Lack of Allelic Exclusion in B Cell Chronic Lymphocytic Leukemia

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Summary

We determined the immunoglobulin (Ig) V<sub>H</sub> subgroup expressed by the leukemia cells of 108 patients with B cell chronic lymphocytic leukemia (CLL). Surprisingly, we found that six samples (5%) each expressed Ig of more than one V<sub>H</sub> subgroup. Southern blot analysis demonstrated that these samples each had rearrangements involving both Ig heavy chain alleles. Nucleic acid sequence analyses of the Ig cDNA revealed each to express two functional Ig V<sub>H</sub> genes: V<sub>H</sub>-2-70, V<sub>H</sub>-3-7, and V<sub>H</sub>-4-59. Despite having more than one Ig heavy chain transcript, each sample was found to express only one functional Ig light chain. From the primary sequence, we deduced that the Ig of some of these CLL samples should react with Lc1, a monoclonal antibody (mAb) reactive with a supratypic cross-reactive idiotypic present on Ig encoded by a subgroup of Ig V<sub>H</sub> genes (namely, V<sub>H</sub>-4-39, V<sub>H</sub>-4-b [DP-67], V<sub>H</sub>-4-59, or V<sub>H</sub>-4-61), and B6, an mAb that reacts with Ig encoded by certain Ig V<sub>H</sub> genes (namely, V<sub>H</sub>-3-23, V<sub>H</sub>-3-30, or V<sub>H</sub>-3-30.3), and/or modified staphylococcal protein A (SpA), a 45-kilodalton bacterial “superantigen” that reacts with most Ig of the V<sub>H</sub>-3 subgroup. Flow cytometric analyses revealed that such samples did in fact react with Lc1 and B6 and/or SpA, but not with control mAbs of irrelevant specificity. This study demonstrates that a subset of CLL patients have leukemic B cells that express more than one functional Ig heavy chain.

Igs are comprised of heavy and light chains that are encoded by genes that rearrange during B cell ontogeny. In the Ig heavy chain gene complex on chromosome 14, there are ~50 functional Ig V<sub>H</sub> genes, 30 diversity segments, and 6 J<sub>H</sub> minigenes (1-3). The Ig V<sub>H</sub> genes are classified into seven subgroups (V<sub>H</sub>-1-V<sub>H</sub>-7) based on their relative nucleotide sequence homology (4, 5). During B cell development, one or more diversity segments can juxtapose with a J<sub>H</sub> gene segment, forming a DJ<sub>H</sub> complex that then rearranges with an Ig V<sub>H</sub> gene to form a VH, DJ<sub>H</sub> exon that ultimately can encode the Ig heavy chain variable region (6). After successful Ig heavy chain gene rearrangement, the genes encoding the Ig κ and/or λ light chain variable regions undergo similar gene rearrangements.

Each mature B cell ordinarily expresses only one Ig heavy chain and one Ig light chain allele (7). This phenomenon, called allelic exclusion, is thought to reflect the relative infrequency of productive Ig gene rearrangements and the fact that expression of a productive Ig heavy chain can suppress subsequent gene rearrangement on the allelic Ig heavy chain complex (8). Similarly, expression of intact Ig generally precludes subsequent Ig light chain gene rearrangement. Allelic exclusion prevents each B cell from expressing Ig with varied combinations of different heavy and light chains, each having potentially distinct binding specificities. This allows for selection of B cells that express Ig with desired binding properties, thus providing a means for generating high-affinity, antigen-specific antibody responses. A similar process governs rearrangement and expression of genes encoding the TCR for antigen (9).

However, several studies have provided evidence that allelic exclusion may not be absolute, at least for the TCR. Rearrangements of both TCR V<sub>α</sub> alleles have been detected in T cell clones (10, 11) and in TCR V<sub>α</sub> transgenic mice (12). Dual V<sub>α</sub> chains also have been detected on the surface of T cells of transgenic mice (13) and on normal human T cells (14). In addition, there also are exceptions to TCR V<sub>β</sub> allelic exclusion. Balomenos et al., for example, demonstrated that a small portion (1%) of thymocytes escape TCR V<sub>β</sub> allelic exclusion in both transgenic and normal mice (15). These dual V<sub>α</sub>-V<sub>β</sub>-expressing cells increase with age and can account for a sizable proportion of the T cells in the periphery. Dual TCR expression also has been observed for a subset (1%) of human α/β T cells (16), and γδ T cells (17).

Conceivably, a small proportion of B lymphocytes also may lack allelic exclusion in their expression of Ig genes. To evaluate this, we examined the fidelity of allelic exclusion...
in B cell chronic lymphocytic leukemia (CLL)\textsuperscript{1}, a monoclonal B cell malignancy. Because the blood lymphocytes of patients with this disease primarily are derived from the leukemic clone, we could screen for leukemia cell expression of more than one Ig V\textsubscript{H} gene subgroup using an anchored reverse transcriptase (R T)-PCR - ELISA assay (18). Our studies indicate that a small subset of patients have leukemia cells that express more than one Ig heavy chain allele, indicating that B cell allelic exclusion of Ig heavy chains is not absolute.

Materials and Methods

Patient Material. Blood samples were obtained from 108 patients from the University of California at San Diego Medical Center, the Veteran’s Administration Hospital at San Diego, or the Scripps Clinic (La Jolla, CA) who satisfied diagnostic criteria for B cell CLL (19). PBMC were prepared by Ficoll-Hyphaque density gradient centrifugation, washed twice, and analyzed directly or suspended in fetal calf serum containing 10% dimethylsulfoxide for frozen storage in liquid nitrogen.

DNA Isolation and Southern Blotting. Genomic DNA was isolated from PBMC as described (20). 10 μg of genomic DNA was digested with a fivefold excess of BamHI and HindIII, EcoRI and HindIII alone. Restriction enzyme-digested DNA were separated in an 0.8% agarose gel for analysis by the technique of Southern (21). N ylon membranes with transferred and denatured DNA were probed with a 32P-labeled J\textsubscript{H} probe, consisting of a 2.1-kb Sau3A fragment spanning the genomic DNA of J\textsubscript{H}2-J\textsubscript{H}6, as described (20). Stringent washing conditions consisted of two 20-min washes at 65°C in 0.1× SSC containing 0.1% N aDodSO\textsubscript{4}.

RNA Isolation and cDNA Synthesis. Total RNA was isolated from 5 × 10\textsuperscript{6} PBMC using R N aZol B (C inna/Biotex, Friendswood, T.X.). First strand cDNA was synthesized using 1–5 μg of total RNA, oligo-dT primer, and Super script RT (G IBCO B RL, Gaithersburg, M.D.). The remaining RNA was removed with R N aseH and the cDNA was purified using Q I Aquick purification columns (Q iagen, Chatsworth, C A).

Poly-dG-tailing of DNA and Anchored PCR. The purified cDNA was poly-dG-tailed using DGTP and terminal deoxynucleotransferase (Boehringer Mannheim, Indianapolis, I N.) and purified using Q I Aquick purification columns (Q iagen). One fourth of the sample was subjected to primary-anchored PCR amplification using an antisense oligonucleotide primer specific for the constant region of human IgM (C\textsubscript{\mu}) and a 9:1 mixture of two anchor sense-strand primers, as previously described (18). The PCR products were purified using the Q I Aquick purification columns and one third of this product was used as a template for a nested PCR. This second PCR reaction was the same as the primary anchored PCR except a 5′ biotinylated C\textsubscript{\mu}-antisense primer was used that was upstream of the initial C\textsubscript{\mu} primer.

RT-PCR-ELISA. The nested PCR product was purified using Q I Aquick purification columns and distributed onto ELISA wells that had been precoated with streptavidin (Sigma Chemical Co., St. Louis, M O), as described (18). Oligonucleotide corresponding to the Ig V\textsubscript{H} subgroup-specific sense-strand sequences of the leader region were labeled with digoxigenin and terminal deoxynucleotransferase (Boehringer Mannheim). The double-stranded PCR product was denatured with 0.1 N AOH and, after washing, it was incubated with each of the digoxigenin-labeled oligonucleotide probes for 20 min at 65°C, followed by 90 min at 42°C, as described (18). A peroxidase-conjugated antidigoxigenin antibody was used to detect the bound probe. The wells were subsequently washed and then incubated with tetramethylbenzidine and peroxidase (K Irkegaard & Perry Labs. Inc., Gaithersburg, M D). The reaction was stopped with 1 M 0-phosphoric acid (Fisher Scientific, Pittsburgh, PA) and the optical densities were measured at 450 nm using an ELISA microplate reader (M olecular Devices Corp., M enilo, P ark, C A).

Cloning and Nu d e c A od Sequence Analysis. The cDNA from each patient was amplified using primers specific for the sense strand of the leader sequence of the Ig V\textsubscript{H} gene of interest and an antisense J\textsubscript{\mu} consensus sequence containing flanking restriction sites for EcoRI or HindIII, as described (22). The fragments were digested with EcoRI and HindIII, purified, ligated into pUC19 vector (Bluescribe\textsuperscript{TM}; Stratagene Corp., La Jolla, C A) that had been similarly digested. The ligated plasmid was used to transform competent E. coli (Stratagene Corp.). The plasmids containing the insert were isolated for double-stranded DNA sequence analysis (Pharmacia Biotech, Piscataway, N J) on an automated nucleic acid sequence analyzer (Applied Biosystems, Foster City, C A). Sequences were analyzed using the EM BL/GenBank/DDB database.

Flow Cytometric Analysis. Immunofluorescence analyses of PBMC were performed on a FACS\textsuperscript{4}-Calibur flow cytometer (B ecton Dickinson, San Jose, C A), as described (23). In addition to labeling cells with fluorochrome-conjugated mAbs, the leukemia cells also were stained with propidium iodide to label dead cells red when excited by the argon laser. This allowed for electronic gating on live cells during the analysis. Overall, cell viability generally exceeded 95%. B6, a murine IgG\textsubscript{1} mAb that reacts with a major cross-reactive idiotype (C R I) present on a subset of Ig encoded Ig V\textsubscript{H}3 genes (24), originally was provided by R. M aged and R. J effers (University of Birmingham, Birmingham, U.K.). Lc1, a murine IgG\textsubscript{1} mAb that reacts with a supratypic cross-reactive idiotype present on a large subset of Ig encoded by Ig V\textsubscript{H}4 genes (25), was originally obtained from I.G. Sions (Cambridge University, Cambridge, U.K.). 26. The nonspecific mouse IgG\textsubscript{1} (M O P C 21) was purchased from C A LT AG (South San Francisco, C A). H B-57 (D A 4-4), a murine IgG\textsubscript{1} anti-human (anti-hu) heavy chain mAb producing hybridoma (27), was obtained from the American Type Culture Collection (R ockville, M D). The H B-57 (anti-hu IgM) mAb was produced and purified from ascites, as described (23). M odified staphylococcal protein A (S pA), a 45-kD bacterial “superantigen” that reacts with most Ig of the V\textsubscript{H}3 subgroup (28), was provided by G. Silverman (University of California at San Diego, La Jolla, C A). B6 and S pA were conjugated to biotin, allowing for detection of cell-bound reagent with avidin-biotinylated horseradish peroxidase. Lc1, H B-57, and M O P C 21 were conjugated to FIT C, as described (23). To compare the fluorescence-staining intensities of leukemic cells from different patients, we computed the mean fluorescence intensity ratio (M F I R). The M F I R for a given antigen was defined as the mean fluorescence intensity of gated B cells stained with an antigen-specific fluorochrome-conjugated mAb divided by the mean fluorescence intensity of such cells stained with a fluorochrome-conjugated isotype control mAb or reagent of irrelevant specificity.

\textsuperscript{1}Abbreviations used in this paper: anti-hu, anti-human; C D R, complementarity determining region; C L L, chronic lymphocytic leukemia; C R I, cross-reactive idiotype; M F I R, mean fluorescence intensity ratio; R, replacement mutation; R T, reverse transcriptase; S, silent mutation; S pA, staphylococcal protein A.
Table 1. IgVH Subgroup OD (± SD)

| Group | VH1  | VH2  | VH3  | VH4  | VH5  | VH6  |
|-------|------|------|------|------|------|------|
| I     | 0.470 (+0.238) | 0.002 (+0.004) | 0.000 (+0.005) | 0.002 (+0.002) | 0.001 (+0.003) | 0.001 (+0.001) |
| II    | 0.002 (+0.001) | 0.282 (+0.069) | 0.001 (+0.002) | 0.000 (+0.000) | 0.001 (+0.000) | 0.001 (+0.002) |
| III   | 0.001 (+0.003) | 0.002 (+0.004) | 0.475 (+0.239) | 0.002 (+0.003) | 0.001 (+0.002) | 0.001 (+0.001) |
| IV    | 0.002 (+0.002) | 0.001 (+0.002) | 0.001 (+0.001) | 0.553 (+0.268) | 0.001 (+0.002) | 0.001 (+0.001) |
| V     | 0.001 (+0.001) | 0.001 (+0.002) | 0.001 (+0.002) | 0.002 (+0.002) | 0.563 (+0.230) | 0.002 (+0.002) |

The far left column provides the roman numeral designator for each group. The columns to the right are labeled with the Ig VH subgroup oligonucleotide used to probe the tethered Ig cDNA. (Because the Ig VH1 and VH7 subgroup share high homology in their respective leader sequences, the probe for Ig VH1 gene also reacts to the leader sequences of Ig VH7 genes.) The numbers in each column provide the mean OD 450 for all the samples within the group for the indicated oligonucleotide probe. The numbers in parentheses provide the standard deviation about the mean.

Results

Identification of Ig VH Subgroups by Anchored RT-PCR - ELISA. Total RNA was isolated from the blood lymphocytes of 108 patients that satisfied diagnostic and clinical criteria for B cell CLL. More than 90% of the lymphocytes of each sample expressed surface IgM and/or IgM/IgD, and CD5. 61 of these leukemia cell samples expressed κ light chains (56%), whereas the remaining 47 samples expressed λ light chains (44%).

We determined the Ig heavy chain subgroup expressed by these cells using an anchored RT-PCR - ELISA (18). This technique generates Ig cDNA from each sample with biotin attached to the 5’ end of the antisense strand, allowing this strand to become tethered to a plastic plate coated with streptavidin. Equal amounts of the Ig cDNA from each sample were dispensed into separate wells of a streptavidin-coated ELISA microtiter plate. After washing away the denatured and unbound sense strand, each well received a digoxigenin-labeled oligonucleotide probe corresponding to one of the major Ig VH gene subgroups. The bound oligonucleotide probes were detected using an alkaline-phosphatase-conjugated antidigoxigenin antibody that was developed with a chromogenic substrate. After a 15-min incubation at room temperature, the optical densities at 450 nm (OD450) of each well were recorded using an ELISA plate reader. Through this method, we simultaneously could evaluate the relative contributions of each Ig VH gene subgroup to the total Ig cDNA.

Through these analyses we delineated five major subgroups. 40 (37%) of the 108 samples had Ig cDNA that only hybridized with oligonucleotides specific for the VH1 and VH7 subgroups (Table 1, group I, boxed value), these samples having a mean OD450 of 0.470 (+ SD = 0.238). The mean OD450 of wells with oligonucleotide probes for each of the other subgroups were significantly lower (P < 0.001, Bonferroni t test), each value being <0.002 (Table 1). A recent study of the nucleotide sequences of the Ig cDNA expressed by these samples confirmed that each expressed Ig VH1 (39 samples) or VH7 genes (1 sample) (29). 2 (2%) of the 108 samples had cDNA that reacted only with the oligonucleotide probe specific for Ig VH2 genes (Table 1, group II, boxed value). Again, the mean OD450 of 0.282 (+ SD = 0.069) for wells with the Ig VH2 probe was significantly higher than that of any of the other wells with other Ig VH subgroup probes (P < 0.001, Bonferroni t test). A similar result was obtained. 41 (38%) had cDNA that reacted only with probes for the VH3 subgroup (Table 1, group III, boxed value), 13 (12%) generated cDNA that reacted only with probes for the VH4 subgroup (Table 1, group IV), 6 (6%) generated cDNA that reacted only with probes for the VH5 subgroup (Table 1, group V), and none generated cDNA that reacted with the VH6 subgroup probe. Again, for each group, the mean OD450 for wells with probes for one Ig VH subgroup probe (boxed values) was significantly higher than that of wells with any of the other Ig VH subgroup probes (P < 0.001, Bonferroni t test).

6 of the 108 samples (5%), however, had cDNA that reacted with probes for more than one Ig VH gene subgroup in each of three separate experiments (Table 2). 4 had Ig cDNA that reacted equally well with probes for the Ig VH3 and Ig VH4 subgroups (P1, P2, P4, and P5), but not with probes specific for any of the other Ig VH subgroups (Table 2). One sample (P4) had Ig cDNA that reacted equally well with probes specific for the Ig VH2 and Ig VH3 subgroups, but not probes for any of the other Ig VH gene subgroups (Table 2). Finally, one sample (P3) had Ig cDNA that reacted equally well with probes specific for the Ig VH2 and VH4 subgroups, but reacted twice as well with probes specific for the Ig VH3 subgroup (Table 2). The Ig cDNA for this sample, however, did not react with oligonucleotide probes specific for any of the other Ig VH gene subgroups.

Southern Blot Analysis. To evaluate the clonality of these leukemia populations, genomic DNA was digested with BamHI and HindIII restriction enzymes and analyzed by Southern blotting using a radiolabeled JH-specific probe. Five of the six samples had Ig gene rearrangements involving both alleles (Fig. 1, lanes 1, 2, and 4–6) and one sample, P3, had evidence for three Ig gene rearrangements (Fig. 1, lane 3). The genomic DNA of each leukemic cell sample lacked a strong band corresponding to the germline nonrearranged DNA present in human placenta DNA (Fig. 1, P).
Table 2. Ig V<sub>H</sub> Subgroup OD (± SE)

| Sample | V<sub>H</sub>1 | V<sub>H</sub>2 | V<sub>H</sub>3 | V<sub>H</sub>4 | V<sub>H</sub>5 | V<sub>H</sub>6 |
|--------|--------------|--------------|--------------|--------------|--------------|--------------|
| P 1    | 0.002 (±0.001) | 0.001 (±0.000) | 0.633 (±0.287) | 0.733 (±0.287) | 0.001 (±0.001) | 0.001 (±0.001) |
| P 2    | 0.001 (±0.000) | 0.002 (±0.001) | 0.600 (±0.082) | 0.600 (±0.141) | 0.001 (±0.002) | 0.001 (±0.001) |
| P 3    | 0.001 (±0.001) | 0.361 (±0.060) | 0.667 (±0.067) | 0.315 (±0.015) | 0.001 (±0.001) | 0.001 (±0.001) |
| P 4    | 0.002 (±0.001) | 0.000 (±0.001) | 0.840 (±0.118) | 0.859 (±0.181) | 0.001 (±0.001) | 0.001 (±0.000) |
| P 5    | 0.001 (±0.002) | 0.001 (±0.000) | 0.610 (±0.271) | 0.663 (±0.187) | 0.001 (±0.001) | 0.001 (±0.000) |
| P 6    | 0.001 (±0.000) | 0.847 (±0.152) | 0.943 (±0.222) | 0.001 (±0.001) | 0.001 (±0.001) | 0.001 (±0.001) |

The far left column (Sample) provides the designator for each patient sample. The columns to the right are labeled as in Table 1. The numbers in each column provide the mean OD<sub>450</sub> for the sample in each of three separate experiments for the indicated oligonucleotide probe. The numbers in parentheses provide the standard error about the mean.

Nucleic Acid Sequence Analyses. Nucleic acid sequence analyses of Ig cDNA demonstrated that each sample had two distinct and productively rearranged Ig V<sub>H</sub> genes that corresponded to the subgroups detected in the anchored RT-PCR-ELISA (Table 1). Each of the isolated V<sub>H</sub> genes had productive gene rearrangements without introduced frameshift or nonsense mutations. Comparison of the nucleic acid sequence of the cloned Ig V<sub>H</sub> genes to known germline genes revealed that sample P4 expressed Ig V<sub>H</sub> with highest homology to V<sub>H</sub>3-30 (99% homology) and V<sub>H</sub>4-6b (90% homology), sample P6 expressed V<sub>H</sub> genes that were most homologous to V<sub>H</sub>2-70 (90% homology) and V<sub>H</sub>3-30.3 (88% homology), sample P1 used V<sub>H</sub> genes with highest homology to V<sub>H</sub>3-33 (99% homology) and V<sub>H</sub>4-4-39 (98% homology), sample P2 had V<sub>H</sub> with highest homology to V<sub>H</sub>3-7 (97% homology) and V<sub>H</sub>4-4-39 (99% homology), and sample P5 expressed Ig V<sub>H</sub> genes with 98% homology to V<sub>H</sub>3-2 and 96% homology to V<sub>H</sub>4-61. From sample P3 we isolated three V<sub>H</sub> genes that had highest homology to V<sub>H</sub>2-70, V<sub>H</sub>3-7, and V<sub>H</sub>4-4-59 (88, 100, and 99% homology, respectively; Fig. 2). We did not detect any intraclonal diversity when we sequenced two separate clones from each RT-PCR reaction.

Evaluation of the sequence data allowed us to deduce the probable D and J<sub>H</sub> segments used to generate the sequence encoding the third complementarity determining region (CDR 3) of each Ig gene. We deduced that the D<sub>H</sub>-containing irregular spacer signal 5 (DIR 5) gene segment was probably used to generate the sequence encoding the CDR3 of the two V<sub>H</sub>3-encoded genes of samples P1 and P2 (Fig. 2) or the V<sub>H</sub>2-70-encoded genes of P6 (Fig. 2). The DXP4 gene segment was probably used to generate the CDR3-encoding sequence detected in the rearrangements involving two of the six patient samples (Fig. 2, P3 and P2). In addition, the DN1 gene segment was rearranged to each of the V<sub>H</sub>4 genes expressed by three patient samples (Fig. 2, P3, P1, and P5). We deduced that either J<sub>H</sub>4B or J<sub>H</sub>3B were used in each of the rearranged Ig genes except for the rearranged V<sub>H</sub>3 genes of samples P1 or P2 (Fig. 2). The latter two apparently used the J<sub>H</sub>6B minigene segment (Fig. 2).

Despite expressing two, or in one case three, Ig heavy chains, each sample was found to express only one functional Ig light chain gene. To determine the rearranged Ig light chain gene of each sample, we performed RT-PCR and sequence analysis directly on the poly-dG-tailed cDNA template isolated from each light chain-expressing patient sample using an anchor sense-strand primer and an antisense-strand primer specific for the constant region of the Ig k light chain gene. The cells from patients P1 or P5 expressed Ig light chain genes belonging to the V<sub>k</sub>1 family (96% homology to DPK5 and 92% to DPK7, respectively) rearranged to the J<sub>k</sub>5 and J<sub>k</sub>2 minigenes, respectively. Samples P3 or P2 had B cells that rearranged Ig light chain genes belonging to the V<sub>k</sub>2 subgroup (94% homology to DPK18 and 86% homology to DPK16, respectively), and to J<sub>k</sub>2 and J<sub>k</sub>1, respectively. The B cells of patient P4 expressed rearranged Ig light chain genes belonging to the V<sub>k</sub>4 subgroup (95% sequence homology to DPK24). This V<sub>k</sub> gene rearranged to J<sub>k</sub>2 minigene (Fig. 3).
Flow Cytometric Analyses. From the primary sequence data, we deduced that the Ig expressed by some of these CLL samples should react with Lc1, a mAb reactive with a CRI present on Ig encoded by a subgroup of Ig VH4 genes such as VH4-39, VH4-59, VH4-61 (25, 30), and B6, an mAb anti-CRI present on Ig encoded by certain Ig VH3 genes, namely VH3-23, VH3-30, or VH3-30 (24), and/or modified SpA, a 45-kD bacterial superantigen that reacts with the variable region of most Ig heavy chains belonging to the VH3 subgroup (28).

The Ig molecules encoded by the VH genes of patients P4, P3, P6, P1, P2, and P5 were expected to react with SpA and/or B6, whereas the Ig encoded by the VH4-39, VH4-59, VH4-39, or VH4-61 genes expressed by the B cells of patients P4, P3, P1, P2, and P5 were expected to react with Lc1 (Table 3). Flow cytometric analyses revealed that the leukemia cells from these samples reacted specifically with Lc1 and SpA and/or B6, but not with control mAbs of irrelevant specificity, as predicted from the sequence analysis (Fig. 4, P3, P5, P4, and P2, and Table 3).

The MFI values of these samples were compared with those of control leukemia cell populations that expressed Ig VH genes belonging to only one subgroup. Analyses of four leukemia cell samples that expressed only Ig VH3 genes revealed these samples to have a mean MFI with SpA of 3.9 (± SD = 1.8), and a mean MFI with Lc1 of 1.1 (± SD = 0.2). For example, the leukemic B cells from patient P8 expressed only Ig VH3 genes of the VH3 subgroup and with SpA had a MFI of 5.2 (Fig. 4, row A, far left). In contrast, this sample did not react with B6 or Lc1 (Fig. 4, rows B and C, far left), having MFI values with each of these reagents of 0.9 or 1.0, respectively. Similarly, analyses of four different leukemia cell samples that expressed only Ig VH4 genes, encoding Lc1-reactive Ig, revealed these samples to have a mean MFI with SpA of 1.1 (± SD = 0.1), a mean MFI with B6 of 1.1 (± SD = 0.2), and a mean MFI with Lc1 of 5.0 (± SD = 2.2). For example, the B cells from sample P9 stained only with Lc1 (Fig. 4, row C, far right), having a MFI for Lc1 of 5.0, but MFI of 1.1 or 1.0 with SpA or B6, respectively (Fig. 4, rows A and B, far right). In contrast, samples P2, P3, P4, or P5 had MFI values >1 after staining with either SpA (Fig. 4, row A), B6 (Fig. 4, row B) or Lc1 (Fig. 4, row C) (Table 3). Moreover, the mean MFI with SpA for these samples (2.3 ± 0.6, SD) is significantly higher than that with SpA for the four leukemia samples tested that expressed only Ig VH4 genes (P <0.01, Student’s t test). Also, the mean MFI with Lc1 for P2, P3, P4, and P5 (6.5 ± 0.4, SD) is significantly higher than that with Lc1 for the four leukemia samples tested that expressed only Ig VH3 genes (P <0.05, Student’s t test). However, the mean MFI observed with the anti-hu IgM mAb with the leukemia cell samples that exclusively expressed Ig VH4 or Ig VH3 (namely, 7.0 ± 0.2 (SD) or 8.7 ± 1.3 (SD), respectively) were not significantly different from that observed with anti-IgM for these variant leukemia cell samples (6.5 ± 3.2 (SD), P >0.05, Bonferroni t test).

In each case, the relative staining intensity observed with the anti-hu IgM mAb correlated with that observed with the subgroup reagents found to react with the leukemia cells (Fig. 4, row D, and Table 3). Moreover, the low staining intensities observed with any of the anti-Ig reagents reflects the low level expression of surface Ig that is a noted characteristic for B cell CLL (19).

D i s c u s s i o n

We examined the Ig VH genes expressed by the leukemia cells of 108 patients by an anchored RT-PCR–ELISA technique. In most cases, we detected expression of only one Ig VH subgroup, consistent with previous findings that each leukemia cell population uses only one Ig heavy chain.

However, in six samples (5%) we detected Ig VH gene transcripts of more than one Ig VH subgroup. Conceivably, this may have been secondary to an artifact of the anchored RT-PCR–ELISA technique. Although we used probes specific for each of the major Ig VH gene subgroups, these exceptional leukemia cell samples may have had unusual VH gene sequences that hybridized with probes for more than one Ig VH gene subgroup. Alternatively, our technique may have detected expression of VH genes from bystander B cells that were not related to the leukemia B cell clone. Finally, each of these leukemic populations itself may have been heterogeneous.

### Table 3. CLL Samples That Lack Ig Heavy Chain Allelic Exclusion

| Sample V H gene | Percent homology | Anticipated phenotype | SpA | B6 | Lc1 | μ |
|----------------|------------------|-----------------------|-----|----|-----|---|
| P1 VH3-33      | 99               | SA +                  | N.T.|     |     |   |
| VH4-39         | 98               | Lc1 +                 | N.T.|     |     |   |
| P2 VH3-7       | 97               | SA+/B6+               | 2.4 | 1.4|     |   |
| VH4-39         | 99               | Lc1 +                 | 10  | 9.5|     |   |
| P3 VH2-70      | 88               | −                     |     |    |     |   |
| VH3-7          | 100              | SA+/B6+               | 1.9 | 1.4|     |   |
| VH4-59         | 99               | Lc1 +                 | 1.6 | 1.9|     |   |
| P4 VH3-30      | 99               | SA+/B6+               | 3   | 1.8|     |   |
| VH4-b          | 90               | Lc1 +                 | 9.4 | 7.2|     |   |
| P5 VH3-23      | 98               | SA+/B6+               | 1.8 | 2.1|     |   |
| VH4-61         | 96               | Lc1 +                 | 4.9 | 7.4|     |   |
| P6 VH2-70      | 90               | −                     |     |    |     |   |
| VH3-30.3       | 88               | SA+/B6+               | N.T.|     |     |   |

The far left column (Sample) provides the designator for each sample (P1-P6). The second column (VH gene) lists the germline Ig VH genes that have the highest homology with the Ig VH genes isolated from each sample. The percent homology column lists the percent sequence homology between the isolated VH gene and the deduced germline VH gene having the highest base sequence homology. The Anticipated Phenotype column provides the expected reactivity of the surface Ig with the anti-CRI or anti-VH subgroup reagents. The columns labeled SpA, B6, Lc1, or μ provide the observed MFI of the leukemia cell population stained with each of these reagents relative to that of the cells stained with an isotype control mAb. N.T., sample was not tested.
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Figure 2. Nucleic acid sequence data of the rearranged Ig V Heavy (VH) genes isolated from the CLL samples lacking Ig heavy chain allelic exclusion. The deduced amino acid sequence using the 20-letter code is provided at the top of each sequence, along with descriptors marking the deduced CDR1 and CDR2 for each isolated VH gene. Each sequence is compared to germline VH gene, D segment, or JH segment having the highest nucleic acid sequence homology, indicated on the far left-hand side of the figure. (Dot, base identity). These sequence data are available from EMBL/GenBank/DDBJ under the following accession numbers: P1, U96277 and U96278; P2, U96279 and U96280; P3, U96281, U96282, and U96283; P4, U96284 and U96285; P5, U96286 and U96287; P6, U96288 and U96289.
However, we found that none of these explanations could describe our findings. First, although the RT-PCR-ELISA revealed the Ig cDNA from these samples reacted with oligonucleotide probes for two or, in one case, three Ig VH gene subgroups, the Ig cDNA of each sample did not hybridize with all probes for each of the Ig VH gene subgroups, including those specific for major Ig V\text{H} gene subgroups, such as Ig V\text{H}1 (Table 2). These hybridization patterns are very distinct from those observed with Ig cDNA generated from heterogeneous populations of normal B cells (18).

Second, Southern blot analyses of the Ig heavy chain genes rearranged in each sample revealed each to have two or, in one case (P3), three Ig rearrangements (Fig. 1). Furthermore, the absence of a strong germline band in the genomic DNA of these blood mononuclear cell samples argued that each was comprised mostly of leukemic B cells.

Figure 3. Nucleic acid sequence data of the rearranged light chain genes isolated from the CLL samples lacking Ig heavy chain allelic exclusion. The deduced amino acid sequence using the 20-letter code is provided at the top of each sequence, along with descriptors marking the deduced CDR1 and CDR2 for each isolated V\text{k} gene. Each sequence is compared to germline V\text{k} gene or J\text{k} segment having the highest nucleic acid sequence homology, indicated on the far left-hand side or top of the sequence, respectively. (Dot, base identity). These sequence data are available from EMBL/GenBank/DDBJ under the following accession numbers: P1, U96290; P2, U96291; P3, U96292; P4, U96293; P5, U96294.
Third, nucleic acid sequence analyses of the Ig cDNA revealed that each of these samples had disparate functional V\(\text{H}\) gene transcripts that corresponded to the V\(\text{H}\) gene subgroups detected in the RT-PCR-ELISA. Finally, we found that each sample expressed only one functional light chain gene. Collectively, these findings indicate that the two or, in one case, three different Ig V\(\text{H}\) gene cDNA isolated from each of these samples were derived from a single clone of leukemic B cells.

Flow cytometric analyses provided evidence that each of these leukemic B cell populations expressed more than one Ig heavy chain protein. We noted that several samples expressed Ig V\(\text{H}4\) or V\(\text{H}3\) genes that were highly homologous to germline genes known to encode Ig molecules that could react with either Lc1, B6, and/or SpA, respectively. Lc1 is a mAb that reacts with a CRI present on a subset of V\(\text{H}4\) genes (25, 30), whereas modified SpA is a 45-kD bacterial superantigen that reacts with Ig encoded by most V\(\text{H}3\) genes (28). B6 is another mAb anti-CRI that reacts with Ig encoded by only a subset of V\(\text{H}3\) genes (24). Flow cytometry analysis revealed that the leukemia cell populations indeed reacted with these reagents, as predicted from the primary sequences of the isolated Ig heavy chain genes (Fig. 4, rows A–C, columns 2–5, and Table 3). In contrast, such double staining was not observed for other leukemic cells samples that expressed Ig V\(\text{H}\) genes of only one subgroup (Fig. 4, rows A–C, columns 1 and 6, and data not shown). Collectively, these data reveal that a subset of patients with CLL have leukemic B cells that express more than one Ig heavy chain.

In one sample, P4, the shape of the fluorescence histogram of cells stained with Lc1 revealed a shoulder of dull-staining cells (Fig. 4, row C, column 4) that was not observed in the fluorescence histograms of these same cells stained with SpA (Fig. 4, row A, column 4) or B6 (Fig. 4, row B, column 4). This discrepancy makes it unlikely that the noted staining with SpA or B6 is due to cross-reactivity of these reagents for the Lc1-positive surface Ig expressed by these cells. The fluorescence histogram of these same cells stained with the anti-hu IgM mAb also failed to demonstrate this shoulder (Fig. 4, row D, column 4), possibly reflecting an averaging of surface densities of both types of surface Ig on this leukemia cell population. Nevertheless, for the other variant leukemia cell samples, the noted shift in the overall fluorescence histograms of the positively stained cells also correlated with that noted for cells stained with anti-IgM mAb, making it unlikely that only a subset of the cells in each sample expressed only Ig of one or the other Ig V\(\text{H}\) subgroup.

These findings indicate that Ig heavy chain allelic exclusion is not absolute and suggest that a subset of normal human B cells also may express dual Ig receptors. Brezinschek et al. noted in a recent study of the Ig heavy chains expressed by isolated B cells using single-cell Ig PCR that 1 of the 74 single-cell samples had two productively rearranged PCR products (31). Although this was explained as being secondary to possible sample-well contamination, it is conceivable that this well instead contained a single B cell that expressed two Ig heavy chains. Furthermore, in a recent study using sensitive multiparameter flow cytometric analyses, Giachino et al. estimated that 0.2–0.5% of human blood B cells from healthy adults may express both k and \(\lambda\) light chains (32), suggesting that light chain allelic exclusion also may not be absolute. Although we did not detect any k and \(\lambda\) light chains expressing leukemia cell samples in our study, it is possible that a critical analysis of the light chain V\(\text{H}\) gene subgroups in all our samples also may have detected a few cases that expressed more than one type of k or \(\lambda\) light chain. This was not found to be the case, however, for those samples found to express more than one Ig heavy chain, arguing against the possibility that these samples lacked the normal regulatory mechanisms governing both light and heavy chain allelic exclusion.

Rather, the structural analyses of the Ig V\(\text{H}\) genes expressed by the CLL samples lacking allelic exclusion suggest that there may be selection for expression of two antibodies with different binding specificities. We deduced that one of the expressed Ig V\(\text{H}\) genes in several cases (P1, P3, P4, P5, or P6) had incurred somatic mutations resulting in a relatively high ratio of deduced replacement mutations (R) to
silent mutations (S) in the CDRs. For example, an R/S ratio of six or seven for the deduced mutations in the CDR1 and CDR2 of the V\(_{\mu}\)2 genes, respectively expressed by P3 or P6, are higher than the innate R/S ratio of 3.8 predicted for random base substitutions in the CDR of these V\(_{\mu}\) genes (33). Mutations resulting in higher R/S ratios in the CDR than expected by chance commonly are noted in the Ig V genes expressed by B cells selected in a secondary antigen-driven immune response (34–37). Conceivably, the allele with the deduced somatic mutations (the V\(_{\mu}\)2 gene of P3 or P6, the V\(_{\mu}\)3 gene of P5, or the V\(_{\mu}\)4 gene of P1) had undergone rearrangement first and was expressed by the B cell clone during an antigen-driven immune response. Should mutations within the expressed Ig V\(_{\mu}\) gene result in an Ig that reduced the fitness of the B cell clone, then there may have been selective advantage for cells that could rearrange and express the other nonmutated Ig heavy chain allele (e.g., the V\(_{\mu}\)3 genes of P1, P3, and P5, or the V\(_{\mu}\)4 genes of P2 and P3).

A similar model has been used to explain the findings of studies on mice engineered to express transgenic Ig reactive with self antigens (38–40). These mice may generate transgene-expressing B cells that also rearrange and express their endogenous Ig genes, thereby presumably allowing these cells to escape negative selection. This mechanism also has been used to explain the observed examples of Ig-receptor editing (41, 42), in which autoantibody-expressing B cells undergo secondary Ig heavy chain rearrangements that allow them to express new Ig molecules with altered antigen-binding specificities. In this regard, it is noteworthy that the CLL B cells are frequently found to make IgM autoantibodies (reviewed in reference 43). Conceivably, expression of such autoantibodies also may favor selection of cells that undergo such reiterative Ig gene rearrangements, particularly if their original Ig receptors develop enhanced binding activity for self antigen through somatic mutation. On the other hand, there may be selection to maintain these B cells that manifest multiple binding specificities. In either case, B cells lacking allelic exclusion may have a selective advantage.

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