Lack of Allelic Exclusion in B Cell Chronic Lymphocytic Leukemia

By Laura Z. Rassenti and Thomas J. Kipps

From the Division of Hematology/Oncology, Department of Medicine, University of California at San Diego, La Jolla, California 92093-0663

Summary

We determined the immunoglobulin (Ig) V_H 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_H 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_H genes: V_H3-33 and V_H4-39; V_H3-7 and V_H4-39; V_H3-23 and V_H4-61; V_H2-70 and V_H3-30.3; or V_H3-30 and V_H4-b (DP67). One sample expressed three Ig V_H genes: V_H2-70, V_H3-7, and V_H4-59. 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 idiotype present on Ig encoded by a subgroup of Ig V_H4 genes (namely, V_H4-39, V_H4-b [DP-67], V_H4-59, or V_H4-61), and B6, an mAb that reacts with Ig encoded by certain Ig V_H3 genes (namely, V_H3-23, V_H3-30, or V_H3-30.3), and/or modified staphylococcal protein A (SpA), a 45-kilodalton bacterial "superantigen" that reacts with most Ig of the V_H3 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.

Ig 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_H genes, 30 diversity segments, and 6 J_H minigenes (1–3). The Ig V_H genes are classified into seven subgroups (V_H1–V_H7) based on their relative nucleotide sequence homology (4, 5). During B cell development, one or more diversity segments can juxtapose with a J_H gene segment, forming a DJ_H complex that then rearranges with an Ig V_H gene to form a $V_H DJ_H$ 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_α alleles have been detected in T cell clones (10, 11) and in TCR V_α transgenic mice (12). Dual V_α 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_β allelic exclusion. Balomenos et al., for example, demonstrated that a small portion (1%) of thymocytes escape TCR V_β allelic exclusion in both transgenic and normal mice (15). These dual V_β-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 γ/δ 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)[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_H gene subgroup using an anchored reverse transcriptase (RT)-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-Hypaque 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, or BamHI, EcoRI, or HindIII alone. Restriction enzyme–digested DNA were separated in an 0.8% agarose gel for analysis by the technique of Southern (21). Nylon membranes with transferred and denatured DNA were probed with a ^{32}P-labeled J_H probe, consisting of a 2.1-kb Sau3A fragment spanning the genomic DNA of J_H2–J_H6, as described (20). Stringent washing conditions consisted of two 20-min washes at 65°C in 0.1× SSC containing 0.1% NaDodSO4.

RNA Isolation and cDNA Synthesis. Total RNA was isolated from 5×10^6 PBMC using RNAzol B (Cinna/Biotex, Friendswood, TX). First strand cDNA was synthesized using 1–5 μg of total RNA, oligo-dT primer, and Superscript RT (GIBCO BRL, Gaithersburg, MD). The remaining RNA was removed with RNaseH and the cDNA was purified using QIAquick purification columns (Qiagen, Chatsworth, CA).

Poly-dG–tailing of cDNA and Anchored PCR. The purified cDNA was poly-dG–tailed using dGTP and terminal deoxytransferase (Boehringer Mannheim, Indianapolis, IN) and purified using QIAquik purification columns (Qiagen). 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_μ) and a 9:1 mixture of two anchor sense-strand primers, as previously described (18). The PCR products were purified using the QIAquik 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_μ-antisense primer was used that was upstream of the initial C_μ primer.

RT-PCR–ELISA. The nested PCR product was purified using QIAquik purification columns and distributed onto ELISA wells that had been precoated with streptavidin (Sigma Chemical Co., St. Louis, MO), as described (18). Oligonucleotides corresponding to the Ig V_H subgroup-specific sense-strand sequences of the leader region were labeled with digoxigenin and terminal deoxytransferase (Boehringer Mannheim). The double-stranded PCR product was denatured with 0.1 N NaOH 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 (Kirkegaard & Perry Labs. Inc., Gaithersburg, MD). The reaction was stopped with 1 M *O*-phosphoric acid (Fisher Scientific, Pittsburgh, PA) and the optical densities were measured at 450 nm using an ELISA microplate reader (Molecular Devices Corp., Menlo Park, CA).

Cloning and Nucleic Acid Sequence Analysis. The cDNA from each patient was amplified using primers specific for the sense strand of the leader sequence of the Ig V_H gene of interest and an antisense J_H 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™; Stratagene Corp., La Jolla, CA) that had been similarly digested. The ligated plasmid was used to transform competent *Escherichia coli* (Stratagene Corp.). The plasmids containing the insert were isolated for double-stranded DNA sequence analysis (Pharmacia Biotech, Piscataway, NJ) on an automated nucleic acid sequence analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed using the EMBL/GenBank/DDBJ database.

Flow Cytometric Analysis. Immunofluorescence analyses of PBMC were performed on a FACS®-Calibur flow cytometer (Becton Dickinson, San Jose, CA), 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_1 mAb that reacts with a major cross-reactive idiotype (CRI) present on a subset of Ig encoded Ig V_H3 genes (24), originally was provided by R. Mageed and R. Jefferis (University of Birmingham, Birmingham, U.K.). Lc1, a murine IgG_1 mAb that reacts with a supratypic cross-reactive idiotype present on a large subset of Ig encoded by Ig V_H4 genes (25), was originally obtained from J.G. Sissons (Cambridge University, Cambridge, U.K.; 26). The nonspecific mouse IgG_1 (MOPC21) was purchased from CALTAG (South San Francisco, CA). HB-57 (DA4-4), a murine IgG_1 antihuman (anti-hu) μ heavy chain mAb producing hybridoma (27), was obtained from the American Type Culture Collection (Rockville, MD). The HB-57 (anti-hu IgM) mAb was produced and purified from ascites, as described (23). Modified staphylococcal protein A (SpA), a 45-kD bacterial "superantigen" that reacts with most Ig of the V_H3 subgroup (28), was provided by G. Silverman (University of California at San Diego, La Jolla, CA). B6 and SpA were conjugated to biotin, allowing for detection of cell-bound reagent with avidin-phycoerythrin. Lc1, HB-57, and MOPC21 were conjugated to FITC, as described (23). To compare the fluorescence-staining intensities of leukemic cells from different patients, we computed the mean fluorescence intensity ratio (MFIR). The MFIR for a given antigen is 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.

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[1] *Abbreviations used in this paper:* anti-hu, anti-human; CDR, complementarity determining region; CLL, chronic lymphocytic leukemia; CRI, cross-reactive idiotype; MFIR, mean fluorescence intensity ratio; R, replacement mutation; RT, reverse transcriptase; S, silent mutation; SpA, staphylococcal protein A.

Table 1. *IgV$_H$ Subgroup OD ($\pm$ SD)*

Group	V$_H$1	V$_H$2	V$_H$3	V$_H$4	V$_H$5	V$_H$6
I	0.470 ($\pm$0.238)	0.002 ($\pm$0.004)	0.000 ($\pm$0.005)	0.002 ($\pm$0.002)	0.001 ($\pm$0.003)	0.001 ($\pm$0.001)
II	0.002 ($\pm$0.001)	0.282 ($\pm$0.069)	0.001 ($\pm$0.002)	0.000 ($\pm$0.000)	0.001 ($\pm$0.000)	0.001 ($\pm$0.002)
III	0.001 ($\pm$0.003)	0.002 ($\pm$0.004)	0.475 ($\pm$0.239)	0.002 ($\pm$0.003)	0.001 ($\pm$0.002)	0.001 ($\pm$0.001)
IV	0.002 ($\pm$0.002)	0.001 ($\pm$0.002)	0.001 ($\pm$0.001)	0.553 ($\pm$0.268)	0.001 ($\pm$0.002)	0.001 ($\pm$0.001)
V	0.001 ($\pm$0.001)	0.001 ($\pm$0.002)	0.001 ($\pm$0.002)	0.002 ($\pm$0.002)	0.563 ($\pm$0.230)	0.002 ($\pm$0.002)

The far left column provides the roman numeral designator for each group. The columns to the right are labeled at the top with the Ig V$_H$ subgroup oligonucleotide used to probe the tethered Ig cDNA. (Because the Ig V$_H$1 and V$_H$7 subgroup share high homology in their respective leader sequences, the probe for Ig V$_H$1 gene also reacts to the leader sequences of Ig V$_H$7 genes.) The numbers in each column provide the mean OD$_{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 V$_H$ 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 V$_H$ gene subgroups. The bound oligonucleotide probes were detected using an alkaline-phosphatase–conjugated antidigoxigenin antibody that subsequently was developed with a chromogenic substrate. After a 15-min incubation at room temperature, the optical densities at 450 nm (OD$_{450}$) of each well were recorded using an ELISA plate reader. Through this method, we simultaneously could evaluate the relative contributions of each Ig V$_H$ 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 V$_H$1 and V$_H$7 subgroups (Table 1, group *I*, *boxed value*), these samples having a mean OD$_{450}$ of 0.470 ($\pm$ SD = 0.238). The mean OD$_{450}$ of wells with oligonucleotide probes for each of the other subgroups were significantly lower (P <0.001, Bonferroni t test), each value being $\leqslant$0.002 (Table 1). A recent study of the nucleotide sequences of the Ig cDNA expressed by these samples confirmed that each expressed Ig V$_H$1 (39 samples) or V$_H$7 genes (1 sample) (29). 2 (2%) of the 108 samples had cDNA that reacted only with the oligonucleotide probe specific for Ig V$_H$2 genes (Table 1,

group *II*, *boxed value*). Again, the mean OD$_{450}$ of 0.282 ($\pm$ SD = 0.069) for wells with the Ig V$_H$2 probe was significantly higher than that of any of the other wells with other Ig V$_H$ subgroup probes (P <0.001, Bonferroni t test) (Table 1). Similarly, 41 (38%) had cDNA that reacted only with probes for the V$_H$3 subgroup (Table 1, group *III*, *boxed value*), 13 (12%) generated cDNA that reacted only with probes for the V$_H$4 subgroup (Table 1, group *IV*), 6 (6%) generated cDNA that reacted only with probes for the V$_H$5 subgroup (Table 1, group *V*), and none generated cDNA that reacted with the V$_H$6 subgroup probe. Again, for each group, the mean OD$_{450}$ for wells with probes for one Ig V$_H$ subgroup probe (*boxed values*) was significantly higher than that of wells with any of the other Ig V$_H$ 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 V$_H$ gene subgroup in each of three separate experiments (Table 2). 4 had Ig cDNA that reacted equally well with probes for the Ig V$_H$3 and Ig V$_H$4 subgroups (*P1, P2, P4,* and *P5*), but not with probes specific for any of the other Ig V$_H$ subgroups (Table 2). One sample (*P4*) had Ig cDNA that reacted equally well with probes specific for the Ig V$_H$2 and Ig V$_H$3 subgroups, but not probes for any of the other Ig V$_H$ gene subgroups (Table 2). Finally, one sample (*P3*) had Ig cDNA that reacted equally well with probes specific for the Ig V$_H$2 and V$_H$4 subgroups, but reacted twice as well with probes specific for the Ig V$_H$3 subgroup (Table 2). The Ig cDNA for this sample, however, did not react with oligonucleotide probes specific for any of the other Ig V$_H$ 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 J$_H$-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_H Subgroup OD ($\pm$ SE)*

Sample	V_H1	V_H2	V_H3	V_H4	V_H5	V_H6
P 1	0.002 ($\pm$0.001)	0.001 ($\pm$0.000)	0.633 ($\pm$0.287)	0.733 ($\pm$0.287)	0.001 ($\pm$0.001)	0.001 ($\pm$0.001)
P 2	0.001 ($\pm$0.000)	0.002 ($\pm$0.001)	0.600 ($\pm$0.082)	0.600 ($\pm$0.141)	0.001 ($\pm$0.002)	0.001 ($\pm$0.001)
P 3	0.001 ($\pm$0.001)	0.361 ($\pm$0.060)	0.667 ($\pm$0.067)	0.315 ($\pm$0.015)	0.001 ($\pm$0.001)	0.001 ($\pm$0.001)
P 4	0.002 ($\pm$0.001)	0.000 ($\pm$0.001)	0.840 ($\pm$0.118)	0.859 ($\pm$0.181)	0.001 ($\pm$0.001)	0.001 ($\pm$0.000)
P 5	0.001 ($\pm$0.002)	0.001 ($\pm$0.000)	0.610 ($\pm$0.271)	0.663 ($\pm$0.187)	0.001 ($\pm$0.001)	0.001 ($\pm$0.000)
P 6	0.001 ($\pm$0.000)	0.847 ($\pm$0.152)	0.943 ($\pm$0.222)	0.001 ($\pm$0.001)	0.001 ($\pm$0.001)	0.001 ($\pm$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_{450} 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_H genes that corresponded to the subgroups detected in the anchored RT-PCR–ELISA (Table 1). Each of the isolated V_H genes had productive gene rearrangements without introduced frameshift or nonsense mutations. Comparison of the nucleic acid sequence of the cloned Ig V_H genes to known germline genes revealed that sample P4 expressed Ig V_H with highest homology to V_H3-30 (99% homology) and V_H4-b (90% homology), sample P6 expressed V_H genes that were most homologous to V_H2-70 (90% homology) and V_H3-30.3 (88% homology), sample P1 used V_H genes with highest homology to V_H3-33 (99% homology) and V_H4-39 (98% homology), sample P2 had V_H with highest homology to V_H3-7 (97% homology) and V_H4-39 (99% homology), and sample P5 expressed Ig V_H genes with 98% homology to V_H3-2 and 96% homology to V_H4-61. From sample P3 we isolated three V_H genes that had highest homology to V_H2-70,

Figure 1. Southern blot analysis. Placenta and leukemia cell genomic DNA were digested with BamHI and HindIII for Southern blot analysis using a human J_H region probe. The molecular weight markers of λ DNA digested with HindIII are shown at the far left. (*P*, placenta; *1*, P1; *2*, P2; *3*, P3; *4*, P4; *5*, P5; *6*, P6.)

V_H3-7, and V_H4-59 (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_H segments used to generate the sequence encoding the third complementarity determining region (CDR3) of each Ig gene. We deduced that the D_H-containing irregular spacer signal 5 (DIR5) gene segment was probably used to generate the sequence encoding the CDR3 of the two V_H3-encoded genes of samples P1 and P2 (Fig. 2) or the V_H2-70–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_H4 genes expressed by three patient samples (Fig. 2, *P3*, *P1*, and *P5*). We deduced that either J_H4B or J_H3B were used in each of the rearranged Ig genes except for the rearranged V_H3 genes of samples P1 or P2 (Fig. 2). The latter two apparently used the J_H6B 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 κ light chain–expressing patient sample using an anchor sense-strand primer and an antisense-strand primer specific for the constant region of the Ig κ light chain gene. The cells from patients P1 or P5 expressed Ig light chain genes belonging to the $V_\kappa1$ family (96% homology to DPK5 and 92% to DPK7, respectively) rearranged to the $J_\kappa5$ and $J_\kappa2$ minigenes, respectively. Samples P3 or P2 had B cells that rearranged Ig light chain genes belonging to the $V_\kappa2$ subgroup (94% homology to DPK18 and 86% homology to DPK16, respectively), and to $J_\kappa2$ and $J_\kappa1$, respectively. The B cells of patient P4 expressed rearranged Ig light chain genes belonging to the $V_\kappa4$ subgroup (95% sequence homology to DPK24). This V_κ gene rearranged to $J_\kappa2$ 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 V_H4 genes such as V_H4-39, V_H4b (DP67), V_H4-59, or V_H4-61 (25, 30), and B6, an mAb anti-CRI present on Ig encoded by certain Ig V_H3 genes, namely V_H3-23, V_H3-30, or V_H3-30 (24), and/or modified SpA, a 45-kD bacterial superantigen that reacts with the variable region of most Ig heavy chains belonging to the V_H3 subgroup (28).

The Ig molecules encoded by the V_H3-30, V_H3-30.3, V_H3-33, V_H3-7, or V_H3-23 genes expressed by the B cells of patients P4, P3, P6, P1, P2, and P5 were expected to react with SpA and/or B6, whereas the Ig encoded by the V_H4-b, V_H4-59, V_H4-39, or V_H4-61 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 MFIRs of these samples were compared with those of control leukemia cell populations that expressed Ig V_H genes belonging to only one subgroup. Analyses of four leukemia cell samples that expressed only Ig V_H3 genes revealed these samples to have a mean MFIR with SpA of 3.9 ($\pm$ SD = 1.8), and a mean MFIR with Lc1 of 1.1 ($\pm$ SD = 0.2). For example, the leukemic B cells from patient P8 expressed only Ig V_H genes of the V_H3 subgroup and with SpA had a MFIR 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 MFIRs with each of these reagents of 0.9 or 1.0, respectively. Similarly, analyses of four different leukemia cell samples that expressed only Ig V_H4 genes, encoding Lc1-reactive Ig, revealed these samples to have a mean MFIR with SpA of 1.1 ($\pm$ SD = 0.1), a mean MFIR with B6 of 1.1 ($\pm$ SD = 0.2), and a mean MFIR with Lc1 of 5.0 ($\pm$ SD = 2.2). For example, the B cells from sample P9 stained only with Lc1 (Fig. 4, row *C, far right*), having a MFIR for Lc1 of 5.0, but MFIR 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 MFIRs >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 MFIR with SpA for these samples (2.3 $\pm$ 0.6, SD) is significantly higher than that with SpA for the four leukemia samples tested that expressed only Ig V_H4 genes ($P <0.01$, Student's *t* test). Also, the mean MFIR with Lc1 for P2, P3, P4, and P5 (6.5 $\pm$ 4.0, SD) is significantly higher than that with Lc1 for the four leukemia samples tested that expressed only Ig V_H3 genes ($P <0.05$, Student's *t* test). However, the mean MFIRs observed with the anti-hu IgM mAb with the leukemia cell samples that exclusively expressed Ig V_H3 or Ig V_H4 (namely, 7.0 $\pm$ 0.2 (SD) or 8.7 $\pm$ 1.3 (SD), respectively) were not significantly different from that observed with anti-IgM for these variant leukemia cell samples (6.5 $\pm$ 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 antisubgroup 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).

Discussion

We examined the Ig V_H 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 V_H subgroup, consistent with previous findings that each leukemia cell population uses only one Ig heavy chain. However, in six samples (5%) we detected Ig V_H gene transcripts of more than one Ig V_H 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 V_H gene subgroups, these exceptional leukemia cell samples may have had unusual V_H gene sequences that hybridized with probes for more than one Ig V_H gene subgroup. Alternatively, our technique may have detected expression of V_H 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	V_H3-33	99	SA+	N.T.			
	V_H4-39	98	Lc1+			N.T.	
P2	V_H3-7	97	SA+/B6+	2.4	1.4		
	V_H4-39	99	Lc1+			10	9.5
P3	V_H2-70	88	–				
	V_H3-7	100	SA+/B6+	1.9	1.4		
	V_H4-59	99	Lc1+			1.6	1.9
P4	V_H3-30	99	SA+/B6+	3	1.8		
	V_H4-b	90	Lc1+			9.4	7.2
P5	V_H3-23	98	SA+/B6+	1.8	2.1		
	V_H4-61	96	Lc1+			4.9	7.4
P6	V_H2-70	90	–				
	V_H3-30.3	88	SA+/B6+			N.T.	N.T.

The far left column (*Sample*) provides the designator for each sample (*P1–P6*). The second column (V_H gene) lists the germline Ig V_H genes that have the highest homology with the Ig V_H genes isolated from each sample. The percent homology column lists the percent sequence homology between the isolated V_H gene and the deduced germline V_H 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-V_H subgroup reagents. The columns labeled SpA, B6, Lc1, or μ provide the observed MFIR 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.

P1

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                                                                          |---CDR1--|
          Q V H L V E S G G G V V Q P G R S L R L S C A T S G F T F T S Y G M H W V R Q A P G K G L E W V A
P1 VH3    CAGGTGCACCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAACGTCTGGATTCACCTTCACTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCA
VH3-33    ................G.....................................................G.............G..................................................................

          |-------------------CDR2------------------|
          L I W Y D G S N K Y Y A D S V K G R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R
P1 VH3    CTTATATGGTATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA
VH3-33    G...........................................................................................................................................................


          E P L G A R G S Y G D Y G Q D G M D V W G Q G T T V T V S S
P1 VH3    GAACCCCTTGGGGCCGAGGGTCCTACGGTGACTACGGTCAGGACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
DIR5      ........
DA4             .......A..
JH6B                        .............................................

                                                                          |-----CDR1----|
          Q L Q L Q E S G P G L V K P S E T L S L T C T V S G G S I S S S S Y Y W G W I R Q P P G K G L E W I G
P1 VH4    CAGCTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTAGTAGTTACTACTGGGGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGG
VH4-39    ..C.G..............................................................................................................................................................

          |-------------------CDR2------------------|
          S I Y Y S G S T Y Y N P S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R
P1 VH4    AGTATCTATTATTATAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCGGACACGGCCGTGTATTACTGTGCGAGA
VH4-39    ....C.....................................................................................A.....T.....................

          R K D S S G W Y S G Y P D A F D I W G Q G T M V T V S S
P1 VH4DJ  CGGAAGGATAGCAGTGGCTGGTACTCGGGTTACCCTGATGCTTTTGACATCTGGGGCCAAGGGACAATGGTCACCGTCTCCTCA
DN1         .T......CA.........
DK4              ......
JH3B                       ........T....................T...
```

P2

```
                                                                        |---CDR1---|
          E V Q L V E S G G G L V Q P G G S L R L S C A V S G F T F S S Y W M N W V C Q A P G K G L E W V A
P2 VH3    GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGTCTCTGGATTCACCTTTAGTAGTTATTGGATGAACTGGGTCTGCCAGGCTCCAGGGAAAGGGCTGGAGTGGGTGGCC
VH3-7     ..............................................................C...........C........G......C.................G.....................................

          |-------------------CDR2------------------|
          N I K Q D G S E K Y Y V D S V K G R F T I S R D N T K N S L S L Q M N S L R A E D T A V Y Y C A R
P2 VH3    AACATAAAGCAAGATGGAAGTGAGAAATATTATGTGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAACACCAAGAACTCACTGTCTCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGA
VH3-7     ..............................C.........................................G.............A..................................T.....................

          E G Y Y Y Y Y A M D V W G Q G T T V T V S S
P2 VH3DJ  GAGGGTTACTACTACTACTACGCTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
DIR5      .....
JH6B           ................G.................................
```

```
                                                                          |-----CDR1----|
          Q L Q L Q E S G P G L V K P S E T L S L T C T V S G G S I S S S S Y Y W G W I R Q P P G K G L L E W I G
P2 VH4    CAGCTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCAGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTAGTAGTTACTACTGGGGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGCTGGAGTGGATTGGG
VH4-39    .......................................G........................................................................................................................

          |-------------------CDR2------------------|
          S I Y Y S G S T Y Y N P S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A S
P2 VH4    AGTATCTATTATTATAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCCGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCAGACACGGCTGTGTATTACTGTGCGAGT
VH4-39    ..............................................................................................................................................A

          T L R F L E W L F Y P F D Y W G Q G T L V T V S S
P2 VH4DJ  ACATTACGATTTTTGGAGTGGTTATTCTATCCCTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
Dxp4      .....................A..C.
JH4B              ....................................
```

P3

```
                                                                        |------CDR1------|
          Q I T L K E S G P T L V K P T E T L T L T C T F S G F S L T T Y K M G V G W L R Q P P G K S L E W L A
P3 VH2    CAGATCACCCTGAAGGAGTCTGGTCCTACGCTGGTGAAACCCACAGAGACCCTCACGCTGACCTGCACCTTCTCGGGGTTCTCACTGACCACTTATAAAATGGGTGTGGGCTGGCTCCGTCAGCCCCCAGGAAAGTCCCTGGAGTGGCTTCAC
VH2-70    ...G...........................................C.........A.............T.........C.G...AG.GG...C.....A....A................G...G.................

          |-----------------CDR2----------------|
          L I Y W D D D R R Y R P S L K N R L T I S K D I S K N Q V V L T L N N M D P V D T A I Y Y C A H
P3 VH2    TCATTTACTGGGATGATGACAGGCGTTATCGTCCATCTCTGAAAAACAGGCTCACCATCTCCAAGGACATTCCAAAAACCAGGTGGTCCTTACATTGAACAACATGGACCCTGTGGACACAGCCATATATTACTGTGCACAC
VH2-70    G...G.T.........T.AATTC..CA.CA.......G.C...............CC.............A..C.................CG............GG

          D Y Y Y G S G N F D S W G Q G I L V T V S S
P3 VH2DJ  GACTATTATTATGGTTCGGGGAACTTTGACTCCTGGGGCCAGGGAATCCTGGTCACCGTCTCCTCA
DXP'1     .....C..............
JH4B             ........A.............C.................
```

```
                                                                        |---CDR1---|
          E V H L V E S G G G L V Q P G G S L R L S C A A S G F T F S S Y W M S W V R Q A P G K G L E W V A
P3 VH3    GAGGTGCATCTGGTGGAGTCTGGGGGAGGCTTGGTCCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGTAGCTATTGGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTGGCC
VH3-7     .........G....................................................................................................................................

          |-------------------CDR2------------------|
          N I K Q D G S E K Y Y V D S V K G R F T I S R D N A K N S L Y L Q M N S L R A E D T A V Y Y C A R
P3 VH3    ACATAAAGCAAGATGGAAGTGAGAAATACTATGTGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA
VH3-7     .........................................................................................................................................

          D G A I F G V V S H I W G Q G T M V T V S S
P3 VH3DJ  GATGGTGCGATTTTTGGAGTGGTTTCTCATATCTGGGGCCAAGGGACAATGGTCACTGTCTCCTCA
DA5       .......
Dxp4          ...............
JH3B              ...................................C....T...
```

```
                                                                          |-----CDR1----|
          Q V Q L Q E S G P G L V K P S E T L S L T C T V S G G S I S S Y Y W S W I R Q P P G K G L E W I G
P3 VH4    CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTAGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGTAGTTACTACTGGAGCTGGATCCGCCAGCCCCCAGGGAAGGGACTGGAGTGGATTGGG
VH4-59    .......................................G........................................................................................................

          |-------------------CDR2------------------|
          Y I Y Y S G S T N Y N P S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R
P3 VH4    TATATCTATTACAGTGGGAGCACCAACTACAACCCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCGGACACGGCCGTGTATTACTGTGCGAGA
VH4-59    ...............................................................................................T........................................

          A E A A A P Y Y F D Y W G Q G T L V T V S S
P3 VH4DJ  GCGGAAGCAGCAGCCCCGTACTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACTGTCTCCTCA
DN1       .........
JH4B             ..............................C.........
```

P4

```
                                                              |---CDR1---|
          Q V Q L V E S G G G V V Q P G R S L R L S C A A S G F T F S S Y G M H W V R Q A P G K G L E W V A
P4 V_H3   CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCA
VH3-30    ...........................................................................................................................................
```

```
          |-------------------CDR2-----------------|
          V I S Y D G S N K Y Y A D S V K G R F T I S R D N S K N T L Y L Q M N S L R T E D T A V Y Y C A K
P4 V_H3   GTTATATCATATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAACTGAAGACACGGCTGTGTATTACTGTGCGAAA
VH3-30    .....................................................................................................................G....G..................
```

```
          G K V I Q L W L T F D Y W G Q G T L V T V S S
P4 V_H3DJ GGGAAGGTTATACAGCTATGGTTAACCTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
DK4       ..............
JH4B                    ..........................................
```

```
                                                                 |-----CDR1----|
          Q V Q L Q E S G P G L L K P S E T L F L T C T V S G F S I S G G Y Y W G W I R Q P P G K G P E W I G
P4 V_H4   CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGCTGAAGCCCTTCGGAGACCCTGTTCCTCACCTGTACTGTCTGGGGTTTCTCCATCAGTGGTGGTTACTACTGGGGCTGGATCCGGCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGG
VH4-b     .................................C.........CG....T...A.........CA......................................................T.............
```

```
          |-------------------CDR2-----------------|
          S V Y H S G S T Y Y N K S L E S R V T I S I H T S E S Q I S L K L N S V T A A D T A V Y S C A R
P4 V_H4   AGTGTTTACCATAGTGGGAGCACCTACTACAACAAGTCCCTCGAGAGTCGAGTCACCATTTCAATACACACATCCGAGAGCCAGATCTCCCTGAAACTGAACTCCGTGACCGCCGCAGACACGGCCGTCTATTCTTGTGCGAGA
VH4-b     ...A.C..T..........CC.........A...G...G..A..A...T........G....G...T.........................G....AC.........
```

```
          T R F D Y L L S P F D M W G Q G T M V T V S S
P4 V_H4DJ ACCCGTTTTGACTACTTGCTTTCCCCTTTTGATATGTGGGGCCAAGGGACAATGGTCACCGTCTCCTCA
DxP4      ........GG..
JH3B                    .........C......................T...
```

P5

```
                                                          |---CDR1---|
          E V Q L L E S G G D L V Q P G G S L R L S C A A S G I T F S N Y A M C W V R Q A P G K G L E W V S
P5 VH3    GAGGTGCAGCTGTTGGAGTCTGGGGGAGACTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCTGTGCAGCCTCTGGAATCACCTTTAGCAACTATGCCATGTGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCA
VH3-23    ..........................................................................T........G.........A...........................................
```

```
          |-------------------CDR2-----------------|
          A I S G S G D S T Y Y A D S V K G R F T I S R D N S M N T L Y L Q M N S L R A E D T A V Y Y C A R
P5 VH3    GCTATTAGTGGTAGTGGTGATAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCATGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAGA
VH3-23    ..............G..................................A..........................................................................A.
```

```
          D R D T M F R G A N D Y W G Q G T L V T V S S
P5 VH3DJ  GATCGAGATACTATGTTTCGGGGAGCTAATGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
Dxp'1     ........G.........T...
JH4B                .........................................
```

```
                                                                 |-----CDR1----|
          Q L Q L Q E S G P G L V K P S E T L S L T C T V S G G S I S S S Y Y W G W I R Q P P G K G L E W I G
P5 VH4    CAGCTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTAGTAGTTACTACTGGGGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGG
VH4-61    ...G....................................................................G.....G.........A.........G.....................A.............
```

```
          |-------------------CDR2-----------------|
          S I Y Y S G S T Y Y N P S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R
P5 VH4    AGTATCTATTATAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCGGACACGGCCGTGTATTACTGTGCGAGA
VH4-61    TA........C...........A........C...........................................T...........................
```

```
          R K D S S G W Y S G Y P D A F D I W G Q G T M V T V S S
P5 VH4DJ  CGGAAGGATAGCAGTGGCTGGTACTCGGGTTACCCTGATGCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCCTCA
DN1       .T.......CA.........
JH3B                 ...............................T...
```

P6

```
                                                              |------CDR1------|
          Q V T L K E S G P T L V K P T Q T L T L T C A F S G F S L N T G G M R V G W I R Q P P G K A L E W L A
P6 V_H2   CAGGTCACCTTGAAGGAGTCTGGTCCTACACTAGTGAAACCCACACAGACCCTCACGCTGACCTGCGCCTTCTCAGGGTTCTCACTCAATACTGGTGGAATGCGTGTGGGGTGGATCCGTCAGCCCCCAGGGAAGGCCCTGGAGTGGCTTGCA
VH2-70    .............................G.G..G.........A........A......A.....T.......GC..A.........A.C.....................
```

```
          |-------------------CDR2-----------------|
          L I Y W D D D K R Y S P S L R T R L T I M R D T S T N Q A V L L M T N M N S E D T A T Y Y C A H
P6 V_H2   CTCATTTATTGGGATGATGATAAGCGCTACAGCCCATCTCTGAGGACACAGACTCACCATCATGAGGGACACCTCCACAAACCAGGCGGTCCTTCTAATGACCAACATGAACTCTGAGGACACAGCCACCTATTACTGTGCACAC
VH2-70    .G....G..........ATT......A......A....G.........TCC..A..........A......T......AC.......G..C..T.........G..............
```

```
          R L N R G L T D N W N Y G Y L D Y W G Q G T L V T V S S
P6 VH2DJ  AGACTGAACAGAGGATTGACGGATAACTGGAACTACGGATACCTTGACTATTGGGGCCAGGGAACCCTGGTCACTGTCTCCTCA
DIR5      ........
DM1                   .T..............
JH4B                     .T.......C......................C.......
```

```
                                                              |---CDR1---|
          Q V L L V E S G G G V G Q P G R S L R V S C E A S G F H F N Q F A M H W V R Q A P G K G L E W V A
P6 V_H3   CAGGTGCTTCTGGTGGAGTCTGGGGGAGGCGTGGGCCAGCCGGGGAGGTCCCTGAGGGTCTCCTGTGAAGCCTCTGGATTCCACTTCAATCAGTTTGCCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCA
VH3-30.3  ......AG...................................T......T...........AC.........C...........AC.....G.AGC.A..T...............................
```

```
          |-------------------CDR2-----------------|
          M I S K D G S D E E Y A D F V K G R F I I S R D N A E N T L Y L Q M N R L K P G D T A V Y Y C A S
P6 V_H3   ATGATATCGAAAGATGGAAGCGATGAAGAGTACGCAGACTTCGTGAAGGGCCGATTCATCATCTCCAGAGACAATGCCGAGAACACTCTCTATCTGCAAATGAATCGCCTGAAACCTGGGGACACGGCTGTCTATTACTGTGCGAGC
VH3-30.3  G.T.....AT.T.......A..A..T.C.............C.............T..A....G..G...........CA......G.G..A............G..............A
```

```
          V R Y D Y V W G N D R Q A Y Y F D Y W G Q G T L V
          GTTCGTTATGACTACGTTTGGGGTAATGATCGCCAGGCCTACTACTTTGACTACTGGGGCCAGGGAACCCTGGTCA
D21/10    ......T...........
D6                  .......
DIR4-c             .......
JH4B                   ...................................
```

Figure 2. Nucleic acid sequence data of the rearranged Ig V_H 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_H gene. Each sequence is compared to germline V_H gene, D segment, or J_H 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.

P1

DIQMTQSPSSVSASVGDRVTITCRASQGVGSWLAWYQQKPGKAPRLLIY (CDR1)
VκI(L5)

SASSLQSGVPSRFSGSRSGTEFTLTITSLQPEDFATYYCQQAGSFP (CDR2) FTFGPGTKVDVK (Jκ5)
VκI(L5)

P2

DIVMTQTPLSSPLTLAQPASISCRSSQSVVHPDGNTYLDWTQQRPGHLPRVLIH (CDR1)
VκII(A23)

LICNRCFGVPKRVSGGGPGTDFTLKISRLEAGDVGVYYCMRDTQFP (CDR2) WTFGQGTKVEIK (Jκ1)
VκII(A23)

P3

DVVMTQSPLSLAVTLGQPASISCSSSQSLVYSDGDTYLNWFQQRPGQSPRRLIY (CDR1)
VκII(A17)

KVSIRDSGVPDRFSGSGSGTDFTLRISRAGTADVGVYYCNQGTQWLL (CDR2) YTFGQGTKLEIK (Jκ2)
VκII(A17)

P4

IVMTQSPDSLAVSLGERATVNCKSSHSILWSNNKNYLAWYQQKPGQPPKLLIY (CDR1)
VκIV(B3)

WASTRESGVPDRFSGSGFGTHFTLTISSLQAEDVAVYYCQQYYSTP (CDR2) YTFGQGTKVEIE (Jκ2)
VκIV(B3)

P5

DIQMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPGKAPKSLIY (CDR1)
VκI(L15)

KASSLESGVPSRFSGSGLGTEFTLTISSLQPDDFATYYCQQYNSYAP (CDR2) YTFGQGTKLEIK (Jκ2)
VκI(L15)

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 $V_κ$ gene. Each sequence is compared to germline $V_κ$ gene or $J_κ$ 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.

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 V_H gene subgroups, the Ig cDNA of each sample did not hybridize with all probes for each of the Ig V_H gene subgroups, including those specific for major Ig V_H gene subgroups, such as Ig V_H1 (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 4. Flow cytometric analysis of surface Ig. Open histograms depict staining with FITC-conjugated control mAb, whereas shaded histograms depict staining with SpA (*A*), B6 (*B*), Lc1 (*C*), or anti-μ (*anti-IgM, D*). Each column represents the flow cytometric analyses performed on one leukemia cell sample, indicated at the top of each column along with its identified Ig V_H genotype. The histograms at the bottom of each column have abscissas indicating the relative logarithmic fluorescence intensity. Control leukemic B cells of sample P8 stained only with SpA, whereas control leukemia cells of sample P9 stained only with Lc1. B cells from P3, P5, P4, or P2 showed significant staining with Lc1 and SA and/or B6.

Third, nucleic acid sequence analyses of the Ig cDNA revealed that each of these samples had disparate functional V_H gene transcripts that corresponded to the V_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_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_H4 or V_H3 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_H4 genes (25, 30), whereas modified SpA is a 45-kD bacterial superantigen that reacts with Ig encoded by most V_H3 genes (28). B6 is another mAb anti-CRI that reacts with Ig encoded by only a subset of V_H3 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_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_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 κ and λ light chains (32), suggesting that light chain allelic exclusion also may not be absolute. Although we did not detect any κ and λ light chains expressing leukemia cell samples in our study, it is possible that a critical analysis of the light chain V gene subgroups in all our samples also may have detected a few cases that expressed more than one type of κ or λ 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_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_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_H2 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_H 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_H2 gene of P3 or P6, the V_H3 gene of P5, or the V_H4 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_H 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_H3 genes of P1, P3, and P5, or the V_H4 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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