Structural and Functional Features of the B-Cell Receptor in IgG-Positive Chronic Lymphocytic Leukemia

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Abstract

Purpose: To determine the origin and relationship of the rare IgG^+ variant of chronic lymphocytic leukemia (CLL) to the two common IgM^+IgD^+ subsets that are distinguished by expression of unmutated or mutated V_H genes, with the former having a worse prognosis.

Experimental Design: IgG^+ CLL cells were characterized using phenotypic, functional, and immunogenetic analyses.

Results: IgG^+ CLL was phenotypically similar to mutated IgM^+IgD^+ CLL (M-CLL) and variably expressed CD38 (4 of 14). ZAP-70, a tyrosine kinase preferentially expressed in unmutated CLL, was found in only 2 of 14 cases. The ability to signal via surface IgM (sIgM) varies between the main subsets of CLL and is associated with expression of ZAP-70. In IgG^+ CLL, 9 of 14 responded to engagement of sIgG with no apparent requirement for expression of CD38 or ZAP-70. However, signal capacity correlated with intensity of sIgG expression. Most switched immunoglobulin variable region genes were somatically mutated without intraclonal variation, and no case expressed activation-induced cytidine deaminase. Derivation from a postgerminal center B cell is, therefore, likely, and a relationship with M-CLL is suggested. This is supported by a shared biased usage of the *V4-34* gene. Similar bias in normal B cells developed with age, providing an expanded population for transforming events. However, conserved sequences detected in the CDR3 of V4-34-encoded γ chains were not found M-CLL, indicating no direct path of isotype switch from M-CLL.

Conclusion: IgG^+ CLL is likely to arise from an age-related expanded pool of B cells, on a path parallel to M-CLL, and perhaps with a similar clinical course.

Chronic lymphocytic leukemia (CLL) results from the clonal expansion of $CD5^+$ B cells (1). Most cases express surface IgM (sIgM) and sIgD, albeit at a much lower level than found on the surface of normal B cells (2). Patients are now known to fall into one of two major prognostic subsets based on IgM V_H mutational status, with unmutated CLL (U-CLL; ~40%) having a worse prognosis than mutated CLL (M-CLL; refs. 3, 4). The division of CLL into two major clinical subsets raises questions over the biological differences underlying the differential clinical behavior. Phenotypic and gene array analyses indicate that both subsets exhibit features of activated, antigen-experienced B lymphocytes (5–7). Interestingly, U-CLL cases are more likely to signal via sIgM *in vitro* than M-CLL (8) and expression of ZAP-70, a receptor-associated protein tyrosine kinase, correlates strongly with the unmutated status (9–11).

Sole expression of IgG or IgA by CLL cells is relatively rare (12–17), occurring at a frequency of ~6% (18). However, subpopulations of isotype-switched cells can exist within IgM^+IgD^+ cases (19–22). There is also evidence from transcript analysis of isotype switch events in ~50% of IgM^+IgD^+ CLL patients, almost all being U-CLL (18–21, 23). Activation-induced cytidine deaminase (AID), required for isotype switch (24), also tends to be expressed as mRNA in U-CLL (23, 25), although protein is difficult to detect (26). Switch events in U-CLL or M-CLL can be increased by stimulation with CD40L and IL-4 *in vitro* (19) but it is debatable whether CD40L-expressing T cells are available *in vivo*. One study indicated defective up-regulation of CD40L on T cells in CLL (27), but another detected CD40L-expressing T cells in tissues (28). An alternative route of CD40L-independent switch is via BAFF, known to interact with BAFF-R expressed in CLL (29) and capable of inducing switching *in vitro* in the presence of cytokines (30).

The origin and relationship of the isotype-switched variant of CLL to the more common disease subsets is unknown. Here, we have focused on cases expressing $sIgG^+$ with no $sIgM^+IgD^+$. In this rare subset of CLL, we have found that the B-cell receptor

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can frequently mediate signaling *in vitro*, apparently independently of ZAP-70. V_H gene patterns, lack of AID expression, low expression of ZAP-70, and low levels of sIg point to a relationship to M-CLL, with a possible common origin from age-related normal B-cell expansions. However, it is unlikely that sIgG$^+$ CLL has developed directly from M-CLL by switch events. Evidence points to a separate but parallel developmental route possibly from normal B cells expanded in the elderly.

Materials and Methods

Blood sample selection and clinical features. Following Local Research Ethics Committee approvals and informed consent, blood was obtained from patients with CLL who attended the hematology outpatient clinics at the Hammersmith Hospital, Portsmouth Hospital, Royal Bournemouth Hospital, and Southampton General Hospital. To date, 319 samples have been collected, of which 14 (4.4%) solely expressed sIgG. All 14 IgG$^+$ patients fulfilled the National Cancer Institute diagnostic criteria for classic CLL (31). Clinical features are shown in Table 1. The male-to-female ratio is 1:1, similar to that found in mutated IgM$^+$IgD$^+$ CLL (32). Patients were either untreated (10 of 14) or at least 6 months posttreated before blood collection. Cases of typical sIgMD CLL were obtained from our bank of frozen cells from untreated patients (3). Blood samples were also obtained from 59 healthy elderly individuals (>75 years) and 42 healthy young individuals (<35 years). Normal subjects were excluded if they had a history of lymphoproliferative disease, acute infection, or autoimmune disease, or if they were taking immunosuppressive agents. Mononuclear cells were isolated using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and cryopreserved.

Immunophenotypic analysis. For surface staining, previously frozen lymphocytes were thawed, washed, and labeled with anti-CD19-APC, anti-CD5-PerCP-Cy5.5, and a combination of FITC-labeled anti-CD27, anti-IgG (Becton Dickinson Biosciences, Cowley, United Kingdom), or anti-IgA (Fab′)$_2$ and phycoerythrin-labeled anti-IgM (Fab′)$_2$ (DAKO, Glostrup, Denmark), anti-CD23, anti-CD38, or anti-CD69 (BD Biosciences, Oxford, United Kingdom), or with appropriate isotype controls and analyzed on a BD FACSCalibur with BD CellQuestPro software. For intracellular immunoglobulin analysis, cells were initially stained for CD5 and CD19, then fixed and permeabilized (FIX&PERM; Caltag-MedSystems, Ltd., United Kingdom) and stained for IgM, IgG, or IgA. To determine BAFF-R expression, 10^6 cells in 100 μL were incubated with 1 μL anti-BAFF-R (11C1, Alexis, San Diego, CA) for 30 minutes at 4°C, washed, labeled with anti-mouse immunoglobulin FITC (DAKO), washed, and then blocked with 10 μL normal mouse serum before staining for CD19 and CD5.

ZAP-70 status was determined as described by Crespo et al. (10). The percentage ZAP-70 expression of the CD5$^+$CD19$^+$ CLL cells was determined relative to that of the CD3-positive cells, and cases with >20% expressing cells were scored as positive.

In healthy elderly subjects, the number of B cells expressing immunoglobulin derived from the *V4-34* gene was measured using our idiotope-specific rat monoclonal antibody 9G4 (33). Whole blood was treated with 9G4 and subsequently with anti-rat-FITC, and subsequently with CD5-phycoerythrin and CD19-Cy5 (DAKO). Red cells were lysed (FACSLyse; BD Biosciences) and the cells were analyzed on a BD FACSCalibur flow cytometer.

AID expression. AID mRNA expression was evaluated by a standard reverse transcription-PCR assay. The primer pair used is capable of amplifying the entire region of mRNA that encodes the AID protein yielding a wild-type product of 646 bp as well as two splice variants 939 and 530 bp (25). cDNA from the Raji Burkitt's lymphoma cell line and from a known AID$^+$ CLL case were used as positive controls. Following incubation at 95°C for 15 minutes, the cDNA was amplified at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute for 30 cycles for the CLL samples. For all cases, we also amplified β-actin under the same conditions to assess the cDNA amount and integrity.

Analysis of sIg-mediated signaling events. Syk phosphorylation was carried out as previously described (8). Syk phosphorylation was detected by Western blotting using the anti-Syk mouse monoclonal antibody (clone 4D10.1, Upstate, Dundee, United Kingdom), followed by horseradish peroxidase–anti-mouse antibodies. All estimates were derived from at least two separate assays and an increase of >2-fold was taken as a positive signal.

For analysis of sIg-mediated increases in intracellular calcium ($[Ca^{2+}]_i$), 5×10^6 cells were incubated in 500 μL RPMI containing 4 μmol/L Fluo3-AM (Invitrogen Ltd., Paisley, United Kingdom) and 0.02% Pluronic F-127 (Sigma, Poole, Dorset, United Kingdom) for 30 minutes at 37°C. Cells were washed twice in dye-free RPMI and kept at 37°C before analysis. Cells were treated with 5 μg goat anti-human IgG F(ab′)$_2$ (Southern Biotech, Cambridge, United Kingdom). Sample viability was generally high (mean 87 ± 10%); however, all tests were

Table 1. Clinical features of patients with CLL B cells expressing sIgG

Patient	Sex	Age at diagnosis	Binet stage at diagnosis	PB lymphs at diagnosis, 10^9/L	Disease progression	Treatment and timing (y)	Survival to date (y)
1	F	64	A(0)	11	Yes	At progression (8.0)	13
2	F	82	A(0)	8	No	None	11
3	M	59	B	80	No	At diagnosis (0)	5.5
4	F	78	A(0)	8	No	None	8.5
5*	M	56	B	7	Yes	None	2.1
6	M	45	A	18	No	None	8
7*	F	74	A(1)	12	No	None	4
8	M	48	A(0)	22.1	Yes	At progression (10)	14
9	F	55	A(0)	28	Yes	At progression (3.5)	15
10	M	77	A(1)	45.9	No	None	2
11	M	84	A(0)	8.7	No	None	7
12	M	46	A(0)	39	No	None	8
13	F	74	A(0)	11	No	None	1.5
14	F	82	A(0)	71	No	None	3

*Patients with paraproteins.

Table 2. Phenotypic characteristics and signaling capability of IgG^+ CLL B cells

Case	L chain	sIgG MFI	iIgG MFI	%$CD38^+$	%CD69	%ZAP-70	Ca^{2+} flux	$p72^{Syk}$ P-Tyr*
1	λ	18	ND	2	24	0	+	+
2	κ	12	38	1	71	0	+	+
3	κ	32	ND	94	39	4	+	+
4	κ	8	34	2	13	1	–	–
5	λ	44	182	100	25	6	+	ND
6	κ	74	67	68	11	58	+	ND
7	κ	4	23	10	21	6	–	–
8	κ	39	54	2	44	12	+	ND
9	κ	5	2	5	92	6	–	–
10	κ	10	39	0	23	1	+	ND
11	κ	5	5	1	12	15	–	ND
12	κ	8	50	0	13	11	–	ND
13	κ	69	ND	93	23	35	+	ND
14	κ	11	44	1	36	1	+	+

Abbreviations: sIgG, surface IgG; iIgG intracellular IgG; $p72^{Syk}$; ND, not done.
*Tyrosine phosphorylation of $p72^{Syk}$ as determined by immunoprecipitation and immunoblotting.

carried out on the gated live cells according to forward and side scatter characteristics. Ionomycin (1 μmol/L; Sigma) was used as a positive control. Data were acquired on a BD FACSCalibur using CellQuest Pro Software.

To quantify the calcium response to anti-IgG, the percentage of cells at the peak of the response with an increase in fluorescence intensity above the 85th percentile of unstimulated cells was determined. This percentage was corrected to take account of non-CLL cells in the lymphocyte gate. Samples where at least 15% of the CLL cells responded were deemed positive. The analysis was done using FlowJo software (Tree Star, Ashland, OR).

V_H gene segment sequencing. A V_H leader primer mix (34) and Cγ112 (5′-CTGAGTTCCACGACACCGTCA-3′), Cα (5′-TTCGCTCCAG-GTCACACTGAGT-3′), or Cμ100 (5′-GGAGAAAGTGATGGAGTCGG-3′) constant region primers were used to amplify heavy-chain genes from cDNA. Similarly, a V_L leader primer mix and either Cκ69 (5′-AGTTA-TTCAGCAGGCACACAAC-3′) or Cλ85 (5′-CACRGCTCCCGGGTA-GAAGTCACT-3′) constant region primers were used to amplify light chain genes. All nucleotide sequences were aligned to the V-base directory (35). PCR products from two patients (3 and 14) were cloned into pGEMT vector (Promega Corporation, Southampton, United Kingdom) and eight clones per patient were sequenced to determine the presence or absence of intraclonal variation.

For analysis of V4-34 sequences from healthy elderly patients, a V_H4 leader primer (5′-ACATGAAACATCTGTGGTTCTTC-3′) and the Cμ100 constant region primer or a Cγ100 constant region primer (5′-ACAC-CGTCACCGGTTCGG-3′) were used with Pfx platinum polymerase (Invitrogen). V4-34 sequences were amplified by a nested PCR reaction using a V4-34 FR1-specific forward primer (5′-AGCTACAGCAGTG-GGGCG-3′) and either a Cμ10 (5′-ACGAGGAAAAGGGTGG-3′) or a Cγ10 (5′-CAGGGGGAAGACCGATGG-3′) constant region reverse prim-er. The PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen) and transformed into Top10 competent cells (Invitrogen). Individual colonies were picked and prepared for plasmid DNA isolation (Qiagen, Crawley, West Sussex, United Kingdom) and sequencing.

Results

Immunophenotypic analysis. The immunophenotype of $sIgG^+$ CLL was similar to $sIgM^+IgD^+$ CLL (both U-CLL and M-CLL) in terms of expression of CD20, CD19, CD5, and CD23. All cases expressed CD27, a marker of normal memory B cells, and the BAFF-receptor, both also evident on U-CLL and M-CLL (data not shown; ref. 29). Tumor cells expressed variable amounts of $sIgG^+$ (Table 2) in all cases weaker (~10-fold) than normal IgG^+ B cells (data not shown). Nine of 11 IgG^+ cases also expressed significant levels of IgG in the intracellular compartment and two patients (cases 5 and 7) had detectable IgG paraproteins, each of the same light chain isotype as the tumor cells. Expression of CD38 exceeded the cutoff level of 30% (7) in 4 of 14 cases, and ZAP-70 was >20% (10) in two of these, including the single unmutated case (patient 13; Table 2). The activation marker, CD69, was expressed heterogeneously with no tendency to be coexpressed with $CD38^+$ (Table 2).

AID, an enzyme required for somatic mutation and isotype switch, is detectable as mRNA by reverse transcription-PCR, predominantly in IgM^+D^+ U-CLL (25). Our cases of IgG^+ CLL did not express detectable AID mRNA, whereas the control sIgMD CLL case was clearly positive (data not shown).

Signal transduction via sIgG. Engagement of the sIgG by specific $F(ab)'_2$ anti-γled to an increase in $[Ca^{2+}]_i$ in 9 of 14 cases (64%; Table 2). The percentage of responding tumor cells was calculated from the number of cells showing an increase in fluorescence intensity compared with the sample before stimulation. The cutoff between responders and nonresponders was taken as 15% of the tumor population responding. In 5 of 14 cases, the whole tumor population showed an increase in fluorescence, although only ~45% of the tumor cells crossed the 85th percentile line after stimulation (cases 1, 3, 10, 13, and 14). These cases were designated as "responders" and a typical response curve (patient 3) is shown in Fig. 1A. However, in 4 of 14 (cases 2, 5, 6, and 8), heterogeneity was evident with only a subpopulation of the tumor cells increasing in fluorescence. These were termed "partial responders," and an example (patient 2) is shown in Fig. 1A. Both "responders" and "partial responders" were listed as positive in Table 2. A third group of five cases (cases 4, 7, 9, 11, and 12) had no detectable increase in fluorescence and these are the "nonresponders" (patient 4 in Fig. 1A). Responses were reproducible when done on separate

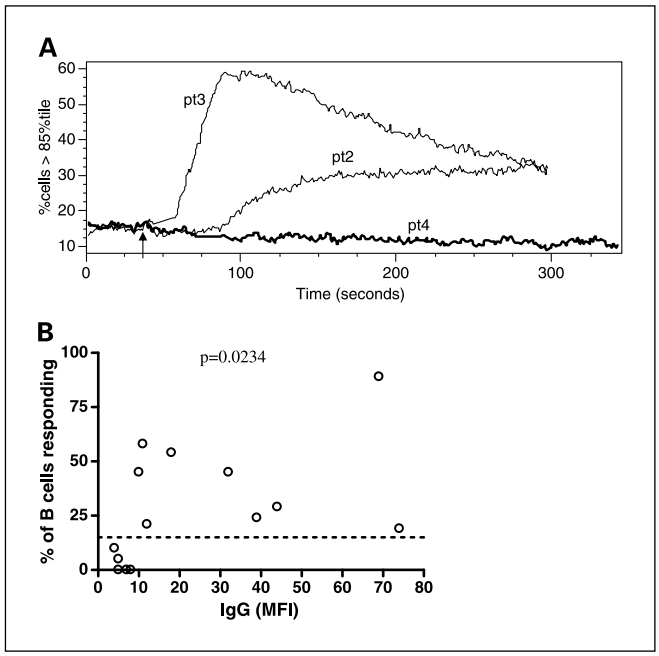

Fig. 1. Detection of intracellular calcium. *A*, anti-IgG F(ab′)$_2$-induced calcium mobilization in IgG-expressing CLL B cells. Arrow, point of addition of anti-IgG F(ab)$_2$. Three types of responses are shown: patient 3 is a typical response curve for "responders"; patient 2 is a typical response curve for "partial responders" where only a proportion of the tumor cell population is responding; and patient 4 is a typical response curve for nonresponders with no detectable response. Because only cells crossing the line of the 85th percentile of unstimulated cells were counted, the percentage response did not reach 100 even when all cells were clearly responding. *B*, influence of sIgG MFI on the signaling ability. The nonparametric (Spearman) correlation gave a *P* value (two-tailed) of 0.0234. All cases having a MFI of ≥10 responded (samples where at least 15% of the CLL cells responded were deemed positive) and all with a MFI < 10 did not respond.

occasions and all samples responded (6.5-fold to 12.5-fold increase) when treated with ionomycin (data not shown). Phosphorylation of p72^{Syk} was also determined on seven cases, with concordant results (Table 2). In a partial responder (case 2), with 21% of tumor cells showing an increase in fluorescence, phosphorylation of Syk was detectable, indicating a comparable sensitivity of the two assays.

There seems to be a clear influence of the level of expression of sIgG on signaling ability [the nonparametric (Spearman) correlation gave a *P* value (two-tailed) of 0.0234], with all cases having a mean fluorescent intensity (MFI) of ≥10 responding, and all with a MFI < 10 not responding (Fig. 1B; Table 2). However, no correlation with expression of ZAP-70, a member of the Syk family of tyrosine kinases, was evident. Although the two cases that express ZAP-70 at the >30% level are both able to signal, several other cases with no detectable expression are also signal competent (Table 2). A similar result is seen for CD38, where the four cases expressing CD38 at >20% can signal, but CD38-negative cases can also signal.

Comparative expression of sIg, ZAP-70, and CD38 in sIgM$^+$IgD$^+$ and sIgG$^+$ CLL. Expression of the key molecules known to vary between the two major subsets of sIgM$^+$D$^+$ CLL was then assessed on the sIgG$^+$ cases. To compare levels of sIg, the 12 of 14 expressing sIgG$^+\kappa$ were directly compared with sIgM$^+$IgD$^+\kappa$ cases in the U-CLL and M-CLL subsets. As seen in Fig. 2A, M-CLL expresses less sIgM$^+$D$^+\kappa$ than U-CLL, and the sIgG$^+\kappa$ cases have low levels comparable with the majority of M-CLL.

In sIgM$^+$IgD$^+$ CLL, ZAP-70 is highly associated with U-CLL and rarely expressed in M-CLL (9–11). This was confirmed in our patients (Fig. 2B). The sIgG$^+$ cases paralleled M-CLL, reaching the current 20% cutoff level in only 2 of 14 cases, including the unmutated case (Fig. 2B). Expression of CD38 is higher in U-CLL than M-CLL (7) and our data support that (Fig. 2C). The sIgG$^+$ cases showed a similar wide scatter in levels of expression but reached the cutoff value of >30% in only 4 of 14 (Table 2). Although there are insufficient numbers for accurate comparison, this trend is similar to that in M-CLL.

V-gene analysis. In the 14 cases sIgG$^+$ cases, VDJ-Cγ transcripts were identified (Table 3), but no VDJ-Cμ transcripts were detectable, consistent with complete deletional recombination (data not shown). In 3 of 14 cases (patients 8, 11, and 14), tumor-derived VDJ-Cα transcripts with identical sequences to the VDJ-Cγ were also detected. IgA protein was not detectable on the cell surface of two of two of these cases (8 and 14), and intracellular IgA was not detectable in one of one case (case 14) tested. V_H-gene usage and mutational levels were closely similar to previous reports (36, 37). The majority (13 of 14) were somatically mutated (>2%) with a biased usage of V_H3 (54%) and V_H4 (46%) families. A predominance (5 of 14) of the V4-34 gene (36%) was confirmed (37).

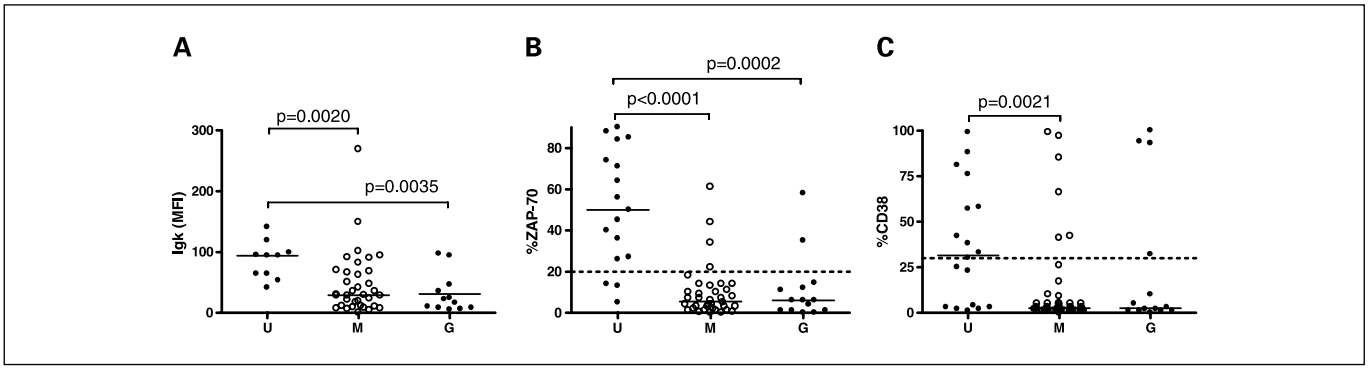

Fig. 2. Comparative phenotype of sIgM U-CLL, M-CLL, and sIgG CLL B cells. *A*, geometric MFI values of sIgκ^+ in U-CLL, M-CLL, and IgG$^+$ CLL samples were compared. *B*, the percentage of cells expressing ZAP-70 in U-CLL, M-CLL, and IgG$^+$ CLL samples were compared. *C*, the percentage of cells expressing CD38 in U-CLL, M-CLL, and IgG$^+$ CLL were compared. Bars, median value. The *P* values are shown where the differences are significantly different using the Mann-Whitney test.

Table 3. Heavy and light chain gene V-gene segment analysis

Patient	VH	D (reading frame)	JH	% VH homology	IgG subclass	CDRH3 sequence	LC	VL*	JL	%VL homology
1	3-07	—	6	93.5	2	DGETVVVWLARVSGG MDV	λ	IGLV10-54 (10a)	3	95.5
2	3-07	—	3	91.2	3	DLPGSGYWWRDA FDM	κ	IGKV1-9 (L8)	2	97.9
3	3-23	2-02 (RF2)	6	95.3	1	RNLLRCSSTSCYGD YYGMDV	κ	IGKV2-28 (A19/A3)	4	98.6
4	3-30.3	—	4	91.8	2	WAGSWAGTWPIVGL FDYP	κ	IGKV3-15 (L2)	2	94.1
5	3-30.3	—	4	93.9	1	DEGGRGAGGGG FDY	λ	IGLV3-25 (3m)	2	97.3
6	3-30.5	4-4/11 (RF3)	2	89.1	1	EGSSVTTSSKRF AFDI	κ	IGKV3-11 (L6)	3	97.5
7	3-64	—	4	94.2	1	DTQWLVK Y	κ	IGKV2-28 (A19/A3)	1	98.2
8	4-34	—	4	92.4	3	REGYQLV Y	κ	IGKV1-39 (O2/O12)	2	96.1
9	4-34	4-17 (RF2)	6	91.0	1	**G**YGD**KR** YYYYGMDV	κ	IGKV2-30 (A17)	2	95.7
10	4-34	5-05 (RF1)	6	93.8	2	**G**YGVD**RR** YYYYGMDV	κ	IGKV2-30 (A17)	2	97.7
11	4-34	5-05 (RF1)	6	92.1	1	**G**YGDTI**KR** YYYYGMDV	κ	IGKV2-30 (A17)	2	98.0
12	4-34	6-13 (RF1)	6	90.7	1	**G**WGSSSQI**RR** YYYYGMDV	κ	IGKV2-30 (A17)	2	97.7
13	4-39	6-13 (RF1)	5	100	1	RDGISSSWYTKKA WFDP	κ	IGKV1-39 (O2/O12)	4	100
14	4-59	—	5	94.5	2	DFYYGDPRG WFDT	κ	IGKV1-39 (O2/O12)	2	92.8
47†	4-34					**G**FPDTDVI**KR** YYYYGFDV				
CLL183†	4-34					**G**YGDTPTI**RR** YYYYGMDV				
CLL240†	4-34					**G**YADTPVF**RR** YYYYGMDV				
CLL342†	4-34					**G**WGDTPML**KR** YYYYGLDV				

NOTE: Amino acids encoded by D gene segments are underlined.
*V_L nomenclature: the IMGT nomenclature for $V\kappa$ (54) and $V\lambda$ (55) genes is used with the Zachau ($V\kappa$; ref. 56) and the Frippiat et al. ($V\lambda$; refs. 57, 58) nomenclatures followed in brackets.
†Data from ref. 41 (cases from the literature).

Features in common with previous studies included a range of immunoglobulin subclasses [IgG1 (8 cases), IgG2 (4 cases), and IgG3 (2 cases)], variable usage of J_H, and detection of a V4-39-D6-13-J_H5/VκO12/O2 combination in the single unmutated case (case 13; Table 3). For IgG$^+$ CLL, V_L usage generally seemed heterogeneous and mutational levels were lower than in V_H as expected (Table 3; ref. 38). No significant levels of intraclonal variation were detected in V_H in two random cases (3 and 14) analyzed by cloning and sequencing of eight clones. Patient 3 had no nucleotide variations out of 2,368 nucleotides sequenced, and patient 14 had 2 of 2,344 nucleotide variations giving an error rate of 0.85×10^{-3} bp$^-$ compared with the Taq error rate of $0.8. \times 10^{-3}$ bp$^-$ (39). The CDRH3 sequences were generally heterogeneous in composition and length, ranging from 8 to 20 amino acids (average 15.6; Table 3). However, conserved sequences were evident among the V4-34-encoded sequences.

V4-34-encoded immunoglobulin in CLL. The bias toward usage of the V4-34 gene in IgG$^+$ CLL (36%) is reminiscent of that observed in M-CLL [11% (15), 9% (40), and 15% in our cohort of 196 cases both published (3)],[5] and possibly even higher. It supports the distinction from U-CLL where there is low usage [3% (40) and our cohort). Strikingly, the V4-34-encoded CDR3 sequences of four of five of our cases (9–12) had identical lengths of 18 amino acids and similar amino acid composition (Table 3). Although four of four used J_H6, different D-segment genes were used, with only two cases (10 and 11) using the D5-05 D-segment gene reported by Messmer et al. (ref. 41; Table 3). The D5-05 assignment was made based on six consecutive nucleotides (VD) in case 10 and seven nucleotides (DT) in case 11, both in reading frame 1. All four had the common features of an aromatic residue (Y or W) following the G residue at the V_H-D junction, which are the products of N additions in cases 10, 11, and 12, but germline encoded by D4-17 in case 9. In our four cases, the Y or W are all followed by a G residue. A pair of basic amino acids (K or R) is located at the D-J_H junction, predominantly encoded by N insertions. Preceding this doublet is a hydrophobic amino acid, either Y or I. The same V_κ gene segment [IGKV2-30 (A17)] was also used (Table 3). The conserved CDRH3 sequence had been observed previously in four cases known to be IgG$^+$ CLL, again all combined with J_H6 and using the IGKV2-30 (A17) V_κ gene segment (41). The conserved sequences of all the known IgG$^+$ cases derived from the *V4-34* gene segment are included in Table 3 for comparison.

However, this conservation was not universal, as case 8 had a completely distinct CDR3 and a different V_L (Table 3). Importantly, a similar conserved sequence was not observed in 27 of our cases of V4-34-encoded CLL (23 M-CLL and 4 U-CLL) or in the public databases of 28 additional V4-34 sequences from typical IgM$^+$IgD$^+$ B-CLL patients. A few cases with similar conserved sequences are in the database, but without isotype designation (42–44).The incidence in known cases of IgM$^+$IgD$^+$ CLL is therefore 0 of 55 cases, indicating a distinction from IgG$^+$ cases.

V4-34-encoded immunoglobulin in normal B cells. To probe the origin of V4-34-encoded CLL, we first analyzed normal blood B cells using our monoclonal antibody (9G4) specific for V4-34-encoded immunoglobulin. Because the B-cell repertoire can change with age (45), we investigated human subjects in

[5] Unpublished data.

two age groups (<35 years or >75 years). Results (Fig. 3) show that expression, and therefore usage of the V4-34 gene, increased markedly in the older age group ($P = 0.0051$).

Analysis of V4-34-encoded IgM or IgG at the transcript level in three selected healthy elderly subjects (Fig. 3, *circled triangles*) revealed that the majority (48 of 55) of the sequences were derived from IgM, and that 43 of the 48 were unmutated (data not shown). This indicates that the expanded population of B cells expressing V4-34-encoded IgM is derived mainly from pregerminal center B cells. Only seven individual IgG^+ sequences could be found, all being mutated. None of the 48 IgM-derived or seven IgG-derived CDR3 sequences from normal elderly subjects had the conserved sequence evident in IgG^+ CLL.

Discussion

In CLL, the proportion of cases expressing isotype switch variants is low, indicating that IgM^+IgD^+ CLL cells rarely undergo further recombination. However, we and others have observed cases that have apparently undergone deletional switch to IgG or IgA, and the question arises over the derivation of these tumors. The clear bias to V4-34 was confirmed in our cohort, which is therefore likely to be representative of this disease subset. The mutational status and immunophenotypic features of IgG^+ CLL point to a developmental pathway shared with M-CLL. This is surprising because U-CLL expresses AID mRNA and often carries transcripts of isotype-switched immunoglobulin, whereas M-CLL generally does neither (18–21, 23). It seems, however, that U-CLL only occasionally completes deletional recombination *in vivo* and that is in a low proportion of the clone (19). There may be an exception in the subset of IgG^+ CLL cases that uses the *V4-39* gene. In our series, we had a single case, but others have found 3 of 44 IgG^+ cases (36) or 5 IgG^+ cases using this gene (37), all being unmutated and having a common D6-13 and J_H5 sequence resulting in the consensus CDRH3 motif XXGYSSWYG/SXXNWFDP (37). These features suggest a common antigenic stimulation as has been described for U-CLL (41, 44) and this could be driving isotype switch events in the absence of somatic mutation.

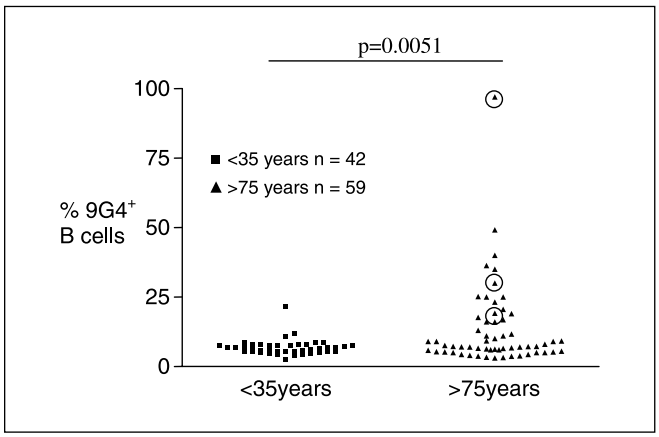

Fig. 3. Percent 9G4 reactive B cells in healthy elderly individuals. Blood samples were obtained from 59 healthy elderly individuals (>75 years) and 42 healthy young individuals (<35 years). The percentages of monoclonal antibody 9G4 reactive B cells were measured and the two sets were shown to be statistically different using the unpaired *t* test. Circles, samples that were used for sequence analysis.

The bias to V4-34 provides insight into the origin of M-CLL and IgG^+ CLL. Shared bias could indicate derivation of IgG^+ CLL from M-CLL by isotype switching within tumor clones. However, if this occurs, it must be rare, because there is no evidence for the conserved CDRH3 sequence characteristic of IgG^+ CLL in IgM^+ CLL. A few cases with similar conserved sequences are in the database but without isotype designation (42–44). It will be interesting to assess if these are in fact isotype switch variants. There is no evidence that isotype switching occurs *in vivo* from M-CLL and the lack of switch transcripts, AID, and a general reluctance of this subset to switch *in vitro* argue against it.

It could be that both tumors derive separately from the expanded V4-34-expressing normal IgM^+ or IgG^+ B cells evident in normal elderly populations. These expansions are likely to be driven by superantigenic stimulation possibly in the context of a cytomegalovirus infection/reactivation (46). It should be noted, however, that to probe age-related changes, our normal elderly cases were selected to be either >75 or <35 years. In fact, two of five of our V4-34-encoded CLL patients were <50 years old and we need to check if expansions also occur at this age. One curiosity about the normal B-cell expansions is that they seem to be mostly derived from pregerminal center B cells and therefore cannot arise from an age-related accumulation of somatically mutated memory B cells. This is consistent with the observation that V4-34-expressing normal B cells are selectively underrepresented in the postgerminal center isotype-switched memory populations (47). If so, mutated IgG^+ tumor cells in CLL must either be derived from a highly unusual subset of normal B cells or a tumorigenic event has changed the rules.

The unique conserved sequence in the CDRH3 of the V4-34-encoded $sIgG^+$ cases points to stimulation by a particular antigen. Although conservation of CDRH3 sequences seems common in IgM^+IgD^+ U-CLL (41, 44, 48), it is unusual to observe this in mutated sequences. However, there is an exception in the mutated V3-21 encoded subset of CLL that does have conserved sequences (44, 49). This subgroup of M-CLL is also unusual in being associated with worse prognosis (49). Generally, it might be expected that antigen selection that follows somatic mutation would tend to lead to specific amino acid changes in the CDRs rather than relying on V-gene sequences generated in the naïve B cell. Interestingly, IgG expressed from three cases of CLL with similar motifs could not recognize ssDNA, dsDNA, or other autoantigens, but acquired these specificities when the somatic mutations in the V4-34 sequence were removed (48). This shows that polyreactivity and/or autoreactivity can be modulated by subsequent somatic mutation.

The ability to signal via sIgM tends to distinguish U-CLL from M-CLL, with the latter exhibiting a more anergic status (8). Both subsets seem activated suggestive of stimulation *in vivo*, and it is possible that M-CLL, being clearly derived from a postfollicular B cell, is more susceptible to induction of anergy. This might also be expected for IgG^+ cases, which are AID negative and postfollicular. In fact, the finding of low levels of sIg with significant levels of intracellular IgG and, in two cases, detection of an IgG paraprotein with a light chain type matching the tumor cell immunoglobulin, suggests that some of the tumor cells could have matured to the stage of secretion of IgG. An

alternative possibility is that the low level of sIgG might reflect an impairment of glycosylation and folding of components of the B-cell receptor, as indicated for $sIgM^+IgD^+$ CLL (50).

However, in contrast to M-CLL, 9 of 14 cases of $sIgG^+$ CLL could signal via sIg *in vitro*, indicating that, although numbers are small, IgG^+ cases might be more able to respond than $sIgM^+IgD^+$ M-CLL (29% positive; $P = 0.037$). The difference might be due in part to the critical influence on signal efficiency by the sIg isotype. Even in M-CLL cases, sIgD is more able to signal than sIgM (8). In normal B cells, sIgG is known to signal more efficiently than either sIgM or sIgD, possibly due to its cytoplasmic tail that prevents phosphorylation of down-regulatory CD22 (51). For IgG^+ CLL, responsiveness to candidate environmental antigen could be greater, and anergy is less easily induced.

Because seven of nine of the signal-competent cases were ZAP-70 negative, it is evident that this molecule is not required for the sIgG-mediated signaling process *in vitro*. However, it remains possible that ZAP-70 is a critical modulator of signaling *in vivo* and that its presence determines the proliferative or apoptotic outcome. It has been reported that ZAP-70 is a more significant prognostic factor than V-gene mutation status (52). Recent studies indicate that ZAP-70 enhances IgM-mediated signaling in CLL regardless of mutational status (53), pointing to signal competence as a major determinant of clinical outcome. The clinical course of the IgG^+ cases can only be estimated, but 10 of 14 have shown no evidence of progression over 0.9 to 10.1 years of follow-up to date. Furthermore, the four patients who have required treatment are alive and well at 5.5 to 15 years following treatment. This indolent pattern of disease behavior and responsiveness to treatment seems to mirror the data for M-CLL as a whole. It has been observed that $sIgG^+$ cases with unmutated V_H genes might have a worse course (37), but our single patient is only 1.5 years postdiagnosis. In general, the disease behavior seems closer to that of M-CLL, even including the single evaluable ZAP-70-positive case. This might suggest that more efficient IgG-mediated signal pathways have a different outcome from ZAP-70-modulated IgM-mediated events. Clearly, the downstream consequences of signaling should now be investigated to probe for further correlations of biology with clinical outcome. Because the number of cases studied is quite small, it will be necessary to confirm both the biological features and patient survival data on a larger cohort of these rare CLL patients whose B cells express IgG.

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