Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia

Fabio Ghiotto,1 Franco Fais,1,2 Angelo Valetto,1 Emilia Albesiano,3 Shiori Hashimoto,1 Mariella Dono,1 Hideyuki Ikematsu,4 Steven L. Allen,1,3 Jonathan Kolitz,1,3 Kanti R. Rai,3,5 Marco Nardini,6 Anna Tromontano,7 Manlio Ferrarini,8 and Nicholas Chiorazzi1,3

1Departments of Medicine, North Shore University Hospital and New York University School of Medicine, Manhasset, New York, USA. 2Department of Experimental Medicine, Section of Human Anatomy, University of Genoa, Genoa, Italy. 3North Shore–Long Island Jewish Research Institute, Manhasset, New York, USA. 4Department of Clinical Research, Hara-Doi Hospital, Higashi-ku, Fukuoka, Japan. 5Departments of Medicine, Long Island Jewish Medical Center and Albert Einstein College of Medicine, New Hyde Park, New York, USA. 6Department of Physics–National Enterprise on Nanoscience and Nanotechnology (INFM) and Center of Excellence in Biomedical Research, University of Genoa, Genoa, Italy. 7Department of Biochemical Sciences “Rossi Fanelli,” University of Rome “La Sapienza,” Rome, Italy. 8Division of Medical Oncology C, National Institute for Cancer Research, Genoa, and Department of Clinical and Experimental Oncology, University of Genoa, Genoa, Italy.

Studies of B cell antigen receptors (BCRs) expressed by leukemic lymphocytes from patients with B cell chronic lymphocytic leukemia (B-CLL) suggest that B lymphocytes with some level of BCR structural restriction become transformed. While analyzing rearranged V_{H}DJ_{H} and V_{L}J_{L} genes of 25 non–IgM-producing B-CLL cases, we found five IgG+ cases that display strikingly similar BCRs (use of the same H- and L-chain V gene segments with unique, shared heavy chain third complementarity-determining region [HCDR3] and light chain third complementarity-determining region [LCDR3] motifs). These H- and L-chain characteristics were not identified in other B-CLL cases or in normal B lymphocytes whose sequences are available in the public databases. Three-dimensional modeling studies suggest that these BCRs could bind the same antigenic epitope. The structural features of the B-CLL BCRs resemble those of mAb’s reactive with carbohydrate determinants of bacterial capsules or viral coats and with certain autoantigens. These findings suggest that the B lymphocytes that gave rise to these IgG+ B-CLL cells were selected for this unique BCR structure. This selection could have occurred because the precursors of the B-CLL cells werechosen for their antigen-binding capabilities by antigen(s) of restricted nature and structure, or because the precursors derived from a B cell subpopulation with limited BCR heterogeneity, or both.

Introduction

B cell chronic lymphocytic leukemia (B-CLL), a monoclonal expansion of mature CD5-expressing B lymphocytes, is a heterogeneous disease that affects primarily individuals over 50 years of age (1). Even though B-CLL is the most common leukemia in the Western hemisphere (2), the events that select out an individual normal B cell clone and usher it toward leukemic transformation remain unknown. Genetic abnormalities probably exist in these cells and represent important inducers; however, no single unifying molecular genetic defect or combination of defects has yet been identified (3).

Studies of the characteristics of the B cell antigen receptors (BCRs) expressed by B-CLL cells imply that precursor B lymphocyte clones that eventually become leukemic exhibit varying degrees of BCR structural similarity (4). This restriction in BCR structure suggests that either the precursors of the leukemic B lymphocytes were selected by specific antigens that have affinity for these BCRs, or they were garnered from a B cell subpopulation with restricted BCR structural heterogeneity.

In the present study, we analyzed the rearranged V_{H}DJ_{H} and V_{L}J_{L} genes of a cohort of 25 B-CLL patients whose leukemic cells express isotype-switched Ig. Our results reveal that a substantial subset of IgG+ cases (∼20%) display strikingly similar Ig V region gene features. These include the use of the same H- and L-chain V gene segments, which are combined in unique ways and exhibit little somatic diversification despite their Ig class–switched nature. These findings are compelling evidence that selection of a specific BCR structure is an important component promoting the development of B-CLL. Preliminary abbreviated reports of these findings have appeared previously (5, 6).

Methods

CLL patients and samples. The Institutional Review Board of North Shore University Hospital (Manhasset, New York) and Long Island Jewish Medical Center (New Hyde Park, New York) approved these studies. From a cohort of 237 patients with clinical and laboratory features of B-CLL, 25 patients with expansions of CD5+/CD19+ B cells expressing surface membrane IgG or IgA were chosen and analyzed. All of the patients with surface membrane IgM+ cells were obtained randomly; some of the IgG+ cases were provided by others because of their surface membrane phenotype and therefore were not randomly acquired. Some patients and the V gene

Nonstandard abbreviations used: arsinate (Ars); B cell antigen receptor (BCR); B cell chronic lymphocytic leukemia (B-CLL); double-stranded DNA (dsDNA); framework region (FR); germinal center (GC); heavy chain third complementarity-determining region (HCDR3); light chain third complementarity-determining region (LCDR3); single-stranded DNA (ssDNA); web antibody modeling (WAM).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J. Clin. Invest. 113:1008-1016 (2004). doi:10.1172/JCI200419399.
sequences of their leukemic cells were described previously (5–9). PBMCs from these patients, obtained from heparinized blood by density gradient centrifugation (Ficoll-Paque; Amersham Biosciences, Piscataway, New Jersey, USA), were used after thawing samples that had been cryopreserved with a programmable cell-freezing machine (CryoMed, Inc., Mt. Clemens, Michigan, USA).

Isolation of DNA. T lymphocytes were purified from PBMCs by negative selection using the Pan T cell isolation kit (Miltenyi Biotec, Auburn, California, USA), and DNA was isolated from these cells with the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California, USA).

Preparation of RNA and synthesis of cDNA. Total RNA was isolated from PBMCs using Ultraspec RNA (Biotecx Laboratories Inc., Houston, Texas, USA) according to the manufacturer’s instructions. RNA (1 μg) was reverse-transcribed to cDNA using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp., Carlsbad, California, USA), 1 U of RNase inhibitor (Eppendorf, Hamburg, Germany), and 20 pmol of oligo dT primer (total volume of 20 μl). These reactants were incubated at 42°C for 1 hour, heated at 65°C for 10 minutes to stop the reactions, and then diluted to a final volume of 100 μl.

PCR conditions for IgV gene DNA sequencing. To determine the IgVH gene family used by various B-CLL cells, cDNA (2 μl) was amplified using sense framework region 1 (FR1) primers specific for the various IgVH gene families in conjunction with an appropriate antisense IgCγ primer (10). Reactions were carried out in 50 μl using 20 pmol of each primer and cycled with a 9600 GeneAmp System (Perkin-Elmer Cetus, Emeryville, California, USA).

The DNA sequence of the B-CLL IgVH gene was determined by re-amplifying the original cDNA (2 μl) using the appropriate IgVH family leader and IgCγ primers defined above (10). PCR products were sequenced directly after purification with Wizard PCR Preps (Promega Corp., Madison, Wisconsin, USA) using an automated sequencer (Applied Biosystems, Foster City, California, USA). In some instances where mutations were detected, an independent PCR product was generated and either sequenced directly or cloned into TA vector (Invitrogen Corp.), processed using Wizard miniprep (Promega Corp.), and then sequenced using M13 forward and reverse primers.

To determine IgVL gene sequences, cDNA (2 μl) was amplified using the leader primers listed in Supplemental Table 1 (supplemental materials available at http://www.jci.org/cgi/content/full/113/7/1008/DC1). For the μA families I, III, and IV, a mixture of primers was used. To amplify μA families II and X, both forward primers were used in a common reaction, since they cross-prime. Reactions were carried out in a 50-μl volume using 20 pmol of each primer, 200 μM dNTPs, 1.5 mM MgCl2, and 1.25 U of Taq Gold (Perkin-Elmer), and cycled with a 9600 GeneAmp System as follows: denaturation at 94°C for 45 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 45 seconds. After 35 cycles, extension was continued at 72°C for an additional 10 minutes. IgVL PCR products were sequenced in the same manner as IgVH PCR products.

To identify potential polymorphisms in the germline VH4–39 gene, PCR was performed on DNA from autologous T cells of two patients (nos. 057 and 114) using VH4–39 CDR2-specific gene primers (forward: 5′-GTTGGGCCTCCTCCAGATG-3′; reverse: 5′-TCACACTCACCTCCCTCAC-3′). PCR products were cloned and sequenced using the TOPO TA cloning kit (Invitrogen Corp.).

Analyses of IgV, D, and J sequences. DNA sequences were compared with those in the V BASE sequence directory (11) using MacVector software, version 6.0 (Accelrys, San Diego, California, USA) as previously described (8). Amino acid sequences were compared with those in GenBank by means of a BLAST search using the tblastn algorithm.

Analyses of heavy chain complementarity region 3 and light chain complementarity region 3 rearrangements. Heavy chain third complementarity-determining region (HCDR3) length was determined according to Kabat and Wu (12) by counting the number of amino acids between position H94 at the 3′ end of FR3 (usually two amino acids downstream of the conserved cysteine) and position H102 at the beginning of FR4 (a conserved tryptophan in all Jγ segments). Light chain third complementarity-determining region (LCDR3) length was determined by counting amino acids beginning at position L89 (preceded by a conserved cysteine) to position L97 (followed by a conserved Phe-Gly pair). Hypervariable loops were defined according to Chothia and Lesk (13); in particular, the third hypervariable loop of the H chain (H3) spans the amino acids 92–104, in the Kabat et al. numbering scheme (14).

Antibody modeling. Three-dimensional models of the V domains of the Ig’s were constructed using the canonical structure model (14) as implemented in the web antibody modeling (WAM) algorithm (15). Models were analyzed using the molecular graphics package Insight II (16).

Results

Identification of five IgG-expressing B-CLL cases with remarkably similar BCR. While determining cDNA sequences of VH and VL rearrangements expressed in 25 isotype-switched B-CLL cases (23 IgG and 2 IgA; see Supplemental Table 2), we identified five IgG-expressing cases with remarkably similar BCRs. These cases (CLL nos. 039, 057, 114, 202, and 209) expressed the same VH (4–39), D [6–13], and Jγ (5b) gene segments (Table 1). For the four cases in which L-chain data were obtained (additional sample on cDNA no. 209 was not available because the patient died in an automobile accident), all used the VkO12/2 gene (Table 1). In cases 057, 114, and 202, this gene recombined with the Jκ1 gene segment; in case no. 039, the VkO12/2 gene was associated with Jκ2. However, the expressed L chains of these four cases, including CLL no. 039, were virtually identical at the amino acid level (see below).

These five patients had several clinical similarities. The subset comprised primarily women with a 1:4 male/female ratio (Table 1), which differs from that in the other isotype-switched cases in this cohort (14:6; see Supplemental Table 2) and in B-CLL cases in general (~2:1 [refs. 1, 2]). Moreover, the patients experienced aggressive clinical courses complicated by severe recurrent infections (no. 039), Richter’s transformation (no. 057), or the occurrence of secondary solid tumors (nos. 114 and 202). There was no familial or consistent ethnic relationship among these cases, and the patients originated from different parts of the world (two from the United States, one from Italy, one from the Caribbean region, and one from Japan).

The majority of the other 20 isotype-switched cases used either a VH3 family gene (50%) or a VH4 family gene (35%; see Supplemental Table 2). All of these VH4–34 gene cases (one case (CLL no. 097) used the VkO12/2 gene, although this Vk gene was mutated and was paired with a mutated Vγ3–73 gene. One of the remaining 20 cases expressed Vk, and six expressed Va genes, with no apparent Va segment biases. All of the Vγ-expressing cases used a VH3 gene; among these, only Jκ2 was used.

Ig V gene mutation status. Although the leukemic cells of these five cases synthesized IgG, their VH and VL genes showed minimal deviation from the germline gene sequence (VH: 0.3–0.7%, and VL: 3–3.5%).
Table 1
Gene segments expressed by subset of IgG+ B-CLL cases

<table>
<thead>
<tr>
<th>CLL no.</th>
<th>Gender</th>
<th>V gene family</th>
<th>Most similar germline V gene</th>
<th>Percent V gene difference from germline</th>
<th>D segment</th>
<th>J gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>039</td>
<td>Female</td>
<td>V4</td>
<td>4–39</td>
<td>0.7</td>
<td>6–13</td>
<td>5b</td>
</tr>
<tr>
<td>057</td>
<td>Female</td>
<td>V4</td>
<td>4–39</td>
<td>0.0</td>
<td>–</td>
<td>k2</td>
</tr>
<tr>
<td>114</td>
<td>Male</td>
<td>V4</td>
<td>4–39</td>
<td>0.0</td>
<td>–</td>
<td>k1</td>
</tr>
<tr>
<td>202</td>
<td>Female</td>
<td>V4</td>
<td>4–39</td>
<td>0.3</td>
<td>6–13</td>
<td>5b</td>
</tr>
<tr>
<td>209</td>
<td>Female</td>
<td>V4</td>
<td>4–39</td>
<td>0.3</td>
<td>6–13</td>
<td>5b</td>
</tr>
</tbody>
</table>

*Based on comparison with patient’s genomic (T lymphocyte) germline V4–39 genes, which differ at one (CLL 057) and three (CLL 114) positions from the canonical germline V4–39 gene in GenBank. NA, not available.

For VH, there were only two amino acid changes among the five cases. CLL no. 057 exhibited a Pro → Thr change at position H63, and no. 039 displayed an Arg → Ser change at position H96; these isolated changes occurred in FR3. The Vλ protein sequences were also virtually identical; only CLL no. 114 showed a Gln → Arg change at position L3 in FR1.

HCDR3 structure. Figure 1A aligns the HCDR3 protein sequences for these five IgG-expressing cases. HCDR3 lengths were very similar (16–17 amino acids). These lengths are longer than most normal B cells (19) and many B-CLL cells (8, 20). The HCDR3 sequences of the five cases were very similar, exhibiting a consensus sequence (XGYSSWSYG/SX(X)NWFDP; Figure 1A) consisting of two N-terminal hydrophilic amino acids, seven invariant amino acids that represent a portion of the D6–13 gene segment read in the hydrophilic reading frame, a glycine or serine that are similar in their small side chains, one to two variable amino acid(s) that lack chemical similarity, and five amino acids corresponding to the 5′ portion of the Jκ5b segment.

LCDR3 structure. The LCDR3 sequences of all cases were identical (Figure 1B), except for a difference in the last amino acid in CLL no. 039; this difference results from the use of the Jκ2 segment in this instance. In each case, an arginine (R) was located at the VK-Jκ junction (position L96). In CLL no. 114 (Figure 2A), CGG, one of the six possibilities that code for arginine, resulted from coding end trimming and recombination of the VK and Jκ germline segments. For cases no. 057 and 202 (Figure 2B), the codon CGA yielded the arginine via a process similar to that for CLL no. 114. However, for CLL no. 039, which used the Jκ2 gene segment (Figure 2C), the combinatorial process that led to the arginine at the VK-Jκ junction was more complex. This required the trimming of one nucleotide from the 3′ end of VL, and the deletion of seven nucleotides from the 5′ end of Jκ2, along with the nontemplated insertion of two G nucleotides, to generate an arginine codon (CGG).

Lack of similar rearranged Ig V genes in other human B cells. We searched GenBank for VH-DJH rearrangements with significant amino acid similarity to the consensus protein sequences of these five B-CLL cases. However, for Vλ, there were only two amino acid changes among the five cases. CLL no. 057 exhibited a Pro → Thr change at position H63, and no. 039 displayed an Arg → Ser change at position H96; these isolated changes occurred in FR3. The Vλ protein sequences were also virtually identical; only CLL no. 114 showed a Gln → Arg change at position L3 in FR1.

HCDR3 structure. Figure 1A aligns the HCDR3 protein sequences for these five IgG-expressing cases. HCDR3 lengths were very similar (16–17 amino acids). These lengths are longer than most normal B cells (19) and many B-CLL cells (8, 20). The HCDR3 sequences of the five cases were very similar, exhibiting a consensus sequence (XGYSSWSYG/SX(X)NWFDP; Figure 1A) consisting of two N-terminal hydrophilic amino acids, seven invariant amino acids that represent a portion of the D6–13 gene segment read in the hydrophilic reading frame, a glycine or serine that are similar in their small side chains, one to two variable amino acid(s) that lack chemical similarity, and five amino acids corresponding to the 5′ portion of the Jκ5b segment.

LCDR3 structure. The LCDR3 sequences of all cases were identical (Figure 1B), except for a difference in the last amino acid in CLL no. 039; this difference results from the use of the Jκ2 segment in this instance. In each case, an arginine (R) was located at the VK-Jκ junction (position L96). In CLL no. 114 (Figure 2A), CGG, one of the six possibilities that code for arginine, resulted from coding end trimming and recombination of the VK and Jκ germline segments. For cases no. 057 and 202 (Figure 2B), the codon CGA yielded the arginine via a process similar to that for CLL no. 114. However, for CLL no. 039, which used the Jκ2 gene segment (Figure 2C), the combinatorial process that led to the arginine at the VK-Jκ junction was more complex. This required the trimming of one nucleotide from the 3′ end of VL, and the deletion of seven nucleotides from the 5′ end of Jκ2, along with the nontemplated insertion of two G nucleotides, to generate an arginine codon (CGG).
NA Antibody library from human B cells (Y. Akahori, et al., unpublished data)

Human monoclonal rheumatoid factor (98)

consensus amino acid isotype (GenBank entry no. Y09250 [ref. 22]).

cDNA library from normal blood B cells (97) (Table 2). One of the κ\textsubscript{4} 3–21 chain of rheumatoid factor bound by 21–46.

Gene rearrangements, with similar LCDR3 lengths and junctional arginine (R) from other human B cells 4 3–21.

1 cases with known companion B-CLL cell (23)


IgG+ B-CLL cases

do not substitute non-coding DNA sequences (96). The LCDR3 length that have an arginine at the VL-JL junction and a specific, unique antigenic reactivity. However, there was a striking enrichment (102/199) for antibodies that react with structures that serve as autoantigens in several autoimmune settings; examples are double-stranded (ds) and single-stranded (ss) DNA, IgG, and thyroid constituents. This was consistent with the autoreactivity identified in the VκO12/2-Jκ listed in Table 2.

Three-dimensional models of the B-CLL BCR. We used the canonical structure method (13) as implemented in the WAM algorithm to build models for the V domains, and then analyzed these structures to deduce characteristics of the antigen-binding sites (Figure 3). The canonical structure model requires that the main chain con-

Figure 2 Different mechanisms generate an arginine at the V\textsubscript{\lambda}-J\textsubscript{\lambda} junctions. The codons for arginine are listed. Color code: identical nucleotides of the arginine codon are in brown, nucleotides deleted are in black, nucleotides probably resulting from nontemplated nucleotide addition are in dark blue, and differences within areas of identity or similarity in red. (A and B) An appropriate codon results from coding end trimming and recombination of the two germline V\textsubscript{\lambda} and J\textsubscript{\lambda} segments. (C) The arginine codon develops via a more complex combinatorial process, requiring the trimming of one nucleotide from the 3′ end of V\textsubscript{\lambda}, the deletion of seven nucleotides from the 5′ end of J\textsubscript{\lambda}, and the nontemplated insertion of two G nucleotides.

Table 2

O12/2-Jκ Gene rearrangements, with similar LCDR3 lengths and junctional arginine (R) from other human B cells

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Percent amino acid difference from consensus O12/2-Jκ\textsuperscript{a}</th>
<th>Jκ</th>
<th>V\textsubscript{\kappa} Gene</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus</td>
<td>–</td>
<td>1</td>
<td>4–29</td>
<td>IgG: B-CLL cases</td>
</tr>
<tr>
<td>BAC01727</td>
<td>0.0%</td>
<td>1</td>
<td>NA</td>
<td>Antibody library from human B cells (Y. Akahori, et al., unpublished data)</td>
</tr>
<tr>
<td>CAAS1109</td>
<td>0.0%</td>
<td>1</td>
<td>NA</td>
<td>Phage library using cDNA from human splenic B cells (94)</td>
</tr>
<tr>
<td>CAD68160</td>
<td>0.9% (1 AA)</td>
<td>1</td>
<td>NA</td>
<td>Antibody reactive with apoptotic cells (R. Gandhi, et al., unpublished data)</td>
</tr>
<tr>
<td>1HEZA</td>
<td>0.9% (1 AA)</td>
<td>1</td>
<td>9.1</td>
<td>k chain of rheumatoid factor bound by k-binding protein (95)</td>
</tr>
<tr>
<td>AAL65713</td>
<td>0.9% (1 AA)</td>
<td>2</td>
<td>1–46</td>
<td>Hybridoma from human tonsil B lymphocytes (M. Vaisbourd, et al., unpublished data)</td>
</tr>
<tr>
<td>CAAS5588</td>
<td>0.9% (1 AA)</td>
<td>1</td>
<td>NA</td>
<td>cDNA library from normal blood B cells (22)</td>
</tr>
<tr>
<td>CAD43018</td>
<td>0.9% (2 AA)</td>
<td>4</td>
<td>3–21</td>
<td>Human anti-La autoantibodies from phage display library (J.M.H. Raats, unpublished data)</td>
</tr>
<tr>
<td>CAD43019</td>
<td>1.8% (2 AA)</td>
<td>4</td>
<td>3–21</td>
<td>Human anti-La autoantibodies from phage display library (J.M.H. Raats, unpublished data)</td>
</tr>
<tr>
<td>CAD43020</td>
<td>1.8% (2 AA)</td>
<td>4</td>
<td>3–21</td>
<td>Human IgG anti-α(IIb)β3 antibody from phage-displayed library (96)</td>
</tr>
<tr>
<td>CAC28926</td>
<td>0.9% (2 AA)</td>
<td>2</td>
<td>1–02</td>
<td>Human IgG anti-α(IIb)β3 antibody from phage-displayed library (96)</td>
</tr>
<tr>
<td>AAD19446</td>
<td>1.8% (2 AA)</td>
<td>1</td>
<td>1–24</td>
<td>cDNA library from normal blood B cells (97)</td>
</tr>
<tr>
<td>1DEEA</td>
<td>1.8% (2 AA)</td>
<td>1</td>
<td>3–30</td>
<td>Human monoclonal rheumatoid factor (98)</td>
</tr>
<tr>
<td>AAN87123</td>
<td>1.8% (2 AA)</td>
<td>4</td>
<td>9.1</td>
<td>Human IgM antibody to capsular polysaccharide of Neisseria meningitidis (63)</td>
</tr>
<tr>
<td>AAC13457</td>
<td>2.6% (3 AA)</td>
<td>4</td>
<td>3–33</td>
<td>Phage-displayed human anti-Rh(D) antibody (99)</td>
</tr>
<tr>
<td>AAF86916</td>
<td>3.5% (4 AA)</td>
<td>2</td>
<td>4–34</td>
<td>B-CLL cell (23)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Denotes percent and number of amino acid differences from consensus VκO12/2-Jκ sequence of IgG+ B-CLL cases.
formation of the hypervariable loops depend solely on length and on the nature of a few specific residues. Antibody specificity is therefore determined by the nature of exposed side chains mounted on the main chain of the hypervariable loops, which in turn is determined by their canonical structure.

In the four available B-CLL L chains (case nos. 039, 057, 114, and 202), the L1 loop is six residues long and contains a conserved isoleucine at position 29. In all known antibody structures, six-residue L1 loops are stabilized by contact of the side chain of this amino acid with residues 2, 25, 33, and 71 (13). These contacts determine the main chain structure of the loop. Each of these five residues is hydrophobic and conserved in the four B-CLL rearranged L chains. Since the exposed amino acids of the loop are also conserved, the contribution of this loop to the antigen-binding site would be virtually identical for all four antibodies. The L2 loop has the same conformation in all known V region structures (13). Since there are no substitutions in the exposed side chains of these cases, the contribution of this loop to the binding site is expected to be the same for all the proteins. Similarly, since the backbone structure of the L3 loop depends on its length and the nature of the residues at positions 90 and 95, which are all identical in the B-CLL sequences, this loop should have the same conformation and solvent-exposed surface in all four cases.

The H1 loop has a conserved hydrophobic residue in position 29 and a conserved glycine in position 26. The former establishes stabilizing interactions with residues 34, 72, and 77. Each of these five residues is conserved among the VH sequences of the five B-CLL cases. Consistent with these observations, the WAM prediction server produces identical models in this region for the five antibodies. The H2 conformation has been shown to depend only on its length and on the residue at position 71 (24); both of these features are conserved in the five B-CLL sequences.

One cannot infer as clear a sequence-structure relationship for H3, since this loop is the most variable in length and sequence. The conformation of H3 is mainly determined by the presence or absence of a β bulge within its structure, in the region closer to the FR. The presence of a β bulge is in turn determined by the amino acid sequence of the loop (25). Based on our modeling analyses, the H3 loop is predicted not to be bulged in CLL nos. 039 and 114 and bulged at residue H101 in CLL nos. 057, 202, and 209. The creation of the bulge in the latter cases implies that residue H101m of the nonbulged loops is in a position equivalent to that of residue H101 of the bulged loops. Thus, the tip of the loop probably contains the same number of residues (n = 14) in all cases, and the residues in the bulged cases can maintain a relationship in space similar to that of the non-bulged cases.

Discussion

In this study we describe five IgG-expressing B-CLL cases with remarkably similar BCRs. These receptors consist of the same VH, D, JH, and VL, JL gene segments, except for a different JK segment in one instance. Despite this difference, all BCRs have identical LCDR3s and very similar HCDR3s, each with unique sequence and junctional motifs. This BCR restriction could be the consequence of either random transformation of a subpopulation of B cells with very limited antigen receptor heterogeneity — determined genetically or by antigen selection; or specific transformation of B cells that were selected by antigen from a BCR-restricted or BCR-heterogeneous subpopulation; or both. Whatever the cause, our findings support the concept that B-CLL develops from a limited set of B lymphocytes of defined BCR structure and imply that selection of B cells with such structures represents an important promoting influence in the evolution of the leukemic cells.
Composition, motifs, and three-dimensional structure models. Developing B lymphocytes do not use all germline IgV gene segments with the same frequency, and biases in gene segment rearrangement do occur (26–29). Nevertheless, considering combinatorial diversity, imprecise joining, nucleotide insertion and deletion, and somatic hypermutation, the probability of finding selectively in one disease, by chance alone, five cell clones with such highly similar rearranged H- and L-chain V region pairs is extremely low. The probability that specific $V_{\text{H}}$, $D$, and $J_{\text{H}}$ genes would be used in the same $V_{\text{H}}D_{\text{H}}J_{\text{H}}$ rearrangement is 1 in 7,128 (1/44 $\times$ 1/27/1/6); for a specific $V_{\text{L}}$ and $J_{\text{L}}$ gene pair the probability is 1/230 (1/46 $\times$ 1/5) or 1/252 (1/36 $\times$ 1/7) for a $\kappa$ versus a $\lambda$ rearrangement, respectively. Only 1 in 1,639,440 B cells would be predicted to randomly express the same $V_{\text{H}}$, $D$, $J_{\text{H}}$, $V_{\text{K}}$, and $J_{\text{K}}$ segments in its BCR. These calculations use the number of germline V segments in IgBLAST and assume that $\kappa$ genes rearrange before $\lambda$ genes.

Considering these estimations, the frequency at which this BCR occurs in our IgG+ B-CLL cases is extraordinary (∼20%). Although this percentage will need confirmation, the frequency of such unique IgG+ cases is very similar to that identified when we first reported on Ig V gene diversification and apparent antigen selection in a smaller number of isotype-switched B-CLL cases (7). This structure was not seen in our IgM+ B-CLL cohort (175 cases for which both the rearranged $V_{\text{H}}D_{\text{H}}J_{\text{H}}$ and $V_{\text{J}}$, are known) or in GenBank, indicating that this BCR is not overexpressed among IgM+ B-CLL cases or in the normal circulating B cell repertoire. We cannot rule out that its frequency increases with age or that it exists among certain distinct noncirculating subpopulations of B cells, although a recent study suggests that the former may be unlikely (30).

Notwithstanding the present limitations of antibody modeling, primarily with respect to the H3 loop, we can reliably conclude that most of the binding site is identical among these IgG+ B-CLL cases. Five of the six $H$ and $L$ loops have the same main chain conformation and differ, in only one case, by a conservative Ser $\rightarrow$ Thr side chain change at position 97 of the $L$ chain. However, this residue does not contact antigen in any known structure (Veronica Morea, personal communication). The H3 loops are also quite similar among the five antibodies, although three are longer by one residue (Figure 1). Since these loops contain a bulge (i.e., they have one residue that is extruded from the regular $\beta$ structure), the residues at the central region of the loop, which are more relevant for antigen binding, can maintain the same relationship in space in all five cases. Therefore, the antigen-binding surfaces of these antibodies are probably very similar, and they likely bind the same epitopic antigen. Nevertheless since some amino acid differences do exist at the $V_{\text{H}}$, and $D_{\text{H}}J_{\text{H}}$ junctions, this remains conjecture.

Restricted IgV gene structural features of antibodies with defined antigenic reactivities. Normal and neoplastic B cells of known antigen specificity can display restricted V (D) J segment use, either at both H and L chain loci or individually at either locus. Murine mAb’s reactive with β-(1,6)-d-galactan (31), α(1 → 6) dextran (32, 33), phosphorylcholine (34), dextrans and fructofuranos (35), and phosphatidylcholine (36–38) pair very restricted and characteristic $V_{\text{H}}D_{\text{H}}J_{\text{H}}$ and $V_{\text{L}}J_{\text{L}}$ gene segments. Human antibodies that exhibit individual H- or L-chain restrictions include mAb’s specific for the capsular polysaccharides of Haemophilus influenzae type b (39–41) and Streptococcus pneumoniae (42, 43) (individual $V_{\text{H}}$,3 genes for both), monoclonal cold agglutinins with anti-I specificity (exclusively $V_{\text{H}}$,3–34 [refs. 44–46]), and monoclonal rheumatoid factors with IgG reactivity (V11–69 [refs. 47–51]). Murine anti-arsonate (52–54) and anti-bacterial polysaccharide mAb’s (55) frequently use specific individual $V_{\text{K}}$ and $J_{\text{K}}$ genes.

In addition, mAb’s reactive with carbohydrates (30, 32, 40, 56), autoantigens (57, 58), and haptenes (52–54) often have characteristic V-J-JL junctional residues. Human anti-H. influenzae mAb’s that use the VKa2 gene segment contain an arginine at position L96, although chain recombination experiments suggest that it is not essential for antigen binding (40, 56). Murine mAb’s reactive with arsonate (Ars), dsDNA, and β-(1,6)-d-galactan exhibit an arginine or an isoleucine at position L96, respectively. These junctional amino acids are essential for binding to Ars (53), but not to β-(1,6)-d-galactan (59).

Nature of the antigen that could have selected these BCRs. Our findings suggest that the B lymphocytes that gave rise to these IgG+ B-CLL cells were selected for a unique BCR structure. If this selection involved antigen binding and triggering through the BCR, the antigen(s) would most likely have been of restricted nature and structure. Although the identity of such an antigen is unknown, we can infer certain features based on our data.

First, the diverse geographic origins of our patients suggests that the putative structure would probably be distributed worldwide. Second, since we could not identify this BCR in B lymphocytes from normal individuals deposited in GenBank. Fourth, the presence of a positively charged center to the antigen-binding pocket, provided by the side chain of arginine L96, and surrounded by an aromatic area and a ridge of polar residues, would imply that a negatively charged or electron-rich group exists in the epitope interactive with these BCRs (Figure 3). This, however, is not a necessity (41, 53, 59). Of the 199 completely sequenced antibodies with an arginine at L96 (14), we found that about 50% react with autoantigens. This is consistent with the association with autoreactivity in the VK012/2/Jk rearrangements listed in Table 2. Autoantigens can be negatively charged molecules (e.g., DNA), and their reactivity with positively charged residues in the CDRs of autoantibodies is enhanced as the number of these latter residues increases (58, 60). Finally, in light of the comparisons with antibodies of known specificities, the epitope could be a carbohydrate that is restricted to a unique molecule or shared by several molecules, an autoantigen that is shared by all individuals or polymorphic among populations, or a carbohydrate determinant of an autoantigen. The possibility that two classes of antigens can bind to the same binding site also should not be ruled out (61).

It is of interest that several relationships exist between carbohydrate reactivity and autoreactivity. Autoantibodies can react with carbohydrates (62–65) and can confer protection against infection with encapsulated bacteria (66, 67). In addition, antipneumococcal polysaccharide antibodies can convert to anti-dsDNA reactivity after minimal amino acid changes in the Ig V region (68). In this regard, it is known that the BCRs of B-CLL clones can be autoreactive (69, 70) and can express cross-reactive idiotypes of dsDNA antibodies (71).

Importance of B cell precursors with constrained BCR structural diversity in the development and progression of B-CLL. Irrespective of the nature of the antigen(s) possibly involved in these cases, these five IgG+ clones are extraordinary examples of the principle that B-CLL progenitors are selected for certain limited BCR structures (4). Sim-
ilar evidence for BCR restriction, possibly attained by antigen or superantigen selection, can be found among the IgM-expressing cases that use a \(\text{V}_{\mu3}–21\) gene with a restricted \(\lambda\) partner (72, 73), and probably in those cases that use an unmutated \(\text{V}_{\mu1}–69\) gene with a characteristic HCDR3 (8, 20).

Although the expression of BCRs of class-switched isotype often suggest involvement in T cell–dependent responses, the lack of significant numbers of IgV gene mutations in B lymphocytes is unusual. IgV gene mutations and the occurrence of a limited degree of isotype class switching also occur during B cell clonal expansion in the absence of T cell help and in response to T-independent antigens (74). Previous studies suggest that antigen-stimulated B cells that participate in a GC reaction lead to B cell lymphoproliferative disorders such as follicular cellular lymphoma and Burkitt’s lymphoma (75). Nevertheless, B cells that follow a different pathway of B cell activation—that is, one not involving a classical GC reaction (76, 77)—could develop into B-CLL cells in certain instances. The marginal zone is considered a possible site for such nonclassical GC reactions (74) and the development of B-CLL from marginal zone B cells has been suggested (4). This suggestion is intriguing because the marginal zone is enriched in B cells that react with carbohydrates (74) and autoantigens (78–80), and its IgV gene repertoire, at least in rodents, is highly restricted (81, 82). This receptor restriction apparently occurs early in life and is based on BCR composition, specificity, and signal-transducing capacity (81).

Finally, since these and other B-CLL clones express BCRs with restricted antigen-binding sites, antigen-driven B cells could promote intracranial evolution leading to accumulation of deleterious DNA mutations by members of the leukemic clone and subsequently to a more aggressive clinical course. Repetitive engagement of the BCRs by either autoantigens or foreign antigens, possibly from microorganisms that are encountered during intermittent or persistent infections, could elicit such effects. In this regard, the cases that have the most convincing molecular evidence for BCR restriction and antigen or superantigen selection (i.e., these IgG\(^+\) cases and those expressing \(\text{V}_{\mu3}–21\) and \(\text{V}_{\mu1}–69\) experience more aggressive clinical courses with shortened mean survival times (72, 83, 84). In addition, ongoing B cell activation and differentiation can occur in B-CLL cells (10, 85–88) and may be initiated or augmented by BCR-mediated signal transduction that is preserved more often in those cases with the worst clinical outcomes (89, 90).

Acknowledgments
The authors appreciate the valuable discussions and suggestions of Martin Weigert (Princeton University) and Michael Potter (National Cancer Institute). These studies were supported in part by RO1 grants from the National Cancer Institute (CA 81544 and CA 87956), General Clinical Research Center grant (M01 RR018535) from the NIH/NCRR, the Joseph Elettro Leukemia Research Fund, the Jean Walton Fund for Lymphoma and Myeloma Research, the Peter Jay Sharp Foundation, and Associazione Italiana Ricerca sul Cancro (AIRC).

Received for publication July 3, 2003, and accepted in revised form January 6, 2004.

Address correspondence to: Nicholas Chiorazzi, North Shore–LIJ Research Institute, 350 Community Drive, Manhasset, New York 11030, USA. Phone: (516) 562-1085; Fax: (516) 562-1022; E-mail: nchizzi@nshs.edu.

Fabio Ghiotto’s present address is: Dipartimento di Medicina Sperimentale, Sezione di Anatomia Umana, Università di Genova, Genoa, Italy.

Angelo Valetto’s present address is: Divisione di Citogenetica e Genetic Molecolare, Azienda Ospedaliera Pisana Santa Chiara, Pisa, Italy.

Shiori Hashimoto’s present address is: Department of Neurology, Neurological Institute, Tokyo Women’s Medical University, Tokyo, Japan.

Mariella Dono’s present address is: Division of Medical Oncology C, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy.


32. Rudikoff, S. 1988. Antibodies to beta(1,6)-D-galact


37. Feeney, A.J. 1992. Predominance of VH-D-JH junctions occurring at sites of short sequence homology results in limited junctional diversity in neona


ger's syndrome and hepatitis C virus infection may share a common pathogenesis: chronic stimu


