Examples of In Vivo Isotype Class Switching In IgM$^+$ Chronic Lymphocytic Leukemia B Cells

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Abstract

Chronic lymphocytic leukemia (CLL) usually involves the expansion of a clone of CD5$^+$ B cells synthesizing IgM antibodies. These B cells appear to be blocked at the antigen receptor–expressing stage of B cell differentiation and are thought not to undergo an isotype class switch to IgG or IgA production. In vivo and in vitro studies suggest, however, that in some instances terminal differentiation and isotype switching can occur.

To test the hypothesis that in vivo isotype class switching occurs in IgM$^+$ B-type CLL cells, we analyzed the PBMC of 19 CLL patients for the presence of transcripts encoding the rearranged CLL V_HDJ_H associated with either γ or α H chains. The molecular data indicate that $\sim$ 50% of B-CLL patients have amplifications of IgM$^+$ B cells that undergo an isotype class switch. Switching to IgA appears to occur more often than to IgG; also, switching can involve different IgG subclasses in individual patients. In many instances, these CLL-related γ and α transcripts are much more plentiful than those of normal B cells that produce the same isotype. These switched transcripts do not reveal evidence for the accumulation of significant numbers of new V_H gene mutations.

The cellular data indicate that B cells with lesser amounts of surface membrane IgD and higher IgM/IgD ratios are more likely to undergo this switching process. Furthermore, B cells expressing IgG and IgA of the same idiotype or V_H family and the same CDR3 length as those of the CLL IgM$^+$ clone can be identified in the blood of patients studied using multiparameter immunofluorescence analyses.

Collectively, these data suggest that not all members of a B-CLL clone are frozen at the surface membrane Ig-expressing stage of B cell maturation, and that some members can switch to the production of non-IgM isotypes. The occurrence of switching without the accumulation of V gene mutations indicates that the processes of differentiation and diversification are not linked. (*J. Clin. Invest.* 1996. 98: 1659–1666.) Key words: surface immunoglobulins • immunoglobulin variable region • point mutation • autoantibodies • autoimmune hemolytic anemia

Introduction

Most patients with B-type chronic lymphocytic leukemia (B-CLL)[1] have clonal amplifications of IgM$^+$ B lymphocytes that are blocked at the surface membrane Ig (smIg)-expressing stage of B cell maturation (1). However, in vitro studies have suggested that B-CLL cells are not frozen permanently at this stage of differentiation, since appropriate stimulation can give rise to terminal differentiation (2, 3) and to isotype class switching (4, 5). Studies of patient samples also support the notion that these processes can occur in vivo. These include the findings of circulating plasma cells that produce Ig of the same isotype and idiotype as the CLL cell (6) and of non-IgM monoclonal serum proteins in the serum of certain IgM$^+$ B-CLL patients (7–9). However, the relationship of these monoclonal Igs to the smIg of the CLL clones is unclear. Finally, the existence of isotype-switched clonal members in other lymphoid malignancies has been documented (10–21).

Recently, our laboratory reported evidence for IgM-expressing progenitors of IgG$^+$ B-CLL cells (22). These progenitors gave rise not only to the IgG$^+$ CLL B cell, but also to IgA-expressing progeny. Both the IgM$^+$ progenitors and their IgA$^+$ progeny were able to accumulate Ig V_H gene mutations, despite evidence suggesting that they might be involved in the leukemogenic process. Since these data suggested that isotype switching could occur in vivo in "preleukemic" B-CLL clones, we investigated whether overt "leukemic" IgM$^+$ B-CLL cells also were able to undergo an Ig class switch.

Our new data indicate that isotype class switching can be detected in $\sim$ 50% of IgM$^+$ B-CLL patients. The B-CLL cells of these patients express lower levels of smIgD. Cellular examples of this switching process can be identified in the blood as smIgG$^+$ and smIgA$^+$ B cells that express the same V_H gene or gene family as those of the CLL B cell. Since significant numbers of new V_H gene mutations are not frequent in these isotype-switched variants, these data support the hypothesis that somatic mutation is downregulated in overt B-CLL cells and their progeny.

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1. *Abbreviations used in this paper:* AIHA, autoimmune hemolytic anemia; B-CLL, B-type chronic lymphocytic leukemia; mSpA, modified Staphylococcal protein A; smIg, surface membrane Ig.

Methods

Patients. 19 patients with B-CLL, 14 males and 5 females, were studied. These patients were selected randomly from a cohort of 158 B-CLL patients seen over the past 8 yr by the members of the Don Monti Division of Medical Oncology of North Shore University Hospital. Monoclonal serum proteins of the IgG or IgA isotypes were not detected in these patients through standard clinical serologic analyses. PBMC obtained from heparinized venous blood by density gradient centrifugation (Ficoll-Paque; Pharmacia LKB Biotechnology, Piscataway, NJ) were used either immediately or after thawing samples that had been cryopreserved with a programmable cell-freezing machine.

Immunofluorescence analyses. The sm phenotypes of the CLL B cells were determined by direct immunofluorescence using a FACScan® (Becton Dickinson & Co., Mountain View, CA). Polyclonal and monoclonal antibodies reactive with the following antigens were used: CD5, CD19, CD23, CD38, CD54, CD58, CD11a, CD18, CD3, CD8, CD16, CD56, Igκ, Igλ (Becton Dickinson & Co.), CD39 (PharMingen, San Diego, CA), IgM, IgD, IgG, IgA, (Southern Biotechnology Associates, Birmingham, AL), and CD10 (Coulter Immunology, Hialeah, FL). As controls, FITC- or PE-conjugated irrelevant antibodies were used (Becton Dickinson & Co.). 4×10^5 cells were incubated with the different antibodies for 30 min at 4°C and washed in cold PBS. Cells then were fixed with 2% formaldehyde for 1 h and analyzed. In some instances, double and triple immunofluorescence analyses were performed using the above antibodies and biotinylated conjugates of modified Staphylococcal protein A (mSpA; gift of Dr. Gregg Silverman, University of California at San Diego) or of the rat mAb 9G4. mSpA reacts with most V_H3 gene products (23) and 9G4 reacts with a private idiotypic determinant of the V_H4-34 ($V_H4.21$) gene (24).

Preparation of RNA and cDNA synthesis. Total RNA was isolated from either fresh or cryopreserved PBMC using Ultraspec RNA (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. 2 μg of RNA were reverse transcribed to cDNA using M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and either a μ, γ, or α H chain–specific 12-mer primer (Table I). These reactions were carried out in 25 μl using 10 pmol of the appropriate primer at 42°C for 1 h.

Conditions for PCR and cDNA sequencing. To determine the V_H gene nucleic acid sequence of the CLL B cells, 3 μl of the μ cDNA were amplified using a sense V_H leader family–specific primer in conjunction with an antisense 19-mer Cμ primer (M5; Table I). The reaction was carried out in 50 μl using 20 pmol of each primer and cycled with a 9600 apparatus (Perkin-Elmer Cetus, Norwalk, CT) as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. After 35 cycles, extension was continued at 72°C for an additional 10 min. The V_H PCR product was either sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI), or cloned into TA vector (Invitrogen, San Diego, CA) and then sequenced using an automated sequenator (Applied Biosystems, Inc., Foster City, CA). In some instances, the products of such PCR were reamplified using a seminested approach in which the H chain isotype–specific antisense primer was maintained and used in conjunction with a V_H FR1 family–specific sense primer. The conditions were the same as for the first round of PCR, except that the reaction was cycled 30 times. These products were cloned in TA vectors. Random colonies were selected and processed using Wizard minipreps (Promega) and then sequenced using M13 forward and reverse primers.

Ig V_H gene fingerprinting analyses. The lengths of the rearranged H chain CDR3 were used as presumptive indicators of transcripts clonally related to the CLL B cell but expressing different H chain isotypes. To analyze CDR3 lengths, the following strategy was used: the μ, γ, and α cDNA were amplified using an appropriate V_H leader–specific sense primer and an isotype-specific H chain antisense primer (Table I). These first PCR products were then reamplified using two nested consensus primers, a sense FR3 and an end-labeled (with $\gamma^{32}P$; Promega) antisense J_H primer. Since the same two primers were used in each reaction, the lengths of the radiolabeled products for an individual V_H CDR3 were identical, regardless of their H chain isotype. The technical details for these reactions have been reported elsewhere (22).

Results

Identification of B-CLL patient-specific, isotype-switched mRNA transcripts. The relevant clinical features and laboratory data for the 19 patients studied are listed in Table II. The V_H gene sequences used by the leukemic cells of these patients were determined in separate analyses (Fais et al., manuscript in preparation). These data (summarized in Table II) allowed us to use the Ig fingerprinting approach (27) to search the blood of these patients for isotype-switched progeny of the CLL B cell clone. This technique takes advantage of the fact that B cells are heterogeneous and relatively unique for their rear-

Table I. Oligonucleotide Primers

V_H leader primers	V_H FR1 primers
V_H1: 5′ ATGGACTGGACCTGGAGGGTCTTCT	V_H1: 5′ GTGAAGGT(TC)TCCTGCAAGGCT [18–24]
V_H2: 5′ ATGGACATACTCTGTTCCACGCTTCC	V_H4: 5′ CTGTCCCTCACCTGC(AG)CTG [18–24]
V_H3: 5′ ATGGAGTTTGGGCTGAGCTGGGTTT	
V_H4: 5′ ATGAAACACCTGTGGTTCTTCCTCC	
V_H5: 5′ ATGGGGTCAACCGCCATCCTCGCCC	
V_H6: 5′ ATGTCTGTCTCCTTCCTCATCTTCTT	FR3 consensus primer
V_H7: 5′ TTCTTGTTGCAGCAGCCACAGG	FR3: 5′ ACACGGC(CT)(AG)TGTATTACTGTGC [94–101}
C_H primers	
M3: 5′ TGGAAGAGGCAC [223c–223]	IgM5: 5′ CCAAGCTTAAGGAAGTCCTGTGCGAG [149–143]
G3: 5′ CCTTGGTGTTGC [218–215]	I_GG: 5′ GTAGGACAGC(C/T)GGGAAGGTGTGCAC [172–164]
A3: 5′ CATCCTGGCTGG [221–216]	IgA: 5′ GAGGCTCAGCGGGAAGACCTT [126–120]
J_H consensus primer	
JH: 5′ CTGAGGAGACGGTGACC [108–113]	

The numbers in brackets represent codon positions according to Kabat et al. (*Sequences of Proteins of Immunological Interest.* U.S. Department of Health and Human Services, National Institutes of Health, Bethesda, MD. 1987).

Table II. Clinical and Laboratory Characteristics of the CLL Patients Studied

CLL patient	Age	Sex	WBC count ($\times 10^3$)	Clinical stage‡	Associated conditions	Past/present therapy	Percent of CD5/CD19 B cells	smIg	V_H family	Germline gene; % similarity of CLL V_H gene§	Isotype-switched variant
002	66	M	86.2	I	Psoriasis	flu, leuk, vcr, ctx	95%	μκ	4	*4-34;* 100%	IgG + IgA
008	60	M	57.0	IV	Hypogammaglobulin, AIHA	ctx, vcr, pred, chlr, ivig	86%	μκ	1	*1-69;* 100%	IgA
011	65	M	40.0	III	None	chlr, pred, pento, ctx	81%	μκ	1	*1-2;* > 99%	IgG + IgA
059	40	M	33.7	IV	None	None	92%	μκ	3	*VH3-8;* 99%	IgG
079	67	M	129.0	I	None	None	74%	μκ	4	*4-30.2;* > 99%	IgA
112	48	M	505.0	III	None	CHOP, flu, pred	98%	μκ	1	*1-69*; 100%	IgA
129	81	M	45.0	III	Peripheral neuropathy	chlr, pred	88%	μλ	4	*4-31*; 100%	IgG + IgA
130	77	F	23.7	II	AIHA	chlr, pred	80%	μκ	1	*1-3*; 100%	IgG + IgA
141	78	M	38.6	III	AIHA, gout	chlr, pred	91%	μκ	4	*4-34*; 100%	IgG + IgA
153	60	F	400.0	IV	AIHA	mine	96%	μκ	3	*YAC-5;* 96%	IgG + IgA
003	70	F	87.0	II	None	None	70%	μκ	3	*DP58;* 95.6%	nd
017	68	M	58.0	IV	Bell's palsy	ctx, vcr, pred, chlr, flu	85%	μκ	1	*YAC-7;* > 99%	nd
018	68	M	12.5	IV	Richter's transformation	ctx, vcr, pred, adra	90%	μκ	3	*3-7;* 9.3%	nd
042	73	F	20.7	IV	Herpes zoster	flu	80%	μκ	1	*1-18;* 100%	nd
047	63	M	36.4	IV	Hypogammaglobulin	chlr, pred, flu	90%	μκ	1	*1-18;* > 99%	nd
058	69	M	22.6	II	None	None	90%	μλ	3	*3-15;* > 99%	nd
093	78	M	26.5	0	None	None	77%	μκ	4	*4-34;* 95.2%	nd
121	87	F	28.6	I	None	None	92%	μκ	3	*3-7;* 91%	nd
122	61	M	47.8	0	None	chlr	62%	μκ	3	*3-7;* 98.3%	nd

PBMC from 19 patients with B-CLL were studied. Institutional Review Board approval was obtained for these studies. ‡Based on the classification of Rai et al. (25). §When possible, genes are identified as suggested by Shin et al. (26), initial descriptions are used otherwise. adria, adriamycin; chlr, chlorambucil; ctx, cyclophosphamide; flu, fludarabine; ivig, intravenous gammaglobulin; mine, mesna, ifosfamide, mitoxantrone, etoposide; pred, prednisone; vcr, vincristine; wbc, white blood cell.
nd, none detected.

ranged V_HDJ_H CDR3 lengths, and therefore, the identification of cDNA with identical CDR3 lengths suggests clonal relatedness.

Total mRNA from the PBMC of the 19 patients were analyzed with this assay using a V_H leader primer corresponding to that of the CLL B cell and C_H primers specific for μ, γ, and α. In ~ 50% of the patients studied, dominant cDNA bands of the γ and/or α isotypes were found that corresponded in size to those of the μ CLL cDNA. In three cases, these bands were solely of the IgA isotype (Nos. 008, 079, and 112), and in one patient, they were only of the IgG isotype (No. 059). In six instances (Nos. 002, 011, 129, 130, 141, and 153), cDNA for both isotypes were found. These data suggest strongly that certain subclones of these CLL B cells have switched isotype production in vivo. Fig. 1 illustrates results for six representative CLL samples, three in which switching has been detected (*left side*) and three in which it has not (*right side*), and a tonsil sample as a polyclonal B cell control.

DNA sequence analyses of the γ and α H chain cDNA. To assure that the products observed in these fingerprinting assays were clonally related to the IgM^+ CLL B cells, the initial PCR products (V_H leader → C_H) of four samples (Nos. 002, 011, 112, and 129) were reamplified using a seminested strategy that yielded products spanning the rearranged V gene from FR1 → C_H1. These products then were cloned and sequenced. For brevity, these sequences are not presented; however, they are available from EMBL/GenBank/DDBJ under accession numbers U71103–U711106.

The data can be summarized by the following points. First, the sequences of the γ and α cDNA were virtually identical to those of the B-CLL μ cDNA, with only rare differences that were consistent with Taq error. In only three instances two nucleotide discrepancies from the μ CLL sequence were found, and these were distributed randomly throughout the rearranged V_H genes. These data confirm the clonal relatedness of the various cDNA. In addition, the high level of sequence similarity between the IgM^+ CLL clone and the clonally related γ- and α-expressing progeny indicates that significant intraclonal diversification had not occurred. Next, since the cDNA that were sequenced spanned the junction between the V_HDJ_H and the C_H, these data confirm that the γ and α H chain gene segments were physically linked to the V_HDJ_H in the initial mRNA transcripts and, therefore, were unlikely the result of PCR priming artefacts. Sequences illustrating this union for the IgG and IgA clones from patient No. 129 are shown in Fig. 2.

Finally, these data demonstrate semiquantitative differences in the numbers and types of H chain switch variants detected in individual CLL patients. Every IgG^+ cDNA clone was found to be related to the corresponding CLL B cell (Table III). Among the IgA clones, however, heterogeneity was observed. In only one patient (No. 112), all the α cDNA were

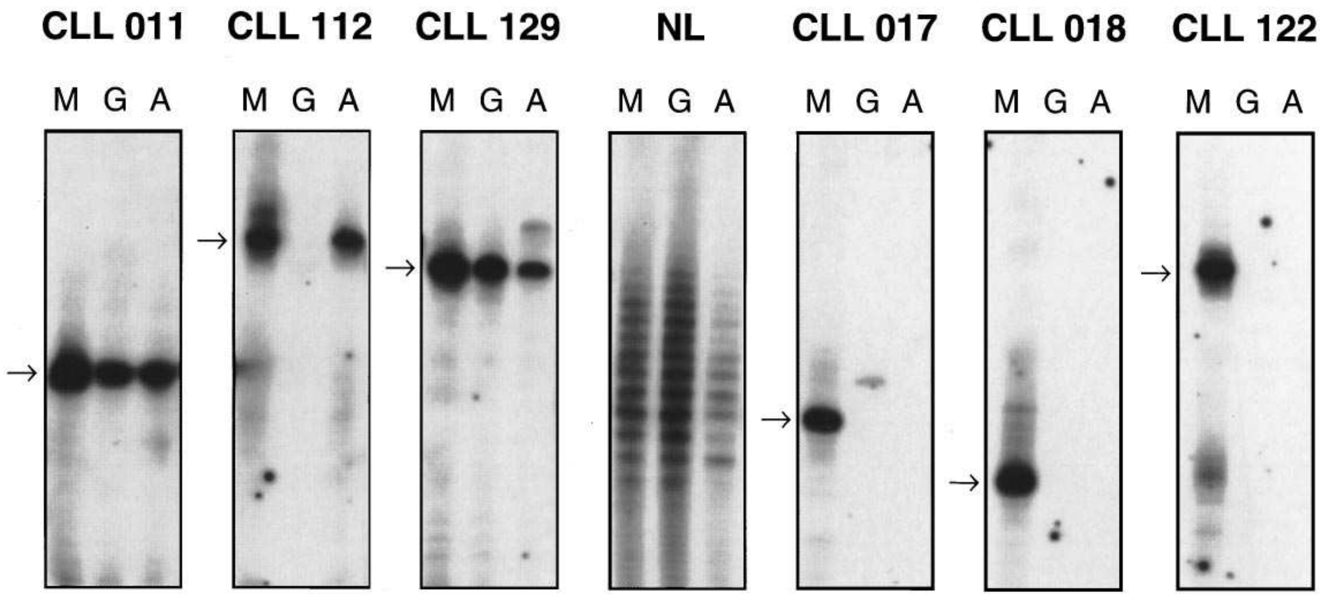

Figure 1. Ig V_H gene fingerprinting analyses. 7.5% acrylamide gel showing the relative lengths of the V_H CDR3 cDNA generated from PBMC RNA of six patients with CLL. The CLL μ bands are indicated by arrows. Note that RNA from patients 011, 112, and 129 yield bands in the γ and/or α lanes with lengths identical to those of the leukemic cells, suggesting clonal relatedness, whereas RNA from patients 017, 018, and 122 yield only the CLL μ band. The center panel represents PCR products generated from tonsillar B cell RNA using a V_H4 leader primer. These illustrate the typical ladder-like appearance of normal polyclonal B cells.

of CLL origin; in patients 002 and 011, 40% of the IgA clones were not related to the CLL. In addition, the data indicate differences in the relative frequencies of the individual IgG subclasses detected within and among patients. Thus, for the two IgG-producing patients studied (Nos. 011 and 129), the majority of the clones expressed IgG1 and/or IgG3 (No. 011, 90% IgG1; No. 129, 63% IgG1 and 30% IgG3); a minority expressed IgG2 and none expressed IgG4. (Table III).

Surface membrane phenotypic analyses. The preceding data indicate that a subpopulation of IgM-expressing B-CLL cells undergoes an isotype class switching event in vivo. Since this phenomenon was observed in only ~ 50% of the patients studied, however, surface phenotyping analyses were performed in an attempt to distinguish those B-CLL clones capable of this process.

Although a total of 16 different markers were analyzed, significant differences among the two groups were found only for the intensity of smIgM and smIgD expression (Table IV). Thus, among the CLL patients in whom switching was not detected, mean channel fluorescence intensity for IgM was 63.50 (SD = 38.007) and for IgD it was 40.75 (SD = 26.926) with an IgM/IgD ratio of 2.00. In contrast, among the CLL patients that did switch, mean channel fluorescence intensity for IgM was 52.714 (SD = 15.524) and for IgD was 22.45 (SD = 19.715); IgM/IgD = 4.60. Statistical comparisons (nested ANOVA) of each of these values between the two groups are significantly different, although the most significant differences are in the levels of smIgD expression ($P < 0.0001$) and the IgM/IgD ratio ($P < 0.0001$).

Detection of circulating isotype class–switched CLL B cells. To confirm that the CLL-related, non-IgM mRNAs were translated into Ig molecules, triple-color immunofluorescence was used to detect $smIgG^+$ and $smIgA^+$ B cells expressing the ap-

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                                        FR3                              N                    D21-9                               N

4-31 gl gene  GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA        G TAT TAC TAT GAT AGT AGT GGT TAT TAC
IgM CLL       ... ... ... ... ... ... ... ... ... ...   TT   . C.. ... ... ... ... ... ... ... ...   CCG GTC CCG T
IgG1 clone    ... ... ... ... ... ... ... ... ... ...   ..   . C.. ... ... ... ... ... ... ... ...   ... ... ... .
IgG2 clone    ... ... ... ... ... ... ... ... ... ...   ..   . C.. ... ... ... ... ... ... ... ...   ... ... ... .
IgG3 clone    ... ... ... ... ... ... ... ... ... ...   ..   . C.. ... ... ... ... ... ... ... ...   ... ... ... .
IgA clone     ... ... ... ... ... ... ... ... ... ...   ..   . C.. ... ... ... ... ... ... ... ...

                                   JH4

AC TAC TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA G
.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .   GG AGT GCA TCC GCC CCA ACC CTT TTC CCC CTC GTC TCC  Cμ
.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .   CC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC  Gγ1
.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .   CC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCG CCC  Cγ2
.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .   CT TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCG CCC  Cγ3
.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .   CA TCC CCG ACC AGC CCC AAG GTC TTC CCG CTG AGC CTC  Cα
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Figure 2. Comparisons of the cDNA sequences derived from the PBMC RNA of the IgM^+ CLL patient No. 129 with representative IgG_1, IgG_2, IgG_3, and IgA related clones. The most homologous V_H, D, and J_H germline gene is listed on top. Note the sequence identity in these five clones. Since the sequences of the V_H segments are identical, only the fragment from FR3 → C_H is shown.

Table III. Summary of the cDNA Sequences of the Isotype-switched CLL Variants

CLL patient	Total number of cDNA clones sequenced		Percentage of γ or α cDNA clones related to the leukemic clone		IgG subclasses of the γ cDNA clones			
	IgG	IgA	IgG	IgA	γ1	γ2	γ3	γ4
002	—	17	0%	58%	—	—	—	—
011	10	12	100%	58%	90%	10%	—	—
112	—	10	0%	100%	—	—	—	—
129	10	10	100%	70%	63%	7%	30%	—

The nucleotide sequences of these CLL cells are available form EMBL/GenBank/DDBJ under accession numbers U71103–U71106.

propriate V_H gene or gene family of the CLL B cell. To address this, we used the 9G4 mAb that reacts with the V_H4-34 (V_H4.21) gene product (24) and mSpA that reacts with V_H3 gene products (23). Preliminary immunofluorescence data using B-CLL cells expressing these two V_H gene families confirmed these selected reactivities (data not shown). Table V and Fig. 3 provide the results obtained when three patients whose CLL B cells use the V_H3 gene YAC-5 (No. 153) and the V_H4-34 gene (Nos. 002 and 141) were studied in this manner.

As would be expected in IgM^+ CLL patients, in each case the majority of the circulating $CD19^+$ cells do not express IgG or IgA (Table V). In all instances, however, there exist a small number of $CD19^+$ B cells that express either $smIgG^+$ (CLL No. 141, 0.37%; No. 153, 1.15%; No. 002, 0.60%) or $smIgA^+$ (No. 141, 1.89%; No. 153, 0.80%; No. 002, 0.32%). Triple-color immunofluoresecence studies indicate that the vast majority of these $CD19^+$ IgG^+ and $CD19^+$ IgA^+ B cells also express the V_H gene products of the corresponding CLL B cell (Fig. 3). When sorted for IgG/IgA expression by FACS®, the V_H gene of these cells had a CDR3 length identical to that of the IgM^+ CLL clone, confirming their leukemic origin (data not shown).

Note, however, that in patient No. 141 there is a relatively large number of IgG-bearing cells (6.52%; Table III) that express the V_H4-34–specific marker (6.39%) but do not express CD19. These IgG^+, $9G4^+$ (non-B) cells coexpress CD16 and CD56, but lack CD3/CD8 coexpression, indicating that they are most likely natural killer cells. Thus, these data suggest that these IgG^+ CLL B cells have secreted sufficient amounts

Table IV. Comparison of smIgM and smIgD Immunofluorescence Intensities of CLL Patients in Whom Switching Was Detected vs Those in Whom it Was Not Detected

Group	smIgM		smIgD		IgM/IgD	
	MCFI	SD	MCFI	SD	MCFI	SD
Switching detected‡	52.71	15.524	22.45	19.715	4.6	4.0
Switching not detected§	63.50	38.007	40.75	26.926	2.0	1.1
Statistical significance	$P = 0.017$		$P = 0.0001$		$P = 0.0001$	

*Values represent mean channel fluorescence intensities determined using direct immunofluoresence and a FACScan® flow cytometer, and were calculated using a nested ANOVA. ‡Mean of 14 determinations of eight samples. §Mean of 16 determinations of seven samples. ‖Statistical significance as calculated by the Student's *t* test. Significant differences in expression were not seen for the following markers: CD5, CD19, CD23, CD38, CD39, CD10, CD54, CD58, CD11a, CD18, CD3, CD8, CD16, and CD56.

Table V. Immunofluorescent Analyses of Circulating IgG^+ and IgA^+ Cells from Three Patients with IgM^+-CLL

	CLL patient number		
	141	153	002
$IgG^+/CD19^+$	0.37%	1.15%	0.60%
$IgG^+/CD19^-$	6.52%	0.95%	0.02%
IgG^+/V_H^+	6.39%	1.52%	0.60%
IgG^+/V_H^-	0.45%	0.91%	0.02%
$IgA^+/CD19^+$	1.89%	0.80%	0.32%
$IgA^+/CD19^-$	1.02%	0.08%	0.00%
IgA^+/V_H^+	1.10%	0.81%	0.34%
IgA^+/V_H^-	0.06%	0.02%	0.01%
$IgG^+/CD16^+$	9.25%	0.64%	0.11%
$IgG^+/CD56^+$	5.62%	0.43%	0.11%

PBMC from three patients were stained with the marker combinations listed and then analyzed by flow cytometry. Results of sm expression of CD19, IgG or IgA, and V_H for patients Nos. 153 and 141, as determined by triple immunofluorescence, are shown in Fig. 3. For patient 141, V_H was determined by the V_H4-34–specific mAb 9G4; for patients 153 and 002, V_H was determined by the V_H3 family–specific reagent mSpA.

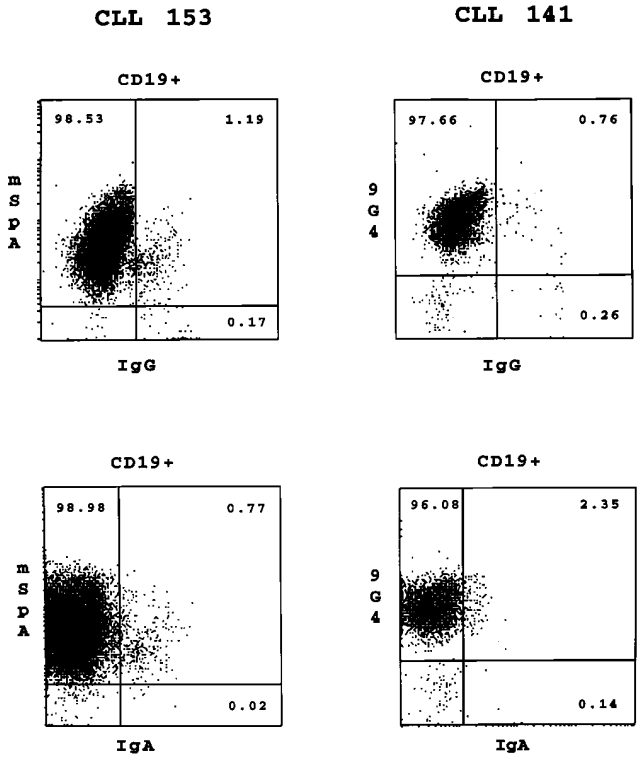

Figure 3. Three-color immunofluorescent analyses of $smIgG^+$ and $smIgA^+$ PBMC from two patients with IgM^+ B-CLL. PBMC were incubated with mSpA-biotin or mAb 9G4-biotin followed by streptavidin-PE, anti–IgG- or anti–IgA-FITC, and CD19-PerCP. Dot plots indicate results obtained by gating selectively on $CD19^+$ cells.

of Ig to permit FcγR-mediated loading of the large granular lymphocytes (LGL) with the V_H4-34 IgG. A similar, albeit less prominent example of this phenomenon is seen in patient No. 153 (Table V).

Discussion

The preceding data indicate that isotype class switching can be detected in ~50% of IgM^+ CLL patients (Table I; Fig. 1). This switching appears to occur more frequently to IgA than to IgG, inasmuch as 9 of the 10 patients in whom switching was detected involved this isotype. Although switching to IgG was found in five patients, in four of these cases switching to IgA occurred also. In one patient, switching was restricted to IgG (Table I). In three instances, serial samples spanning several years were analyzed. In two cases (Nos. 002 and 003), the identical isotype expression pattern was found, whereas in the third patient (No. 112), a new IgG band appeared. Thus, these findings suggest that this switching is an ongoing, nonrandom event that may be influenced by B cell, accessory cell, and/or antigenic signals.

The notion that switching is not random is supported by the differences in the expression of CLL-related transcripts that are associated with the various IgG subclasses. Consistent with our previous studies of 10 IgG^+ B-CLL cases (28), the CLL-related clones were enriched in IgG1 and IgG3 expression (Nos. 011 and 129; Table III). It is possible that our inability to detect transcripts for every isotype in each patient may be a function of the sensitivity of the PCR assay and the primers used. Nevertheless, our data suggest a differential level of mRNA expression for the various isotypes within an individual patient. Supportive of this contention is our recent finding that the PBMC of only three (Nos. 11, 79, and 141) of six patients who were found to switch to IgA and/or IgG (Nos. 11, 79, 141, 129, 130, and 153) were found to also express CLL-specific transcripts associated with the ε H chain (data not shown).

Our Ig V_H fingerprinting and sequencing data indicate two points about the numbers of the clonally related CLL vs. normal B cells in the blood of these patients. First, in many instances, the CLL-related α and γ clones were expressed in significant excess over the normal B cell clones. Indeed, in most cases, radiolabeled bands representative of normal B cells were barely visible (Fig. 1) unless the radiographs were exposed for longer periods of time (data not shown). These data suggest an in vivo amplification of the isotype-switched CLL clones. Indeed, this impression is supported by the immunofluorescent analyses that document that the majority of the IgG^+ and IgA^+ B cells in the blood of these patients express the CLL V_H gene (Fig. 3) and have a CDR3 length identical to that of the IgM^+ CLL clone. The frequency of B cells expressing these switched Ig molecules in relation to those expressing IgM, however, appears to be very small, approximating a 98:1:1 IgM/IgG/IgA ratio (Fig. 3).

Second, there appear to be more residual normal B cells producing IgA than IgG in the blood of these patients (Table III and data not shown). Similar phenomena were seen in our previous study of IgG^+ B-CLL cells (22). This may be a reflection either of an increased number of IgA precursors in the blood of CLL patients and normal subjects or of specific types of antigenic stimuli in these patients. In this regard, previous studies have suggested that IgA is the dominant isotype produced in vivo and in vitro by normal B cells (29, 30).

Decreased expression of smIgM and smIgD characterized those CLL B cells in which switching was detected (Table IV). Since the diminution in smIgD far exceeded that of smIgM, these cells had higher IgM/IgD ratios. Analyses of 14 surface markers, including adhesion molecules, did not reveal other significant differences between these two groups. Since stimulation and maturation of normal B cells results in decreased expression of smIgD (31), these B-CLL cells may have received or continue to receive antigenic and/or accessory cell signals driving them to mature and undergo isotype class switching. Although the antigens driving these cells are unknown, they may be autoantigens, based on the known propensity for B-CLL cells to produce Ig molecules with autoreactivity (32–34, reviewed in 35).

Not infrequently, antigenic stimulation of normal B cells results in the accumulation of mutations in their Ig V_H and V_L genes. Therefore, those B-CLL cells with diminished smIgD and evidence of isotype switching might be expected to have a greater likelihood of having accumulated somatic mutations in these genes. Such a correlation was not found in these cases, since the V_H genes of these IgM^+ B-CLL cells and their switched progeny were virtually identical to those of the corresponding germline gene (Table II). It is unlikely that these switched transcripts were generated from a preleukemic progenitor cell that gave rise to the IgM^+ B-CLL cell and other normal cells, since our previous data suggest that such progenitors can and do develop somatic mutations that can distinguish them from the B-CLL cell (22).

The finding that isotype class switching was not accompanied by the accumulation of new V gene somatic variants is consistent with a large body of data suggesting that the level of somatic mutation detected in IgM^+ B-CLL cells is minimal (36–38, reviewed in 39). Somatic mutations, however, are more common in B-CLL cells of switched isotype (40–42), even though in several instances similar V_H genes were expressed in these two sets of CLL B cells. We recently demonstrated IgM^+ progenitor cells for IgG^+ CLL B cells that exhibited certain features of "premalignant" B-CLL cells (e.g., in vivo expansion) but retained the ability to undergo an isotype class switch and to accumulate somatic mutations (22). This suggested that these two processes (switching and mutation) were active in the CLL precursors and their clonal relatives, but were downregulated in the overt B-CLL cell. Our present findings further refine this notion by indicating that the B-CLL cell retains the ability to switch isotype classes while downregulating the accumulation of significant numbers of new V_H mutations.

Finally, the data presented here may have clinical relevance to the autoimmune phenomena that are seen in some patients with B-CLL. Studies have suggested that the antibodies responsible for autoimmune hemolytic anemia (AIHA) in CLL usually do not originate from the B-CLL cell, since the antibodies bound to the erythrocytes are "warm reactive" (i.e., usually non-IgM), whereas most B-CLL cells express smIgM (43). However, since our studies indicate that isotype class switching to IgG and IgA occurs in a subset of the B-CLL patients (Figs. 1 and 3; Table II), and that in some cases secretion of these molecules is suggested by their detection on the surface of circulating natural killer cells (Table V), the CLL cell and its switched progeny could be a source of pathologic warm-reactive antibodies. Indeed, the B cells from every patient analyzed in this study that had associated AIHA switched

to IgG and/or IgA production (Nos. 008, 130, 141, and 153). Since studies of patients with AIHA have identified warm-reactive antierythrocyte antibodies of all three major isotypes (43), one must consider the possibility that in certain patients the CLL B cell may be the source of such antibodies. Several previous studies support such a hypothesis. For example, antibodies eluted from erythrocytes in some AIHA patients may be of restricted heterogeneity, as defined by serologic markers (44), Ig L chain type (45, 46), and electrophoretic mobility (47). In addition, murine $CD5^+$ B cells frequently react with red blood cell antigens (48) and can cause hemolysis in vivo (49). Therefore, it is possible that the causes of AIHA and other autoimmune phenomena in CLL are heterogeneous, and the potential role of CLL-derived autoantibodies in these conditions requires further study.

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