-
Table 1. Phenotype of CLL Cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>P87 (%)</th>
<th>P103 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>97.3</td>
<td>96</td>
</tr>
<tr>
<td>CD5</td>
<td>80.8</td>
<td>75</td>
</tr>
<tr>
<td>Sκ</td>
<td>1</td>
<td>65</td>
</tr>
<tr>
<td>Sλ</td>
<td>70.8</td>
<td>0</td>
</tr>
<tr>
<td>Sμ</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sγ</td>
<td>94.3</td>
<td>75</td>
</tr>
<tr>
<td>Tumor load</td>
<td>139.10⁶/mL</td>
<td>47.10⁶/mL</td>
</tr>
</tbody>
</table>

CLL cells are described by percentage of MNC positive for the indicated markers.

pressed by P87 was amplified using a 5' pan-V₃ primer in combination with a 3' C, primer with attached EcoRI and SalI sites, respectively (Table 2). In each experiment, control amplifications consisted of the reaction mixture without cDNA.

PCR products were digested with the appropriate restriction endonucleases and purified using the MERmaid kit according to the manufacturers instructions (Bio 101 Inc, La Jolla, CA). After ligation of the PCR products into appropriately digested Bluescript vectors (Stratagene, La Jolla, CA) and transformation of competent DH5α cells, bacterial colonies containing plasmid DNA with correctly sized inserts were selected. Nucleotide sequences of V regions were determined by dideoxy sequencing using the T7 Sequencing kit according to the manufacturers instructions (Pharmacia, Uppsala, Sweden). For each patient, V₃ and V₄ nucleotide sequences were determined from PCR amplified material of two independent cDNA reactions.

RESULTS

Cloning of CLL V regions We have cloned and sequenced the V₃ and V₄ regions expressed by two IgG-bearing CD5+ CLL cases, P87 and P103 (Table 1 and Fig 1). Although the tumor load in both patients was very high (Table 1), the cDNA/PCR-based approach could potentially result in the amplification and cloning of V regions from contaminating B cells in the blood samples. To minimize this potential problem, we performed two cDNA/PCR reactions for each sample and obtained nucleotide sequences from independent cDNA clones.

In both CLL cases, a diagnostic DNA fragment of approximately 400 bp was obtained with the V₃₃-specific primer, whereas no amplification products were obtained with primers specific for the other V₃ gene families (results not shown). The amplified products were cloned into Bluescript and sequenced from two directions. In each patient, the VH/DJ₃ joints of cDNA clones from independent cDNA/PCR reactions were identical, rendering it highly likely that they were derived from the tumor.

The V₃ gene expressed in the malignant cells of patient P87 differed by three nucleotides from 38P1, a member of the VH₃ gene family previously obtained from a fetal liver cDNA library (Fig 2). One alteration in VH₃.P87 is silent and localized in framework 2, whereas the other two are replacement mutations in complementarity determining region 2. The D gene segment is most homologous to the germline DN1 gene, whereas the J₃ gene is 100% identical to J₃₆ (Fig 2). The V₃ gene expressed in the malignant cells of patient P103 displayed the highest degree of homology to the germline DN1 gene, whereas the J₃ gene is 100% identical to J₃₆ (Fig 2). The V₃ gene expressed in the malignant cells of patient P103 displayed the highest degree of homology (93.8%) to V₃₃.X15, a V₃ gene expressed by an Epstein-Barr virus-transformed B-cell line derived from a patient with X-linked agammaglobulinemia (release 34). The J₄ gene differed by five nucleotides from J₄₅, whereas the D segment expressed in P103 displayed a moderate degree of homology with DN1 (Fig 3). The V₃ gene expressed by P103 differed by seven nucleotides from the germline HumkV325 gene segment (Fig 4, Table 3). The rearranged J₄ gene differed by four nucleotides from the previously reported germline J₄4 gene seg-
Table 2. Primers Used in First-Strand cDNA and PCR Reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5'-gggCAGGCAGCCCAGGGCCGCTGTGC-3'</td>
</tr>
<tr>
<td>V3</td>
<td>5'-gggaattcGAGTTTGGGCTGAGCGTGG-3'</td>
</tr>
<tr>
<td>C</td>
<td>5'-gggaattcAACAGAGGCAGTTCCAGACTT-3'</td>
</tr>
<tr>
<td>V3</td>
<td>5'-gggaattcTGGAAACCACCAGCCCA-3'</td>
</tr>
<tr>
<td>C</td>
<td>5'-gggaattcTGGGCTCAGTCCAGACTT-3'</td>
</tr>
<tr>
<td>pan-V</td>
<td>5'-gggaattcCTTGTCAGTCCAGACTT-3'</td>
</tr>
</tbody>
</table>

The V<sub>n</sub> and V<sub>l</sub> family-specific primers were designed to anneal to the leader sequences of the V<sub>n</sub> and V<sub>l</sub> genes. The pan-V<sub>n</sub> primer is homologous to a common framework 1 sequence of all V<sub>n</sub> genes. Sequences not homologous to the specified gene segments are depicted in lower case letters. The endonuclease restriction sites are underlined.

The V<sub>n</sub> and V<sub>l</sub> family-specific primers were designed to anneal to the leader sequences of the V<sub>n</sub> and V<sub>l</sub> genes. The pan-V<sub>n</sub> primer is homologous to a common framework 1 sequence of all V<sub>n</sub> genes. Sequences not homologous to the specified gene segments are depicted in lower case letters. The endonuclease restriction sites are underlined.

The V<sub>n</sub> chain used by P87 consisted of J and C regions belonging to subgroup 2, joined to a V<sub>n</sub> gene that displayed 82.4% homology to K6F5, a V<sub>n</sub> gene previously cloned from a follicular lymphoma. The deduced amino acid sequence shows that this V<sub>n</sub> gene belongs to the recently described subgroup IV of V<sub>n</sub> genes (Fig 5). For each CLL, we analyzed four V<sub>n</sub> and three V<sub>l</sub> cDNA clones obtained from two independently performed cDNA/PCR reactions. All cDNA clones of VH3.P87, VH3.P103 and V<sub>n</sub>.P87 were 100% identical, whereas only a single base difference could be detected in the three cDNA clones of VH3.P103 (Fig 2).

**DISCUSSION**

Molecular analysis of the Ig V<sub>n</sub> and V<sub>l</sub> genes expressed in the malignant cells of IgM-bearing CLL have contributed to our understanding of the ontogenic origin and in vivo progression of these tumors. These studies have shown that particular V<sub>n</sub> and V<sub>l</sub> gene segments are recurrently expressed in CD5<sup>+</sup> CLL and that malignant transformation generally occurs at a stage when the V regions have not accumulated somatic mutations except for cases expressing...
Fig 3. Comparison of the D and J genes expressed in P87 and P103 and previously described genes. D segments include putative N insertions; (A) D and J<sub>S</sub> segments identified in the cDNA clones encoding the heavy chains of P87 and P103. The DN1 sequence is derived from Ichihara et al. The J<sub>S</sub> segment used by P87 was 100% identical to J<sub>6</sub> (not shown); (B) J<sub>2</sub> gene expressed by P103 aligned to J<sub>1</sub>. The J<sub>2</sub> segment expressed by P87 was 100% identical to J<sub>2</sub>.

The V<sub>h</sub><sup>5</sup> gene. Furthermore, CD5<sup>+</sup> IgM-bearing CLL tumors display very little intraclonal diversification, suggesting that in at least the majority of the malignant B cells in the population, the somatic hypermutation mechanism is not activated during clonal outgrowth. In that respect they may differ from rare CLL variants that do not express the CD5 molecule.

The V<sub>H</sub> genes expressed in the two IgG-bearing CLL cases analyzed in this study were identified as members of the large V<sub>H</sub>3 gene family, whereas the L chain V regions were encoded by members of the V<sub>III</sub> and V<sub>IV</sub> gene families. Although the sample size is small, these data suggest that V gene expression patterns in IgG CLL resemble those observed in their IgM-bearing counterparts, ie, frequent use of members of the large V<sub>H</sub>3 gene family and overrepresentation of the single Humkv325 gene segment. Recently, a V<sub>H</sub>3 gene was also identified in an IgA-bearing CLL.

The V<sub>H</sub> gene expressed in P87 differed by three nucleotides from 38P1, a V<sub>H</sub>3 gene previously isolated from a second-trimester, fetal liver-derived cDNA library. Considering the source of this cDNA clone, 38P1 likely represents a germline gene. The three nucleotide differences between V<sub>H</sub>3.P87 and 38P1 gene could reflect a polymorphism, a closely related novel member of the V<sub>H</sub>3 gene family and/or somatic mutations. Based on the current data, we cannot discriminate between these alternatives.

The V<sub>H</sub> gene expressed in P103 was identified as a member of the V<sub>III</sub> subgroup. The germline sequences of eight of nine members of this gene family have been characterized. Nucleotide sequence comparison showed seven differences between V<sub>H</sub>3.P103 and Humkv325, the most homologous germline V<sub>H</sub>III gene, whereas other germline V genes display 95.5% or less homology. The Humkv325 gene is highly conserved in the human population, being 100% identical at the nucleotide level in at least nine different individuals analyzed. Although we did not formally

Fig 4. Nucleotide sequences of the V<sub>h</sub> genes expressed by P87 and P103. Sequences were derived from three independent cDNA clones. V<sub>H</sub>3.P103 is compared with the most homologous V<sub>H</sub> gene, Humkv325. Silent mutations are shown in lower case letters and replacement mutations are shown in upper case letters. Dots represent identical nucleotides.
characterize the Humkv325 genes present in this patient’s genome, it seems likely, based on the above considerations, that P103 expresses a somatically mutated variant of the Humkv325 gene.

The nucleotide sequences of V_H.P103 and V_g.P87 differ extensively (93.8% and 82.4%, respectively) from the most homologous V_H and V_g germline genes in the database, precluding definitive conclusions on the origin of these expressed genes.

The analysis of seven cDNA clones from independent cDNA/PCR reactions of each patient showed a very high degree of homogeneity among sets of cDNAs representing each V_H or V_g gene, showing that the cells that belong to the malignant clone in IgG CLL display very little intrachromosomal diversification. In this respect, IgG CLL resembles IgM CLL, a single case of IgA CLL and small cell lymphocytic leukemia, another CD5+ B-cell malignancy that shares many of the characteristics of IgM CLL. This clearly distinguishes these CD5+ B-cell tumors from follicular lymphomas that mutate extensively, and they have been postulated to represent a more mature B-cell phenotype.

Two scenarios may be envisaged to explain the origin of IgG CLL tumors. IgG CLL with mutated V regions may reflect a more mature stage of B-cell differentiation than their IgM-bearing counterparts. Indeed, during a primary immune response, isotype switching may occur in the absence of somatic hypermutation, a pathway perhaps associated with both CD5+ and CD5- B-cells. Alternatively, isotype switching may be an infrequent event associated with transformation, implying that the malignant cells are in fact representatives of fortuitously switched, IgM-bearing B cells. The V_H.P87 gene may be testament to such a scheme. The occurrence of somatic mutations in the V_L region of P103 is not in contradiction with this scenario, because we have recently demonstrated that a major fraction of IgM-bearing B-cell tumors in the blood of healthy adults express mutated V genes.

ACKNOWLEDGMENT

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REFERENCES


Table 3. Homology Comparison Between V_H.P103 and Germline V_g.III Genes

<table>
<thead>
<tr>
<th>Germline V_g.III Gene</th>
<th>Degree of Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humkv325</td>
<td>97.2</td>
</tr>
<tr>
<td>Humkv301</td>
<td>79.9</td>
</tr>
<tr>
<td>Humkv305</td>
<td>95.5</td>
</tr>
<tr>
<td>Humkv3c22*</td>
<td>75.8</td>
</tr>
<tr>
<td>Humkv3g*</td>
<td>91.7</td>
</tr>
<tr>
<td>Humkv3h</td>
<td>91.9</td>
</tr>
</tbody>
</table>

* Humkv3c22 is an orphan gene located on chromosome 22.
Biased V\(\gamma\) gene expression in chronic lymphocytic leukemia. Int Immunol 1:360, 1989


Molecular analysis of VH and VL regions expressed in IgG-bearing chronic lymphocytic leukemia (CLL): further evidence that CLL is a heterogeneous group of tumors

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