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Hepatitis C Virus Drives the Unconstrained Monoclonal Expansion of $V_H1–69$-Expressing Memory B Cells in Type II Cryoglobulinemia: A Model of Infection-Driven Lymphomagenesis[1]

Maurizio Carbonari,* Elisabetta Caprini,† Tiziana Tedesco,* Francesca Mazzetta,* Valeria Tocco,† Milvia Casato,* Giandomenico Russo,† and Massimo Fiorilli[2]*

Chronic hepatitis C virus infection causes B cell lymphoproliferative disorders that include type II mixed cryoglobulinemia and lymphoma. This virus drives the monoclonal expansion and, occasionally, the malignant transformation of B cells producing a polyreactive natural Ab commonly encoded by the $V_H1–69$ variable gene. Owing to their property of producing natural Ab, these cells are reminiscent of murine B-1 and marginal zone B cells. We used anti-Id Abs to track the stages of differentiation and clonal expansion of $V_H1–69^+$ cells in patients with type II mixed cryoglobulinemia. By immunophenotyping and cell size analysis, we could define three discrete stages of differentiation of $V_H1–69^+$ B cells: naive (small, $IgM^{high}IgD^{high}CD38^+CD27^-CD21^{high}CD95^-CD5^-$), "early memory" (medium-sized, $IgM^{high}IgD^{low}CD38^-CD27^+CD21^{low}CD95^+CD5^+$), and "late memory" (large-sized, $IgM^{low}IgD^{low\text{-}neg}CD38^-CD27^{low}CD21^{low\text{-}neg}CD5^-CD95^-$). The B cells expanded in cryoglobulinemia patients have a "memory" phenotype; this fact, together with the evidence for intraclonal variation, suggests that antigenic stimulation by hepatitis C virus causes the unconstrained expansion of activated $V_H1–69^+$ B cells. In some cases, these cells replace the entire pool of circulating B cells, although the absolute B cell number remains within normal limits. Absolute monoclonal $V_H1–69^+$ B lymphocytosis was seen in three patients with cryoglobulinemia and splenic lymphoma; in two of these patients, expanded cells carried trisomy 3q. The data presented here indicate that the hepatitis C virus-driven clonal expansion of memory B cells producing a $V_H1–69^+$ natural Ab escapes control mechanisms and subverts B cell homeostasis. Genetic alterations may provide a further growth advantage leading to an overt lymphoproliferative disorder. ***The Journal of Immunology,*** **2005, 174: 6532–6539.**

Hepatitis C virus (HCV)[3] is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, with a global prevalence of ~3% (1). In addition, HCV causes a number of extrahepatic manifestations, and especially B cell lymphoproliferative disorders that include type II mixed cryoglobulinemia (cryo-II) (2, 3) and non-Hodgkin lymphoma (NHL) (reviewed in Ref. 4). Approximately half of the individuals chronically infected with HCV have asymptomatic cryoglobulinemia (5). A minority of cryo-II patients (<15%) develop NHL over several years, and epidemiological studies have substantiated an association between HCV infection and NHL (4). It has been estimated that HCV may cause 1 of 20 B cell NHL cases in Italy (6).

In cryo-II, chronic HCV infection drives a non-malignant monoclonal B cell expansion (3) that regresses when HCV infection is cleared by antiviral therapy (7, 8). The clonally expanded B cells produce a polyreactive natural Ab of the IgM class with rheumatoid factor (RF) activity. Most commonly, this monoclonal RF bears the cross-reactive Id WA, which is generally encoded by the Ig H chain variable (V_H) gene *$V_H1–69$* and by the L chain variable (V_L) gene *kv325* (9, 10). The $V_H1–69$/kv325-encoded Id is also commonly expressed by B cell NHL associated with HCV infection (11–13). Some evidences suggest that the non-malignant proliferation of $V_H1–69$ B cells (cryo-II) and their eventual transformation into NHL originate from a response to the E2 glycoprotein of the viral envelope (12, 14). Thus, cryo-II provides a valuable tool to investigate the mechanisms of viral Ag-induced lymphomagenesis of human B cells producing natural Ab.

Mature B lymphocytes are currently subdivided into three subsets: B-1, B-2 (or follicular), and marginal zone (MZ) B cells. B-2 cells produce conventional Abs and differentiate within the germinal centers to originate class-switched memory cells (15), whereas B-1 and MZ B cells share the capacity to produce natural Abs (16, 17). These Abs belong to the IgM class, have broad antimicrobial as well as anti-self reactivity, and serve as critical effectors of the first line defense against blood-borne pathogens (16). Because the WA Id-positive monoclonal RF typical of cryo-II has the features of a natural Ab (10), it is tempting to postulate that the B cells producing it may represent the human equivalent of murine B-1/MZ cells.

The development and differentiation of B cells producing natural Abs has been extensively investigated in mice using transgenic models (18–20), but much less information on these cells is available in humans. In the present study, we exploited anti-Id Abs and

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[3] Abbreviations used in this paper: HCV, hepatitis C virus; cryo-II, type II mixed cryoglobulinemia; NHL, non-Hodgkin lymphoma; RF, rheumatoid factor; MZ, marginal zone; FSC, forward scatter; MFI, mean fluorescence intensity.

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immunophenotyping to delineate the steps of activation, clonal expansion, and progression toward neoplastic transformation of $V_H1–69^+$ B cells in HCV-associated cryo-II.

Materials and Methods

Patients

Nine patients with cryo-II secondary to HCV infection were studied. The diagnosis was based on the typical clinical findings of cryoglobulinemic vasculitis, and on the demonstration of cryoglobulins composed of monoclonal IgMk with rheumatoid activity and polyclonal IgG, of anti-HCV Abs and of HCV viremia, as previously described (8). Three patients had, in addition to typical cryo-II, an overt lymphoproliferative disorder characterized by monoclonal B cell lymphocytosis and splenomegaly, clinically resembling splenic MZ B cell lymphoma (21). One of these two patients (no. 8) has been described in detail in a previous report (22). Ten healthy subjects and two individuals with untreated chronic hepatitis B virus infection were studied as controls.

This study was conducted according to the good clinical practice guidelines of the Italian Ministry of Health and to the Helsinki Declaration. Informed consent was obtained from all patients and controls.

Flow cytometry

The mAbs G6 and G8 were kindly provided by Dr. R. Jefferis (University of Birmingham, Birmingham, U.K.), and 17.109 by Dr. S. Albani (University of California, San Diego, La Jolla, CA). G6 and G8 react with different epitopes of the $V_H1–69$ V_H gene product (23) whereas 17.109 reacts with the kv325 V_L gene product (24). Allophycocyanin-conjugated anti-IgM (custom-labeled) and anti-CD5 mAbs, and fluorochrome-conjugated mAbs to IgD, IgG, CD19, CD21, CD23, CD25, CD27, CD38, CD69, and CD95 were purchased from BD Biosciences. Flow analyses and cell sorting were done on a FACSCalibur (BD Biosciences). Analysis of the density of surface molecules was done as described by Carbonari et al. (25). Briefly, assuming the forward scatter (FSC) as a measure of cell size, the size distribution of B cells is subdivided in FSC intervals and, for each interval, the mean fluorescence intensity (MFI) is determined. The apparent surface density of a given surface molecule is then calculated as the ratio between MFI and FSC.

CDR3 spectratyping and sequencing

Size distribution analysis (spectratyping) of the third CDR (CDR3) was done as previously described (26). Briefly, CDR3 segments were amplified by PCR using FAM-labeled oligonucleotide primers specific for the constant μ (Cμ) region and for a conserved sequence in the human V_H framework region 3. Amplification products were electrophoresed on a 310 ABI PRISM automated sequencer, and the CDR3 size distribution profile was analyzed with the Genescan software (Applied Biosystems). For sequencing, the $V_H1–69$ CDR3 regions were PCR amplified with consensus primers for the $V_H1–69$ family and for Cμ, using the cDNA synthesized from the flow sorted $G6^+CD27^-$ and $G6^+CD27^+$ B cells; the PCR fragments were separately inserted into a bacterial plasmid vector (pCRII-TOPO; Invitrogen Life Technologies), cloned into *Escherichia coli*, and plated onto selective medium. For each separated cloning reaction, sequencing grade plasmid DNA was purified from 96 individual colonies grown overnight in a 96 square-well plate using the NucleoSpin Multi 96 Plus Plasmid kit (Macherey-Nagel) following the manufacturer's instructions. Automated sequences were conducted on an Applied Biosystem Sequencer Model 377–96 using the dye terminator chemistry with the PCR reverse oligonucleotide as sequencing primer.

Sequence similarities were identified using the multiple sequence alignment application, Align X, of the Vector NTI Suite 6.0 (InforMax) based on the Clustal W algorithm (27). The identification of the of the V_HDJ_H junctions and CDR3 were performed comparing the sequences with the ImMunoGeneTics database: IMGT, the international ImMunoGeneTics database ⟨http://imgt.cines.fr:8104⟩ (initiator and coordinator: M.-P. Lefranc, Montpellier, France) (28). The deduced CDR3 amino acid sequences were analyzed by the basic local alignment search tool (BLAST) (29) at the GenomeNet service of the Kyoto University Bioinformatics Center (⟨http://blast.genome.ad.jp/⟩).

Results

Memory $G6^+$ B cells supersede the pool of circulating B cells in HCV-associated cryo-II

We used the G6, G8, and 17.109 mAbs to identify by flow cytometry B cells expressing the *$V_H1–69$* and *kv325* gene products. These variable genes are typically expressed by B cells involved in HCV-associated lymphoproliferative disorders (9), and are thought to be implicated in an immune response to HCV (12, 14). G6 and G8 react with different epitopes of the *$V_H1–69$* gene product (23); because we found that these mAbs identified identical B cell populations in patients and in controls, we will show only data obtained with G6.

The percentages of $G6^+$ B cells varied between 18 and 98% of circulating B cells in six patients with uncomplicated cryo-II (patients 1–6 in Table I). By contrast, they were <6% of B cells in normal individuals (Table I) and in two untreated patients with chronic hepatitis B virus infection (not shown).

The proportions of $G6^+$ and 17.109^+ cells were roughly the same in three of the cryo-II patients, indicating that the expanded B cell clone coexpressed $V_H1–69$ and kv325. In the remaining three patients, we could detect a large population of B cells expressing the G6 but not the 17.109 epitope. This could be due either to the usage of *V_L* gene(s) other than kv325 to generate a cryo-related cross-Id, or to the loss of the epitope recognized by the 17.109 mAb on the *kv325* gene product (10).

In the six patients with uncomplicated cryo-II, the absolute number of circulating B cells was within the normal limits, although in some cases B cells were almost completely represented by $G6^+$ monoclonal B cells (Table I). Three additional patients with HCV-associated cryo-II had, in addition to cryo-II, splenic lymphoma with leukemia-like monoclonal expansion of $G6^+$ B cells (Table I).

The $G6^+$ B cells of cryo-II patients were, on average, larger than autologous $G6^-17.109^-$ B cells or $G6^+$ B cells of normal subjects (see for example Fig. 1). To investigate whether the large size of

Table I. *Demographic and immunological data in patients with HCV-associated cryo-II*

Patient	Age (Years)/Sex	Diagnosis	Total B Cells	Percentage of IgMk B Cells	Percentage of $G6^+$ B Cells	Percentage of 17.109^+ B Cells
1	56/F	Cryo-II	132	60	18	18
2	69/F	Cryo-II	160	66	28	32
3	71/F	Cryo-II	52	86	44	7
4	68/F	Cryo-II	252	80	69	61
5	63/F	Cryo-II	285	92	88	16
6	66/F	Cryo-II	231	98	98	4
7	40/M	Cryo-II, splenic lymphoma	9170	100	96	95
8	57/M	Cryo-II, splenic lymphoma	24097	100	100	95
9	52/M	Cryo-II, splenic lymphoma	2640	99	98	98
Normal subjects[a]			203 (127–291)	41 (35–45)	3.9 (2.7–5.7)	5.4 (4.8–6.1)

[a] Median (range).

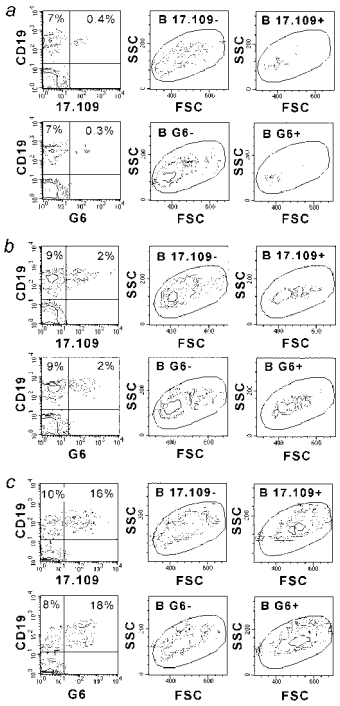

FIGURE 1. Flow cytometric analysis of B cells expressing the G6 and 17.109 idiotypes. *a*, Healthy subject, with $G6^+17.019^+$ cells representing ~5% of total B cells; *b*, Cryo-II patient (no. 1) with mild blood invasion by $G6^+17.019^+$ cells (18% of B cells); *c*, Cryo-II patient (no. 4) with more pronounced blood invasion by $G6^+17.019^+$ cells (69% of B cells). Gated $G6^+17.019^+$ B cells from cryo-II patients display a larger size, as indicated by their higher FSC (*right panels*), than $G6^+17.019^+$ B cells from a normal subjects or autologous $G6^-17.019^-$ B cells.

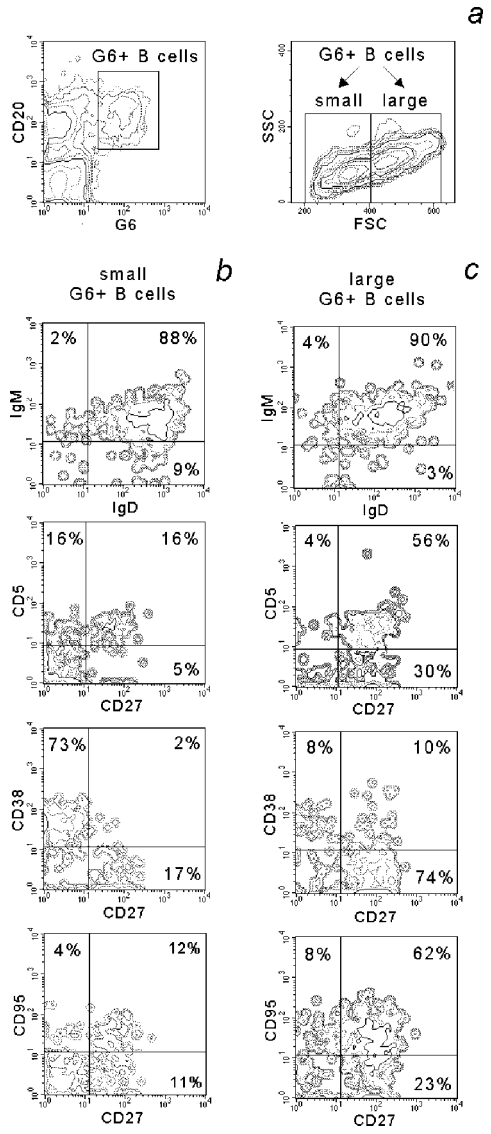

FIGURE 2. Immunophenotype of small and large $G6^+$ B cells from a patient with cryo-II (no. 4). *a*, Gated $G6^+$ B cells display a bimodal distribution of cell size; *b* and *c*, both small and large $G6^+$ B cells are IgM^+IgD^+, but large cells express higher levels of CD5, CD27, and CD95, and largely loose expression of CD38.

$G6^+$ B cells of cryo-II patients reflected their activation, we analyzed the expression of CD27, a general marker of memory B cells (30, 31).

In a patient with a strikingly bimodal cell size distribution (Fig. 2*a*), ~85% of large $G6^+$ cells expressed CD27, compared with 20% of small $G6^+$ cells (Fig. 2, *b* and *c*). This finding suggested that transition of $G6^+$ B cells from naive to memory is accompanied by an increase in cell size. Thus, we systematically analyzed the correlation between cell size, expression of CD27, and level of blood invasion by $G6^+$ B cells. The mean size of $G6^+$ B cells was greater in patients with higher proportions of these cells in the peripheral blood and in the patients with leukemia-like expansion (Fig. 1 and data not shown). We found that the prevalence of $CD27^+$ cells and their mean cell size coincreased linearly with blood invasion (Fig. 3). These findings indicate that a clonal population of steadily larger memory $G6^+$ B cells progressively invades the circulating B cell compartment of patients with HCV-associated cryo-II. Thus, these non-neoplastic B cells appear to evade the homeostatic mechanisms that regulate Ag-driven clonal expansion. Genetic events may cause further escape from control and lead to absolute lymphocytosis. At this regard, molecular cytogenetic studies revealed in two of the three patients with splenic lymphoma (Table I) trisomy of the long arm of chromosome 3 (22, and C. Mecucci, M. Fiorilli, unpublished observations). This anomaly is frequent in low-grade MZ B cell lymphomas (32).

V region gene sequences expressed by monoclonal $G6^+$ B cells display intraclonal variability

Spectratyping of the H chain CDR3 in whole PBMC revealed in all cryo-II patients a predominant band, indicative for a monoclonal B cell expansion (data not shown). To compare the predominance of the monoclonal population within different B cell subsets, we flow sorted B cell subpopulations from a selected patient (no. 1). The spectratyping profiles indicated that the dominant clone was confined to k-expressing B cells (Fig. 4, *a* and *b*). The expanded clone was highly represented among $G6^+CD27^-$ cells and it was virtually the only population present in $G6^+CD27^+$ B cells (Fig. 4, *c* and *d*). $G6^-CD27^+$ B cells displayed an oligoclonal pattern (not shown). This finding is consistent with the earlier observation of oligoclonal B cell expansion in the bone marrow of cryo-II patients (33).

To investigate the role of antigenic stimulation in the clonal expansion of $G6^+$ cells, we sequenced the H chain variable genes from flow-sorted $CD27^-$ and $CD27^+$ cells. The deduced amino acid sequences showed, in both cases, a remarkable prevalence of molecules with a fragment length of 16 aa. They represented 42% (13/31) of the 31 different clonotypes detected among 50 randomly selected CDR3s in $CD27^-$ cells, and over 80% (9/11) in $CD27^+$ cells (Table II). Within this prevalent fraction of CDR3 sequences,

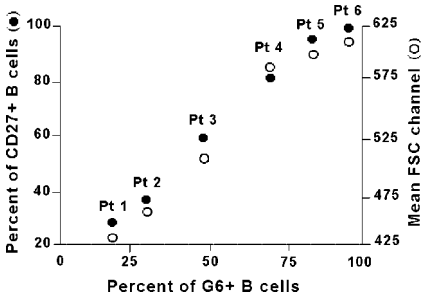

FIGURE 3. The frequency of large memory $G6^+$ B-1 cells increases linearly with the level of blood invasion in patients with HCV-associated cryo-II. The figure shows, for each patient, the correlation between the percentage of $G6^+$ B cells, the percentage of $CD27^+$ B cells (●), and the mean B cell size (○). The B cell size is expressed as the mean FSC channel.

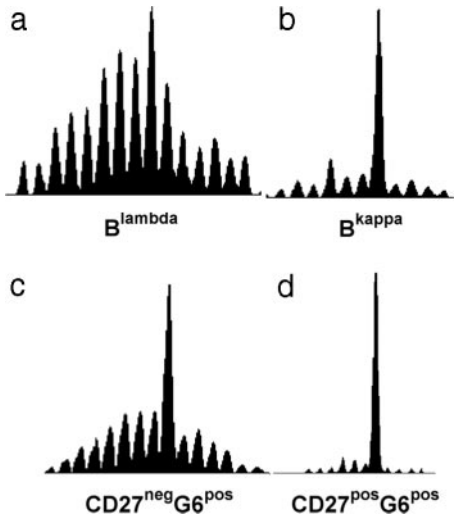

FIGURE 4. Size distribution of CDR3 sequences from flow-sorted B cell subpopulations of a cryo-II patient (no. 1). *a*, λ-Expressing B cells. *b*, κ-Expressing B cells. *c*, $CD27^-G6^+$ B cells. *d*, $CD27^+G6^+$ B cells. The dominant bands present in B^{κ}, $CD27^-G6^+$, and $CD27^+G6^+$ cells have the same size.

one single clonotype appeared particularly abundant in both cell populations, representing 34% of 50 randomly selected CDR3s from $CD27^-$ cells and 62% of 37 CDR3s from $CD27^+$ cells. The sequence of the D and J_H germline genes revealed that all the CDR3 molecules 16-aa long used an exclusive combination of J4 and D5-5 genes. Moreover, the analysis of N-regions and P-nucleotide insertions showed in all these clonotypes highly conserved amino acid motifs: glutamic acid-glycine-lysine-lysine (EGKK) at the VD segment joint and threonine-alanine-isoleucine (TAI) at the DJ junction (Table II). Thus, the observed amino acid substitutions, especially clustered in the D region, suggest a process of Ag-driven intraclonal variation, in agreement with previous reports (12, 13, 34). The fact that the dominant clonotype was much more represented among memory rather than naive $G6^+$ B cells (62 vs 34%) supports a role for antigenic stimulation by HCV in driving memory B cell expansion.

Comparison of the deduced CDR3 amino acid sequences using a BLAST protein database indicates (Table II) that the highest significant homology is, in all cases, with a correspondent region amplified from a monoclonal non-neoplastic B cell expansion of a Sjögren's syndrome patient (GenBank Acc. No. AF303912) (35). This suggests that the stimulatory agents underlying these disorders may share common antigenic determinants.

BCR is down-regulated on memory $G6^+$ B cells

Human memory B cells are subdivided into switched and IgM-expressing cells (36). IgM-expressing memory B cells, which represent about one-fourth of circulating B cells, can in turn be subdivided into IgM^+IgD^+ and IgM-only cells (30). IgM-expressing memory B cells are known to express higher levels of BCR components than naive cells (30, 31, 36). However, memory B cells have a larger size than naive cells. Therefore, the straightforward measurement of MFI is inadequate to compare the actual density of surface molecules on memory and naive cells. In fact, the same MFI generated by a small and by a large cell reflects a higher surface density of the molecule on the former. Thus, we exploited a method of analysis that correlates MFI to the cell size and provides an estimate of the actual density of surface molecules (25). By using this approach, we could confirm (30, 31, 36) that normal IgM-expressing memory B cells have an IgM surface density higher than naive B cells (Fig. 5*a*). The level of IgM expression increases together with the cell size (Fig. 5*a*). By contrast, in cryo-II the IgM surface density is lower on memory than on naive B cells, and does not increase with the cell size (Fig. 5*b*). The expression of CD21 progressively wanes throughout the differentiation of $G6^+$ cells from naive to late memory (data not shown). This finding is in agreement with the notion that surface CD21 expression terminates when B cells are activated and become blast-like (37). The only BCR component expressed at high level on memory $G6^+$ B cells of cryo-II patients is CD19 (Fig. 5*b*). This might be related to the strict dependence of B cells on CD19-mediated signals for maintaining sufficient replication levels (38). The low level of expression of BCR by circulating memory $G6^+$ B cells of cryo-II patients may reflect the fact that these cells have been recently stimulated and are still subjected to antigenic pressure. By contrast, the IgM^{high} memory cells of normal individuals, which display a resting phenotype (30, 36), might represent post-germinal center cells.

Discrete stages of differentiation of $G6^+$ B cells from naive to memory cells

The differential expression of specific surface markers has been instrumental in defining discrete stages of differentiation of human mature B cells, from naive to memory, referred to as Bm1-Bm5 classification (39). We took advantage of this model, integrated by cell size analysis, to provide a description of the stages of differentiation of monoclonal $G6^+$ B cells in HCV-associated cryo-II.

We could delineate three stages of $G6^+$ B cell differentiation: naive, early memory, and late memory. A further stage is represented by transformed cells (Fig. 6).

Naive $G6^+$ B cells are small, $IgM^{high}IgD^{high}CD21^+CD38^+CD27^-$ lymphocytes. The transition from naive to $CD27^+$"early memory" cells is accompanied by increase in cell size, termination of CD38 expression (see, for example, Fig. 2) as predicted by the Bm1-Bm5 model (39), and down-regulation of IgD and CD21 surface density. Transition to "early memory" cells is also accompanied by de novo CD5 expression. The latter issue is illustrated by findings in a representative patient (Fig. 2) showing that the bulk of small $CD27^-$ cells are $CD5^-$, whereas the majority of large $CD27^+$ cells are $CD5^+$. The small population of $CD27^-CD5^+$ cells probably represents a transition stage. The pattern of expression of CD95, an activation molecule functionally related to apoptosis (40), parallels that of CD5 (Fig. 2). Thus, the medium-sized "early memory" $G6^+$ B cells are, on average, $CD27^+CD38^-IgM^{high}IgD^{low}CD21^{low}CD5^+CD95^+$.

Progression from "early memory" to "late memory" cells is marked by further increase of cell size, further down-regulation of

Table II. *Deduced amino acid sequences of CDR3s with a 16-aa length derived from G6$^+$ B cells with a naive (CD27$^-$) and a memory (CD27$^+$) phenotype*

Clonotype No.	Ig_H CDR3 Deduced Amino Acid Sequences[a]	V_H	D	J_H	No. Clonotype/Total Sequences	Length of CDR3 (aa)	*E* Value[b] for the Similarities with the AF303912 Sequence (35)
NAIVE_63	CAREGKKDTAMVTAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.002
NAIVE_21	**CAREGKKETAMVTAIDYWGQG**	V1-69	D5-5	J4	**17/50**	16	0.006
NAIVE_67	CAREGKKEKAMVTAIDYWGQG	V1-69	D5-5	J4	2/50	16	0.028
NAIVE_60	**CAREGKKETTMVTAIDYWGQG**	V1-69	D5-5	J4	**2/50**	16	0.001
NAIVE_17	CAREGKKDTTMVTAIDYWGQG	V1-69	D5-5	J4	2/50	16	5e-04
NAIVE_45	CAREGKKDTAVATAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.016
NAIVE_32	CAREGKKETGMATAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.028
NAIVE_14	CAREGKKHTGMVTPIDYWGQG	V1-69	D5-5	J4	1/50	16	0.003
NAIVE_01	CAREGEKDTGMVTAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.003
NAIVE_27	CAREGKKDGGMVTAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.021
NAIVE_03	CAREGKKETVMVTAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.006
NAIVE_84	CAREGKKETAMETAIDYWGQG	V1-69	D5-5	J4	1/50	16	0.028
NAIVE_57	CAREGKKETAMVTPIDYWGQG	V1-69	D5-5	J4	1/50	16	7e-04
MEMORY_38	CAREGKKDTAMVTAIDYWGQG	V1-69	D5-5	J4	1/37	16	0.002
MEMORY_60	**CAREGKKETAMVTAIDYWGQG**	V1-69	D5-5	J4	**23/37**	16	0.006
MEMORY_29	**CAREGKKETTMVTAIDYWGQG**	V1-69	D5-5	J4	**2/37**	16	0.001
MEMORY_22	CAREGKKETVMVTPIDYWGQG	V1-69	D5-5	J4	1/37	16	7e-04
MEMORY_36	CAREGKKVTAMVTPIDYWGQG	V1-69	D5-5	J4	1/37	16	0.003
MEMORY_45	CAREGKKETAMVTAIDHWGQG	V1-69	D5-5	J4	1/37	16	0.021
MEMORY_31	CAREGKKDRGMVTAIDYWGQG	V1-69	D5-5	J4	2/37	16	0.016
MEMORY_02	CAREGKKDTGMATAIDYWGQG	V1-69	D5-5	J4	2/37	16	0.010
MEMORY_13	CAREGKKHTRMVTAIDYWGQG	V1-69	D5-5	J4	1/37	16	0.016
AF303912	CAREGHKDTTMVTPFDYWGQG	V1-69	D5-5	J4	–	16	1e-06

[a] Sequences in bold represent the clonotypes common to the naive and memory cells; the underlined amino acids derive from N and P nucleotide insertions during the somatic recombinatory process.

[b] Smallest sum probability according to the BLAST similarity search program. The *E* value is inversely related to statistical significance and is considered significant for a value <0.05.

the surface density of BCR components, with the exception of CD19, reduced surface density of CD27, and decreased expression of CD5 and CD95 (from ~50% to ~20% positive cells). Thus, the predominant phenotype of large "late memory" G6$^+$ B cells is $CD27^{low}CD38^-IgM^{low}IgD^{low\text{-}neg}CD21^{low\text{-}neg}CD5^-CD95^-$ (Fig. 6). At none of the stages described did G6$^+$ B cells express the activation markers CD23, CD25, and CD69 (data not shown).

We investigated three patients with cryo-II, splenic lymphoma, and leukemia-like expansion of G6$^+$ B cells carrying chromosomal abnormalities. These "transformed" elements were found to be very large memory cells with extremely low density of IgM and CD21, no expression of IgD and CD95, and high CD19. CD5 was positive in one of three cases (Fig. 6). The fact that splenic lymphoma is a neoplasia of MZ cells (25) reinforces the suggestion

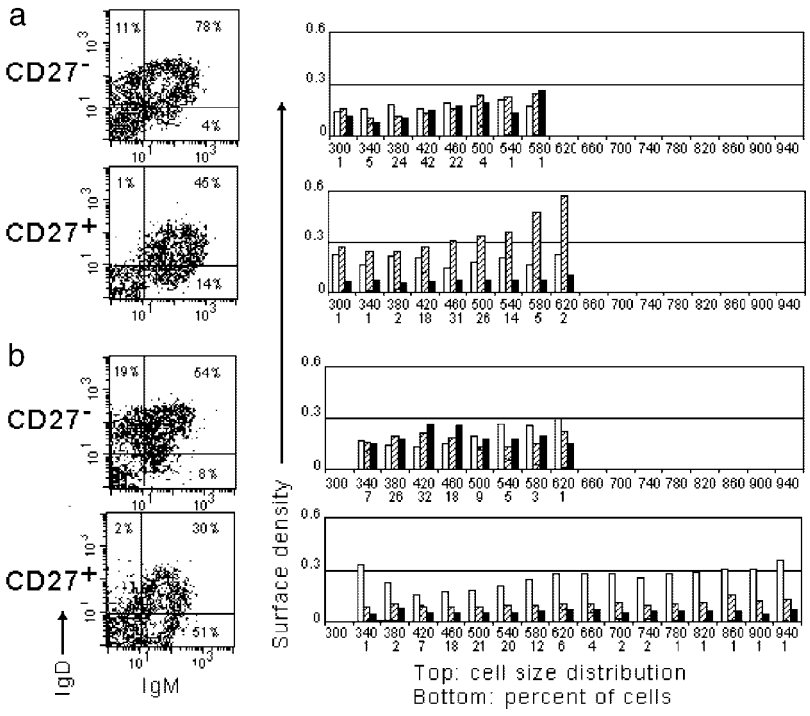

FIGURE 5. Low expression of BCR components by memory G6$^+$ B cells. *a*, Normal subject; *b*, cryo-II patient (no. 4). The raw MFI values (*left panels*) and the cell surface density values (*right panels*), calculated as previously described (25), are shown. □, CD19; ▨, IgM; and ■, IgD. The horizontal axis indicates the cell size distribution as reflected by the distribution of FSC values (top numbering), and the percentage of cells gated in each cell size interval (bottom numbering). Large cells (high FSC) are more prevalent among memory than naive cells. In the cryo-II patient, memory cells are on average larger than those of the normal subject. The MFI values for IgM and IgD are similar in naive and memory cells (*left panels*). However, calculation of the surface density of BCR components (*right panels*) reveals differences between normal and cryo-II memory B cells. The surface density of IgD is lower on memory compared with naive B cells in both cases. By contrast, in the normal subject, memory B cells have higher surface density of IgM than naive cells, whereas in cryo-II memory, G6$^+$ B cells have strongly reduced IgM density compared with naive G6$^+$ B cells. The density of CD19 on memory and naive B cells is similar both in normal subject and in cryo-II.

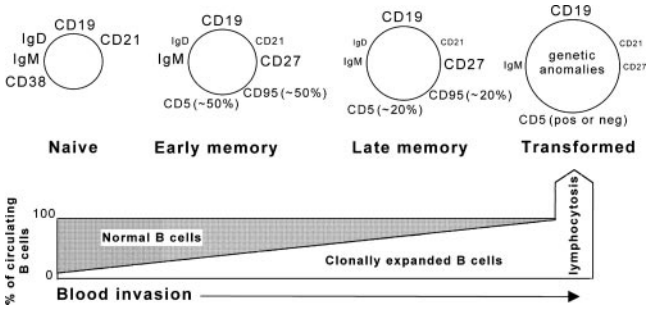

FIGURE 6. A model of differentiation, clonal expansion and transformation of $G6^+$ B cells in HCV-associated cryo-II. These cells produce a natural polyreactive autoantibody that is putatively associated with an immune response to HCV (14). The density of surface molecules, evaluated as previously described (25), is represented proportionally to the size of the font. Naive $G6^+$ B cells are small $IgM^+IgD^+CD21^+CD38^+CD5^-$. Upon initial differentiation, they become medium-sized $CD27^+CD38^-$ early memory cells. At this stage, the surface density of IgM remains high, whereas those of IgD and of CD21 decrease. Nearly half of these cells express CD5 and CD95. At the subsequent late memory stage, the cells further increase their size and down-regulate the expression of IgM, IgD, CD21, CD95, and CD5. Late memory cells represent the predominant B cell type in cases with profound blood invasion, in which nearly all circulating B cells are represented by the monoclonal population. The transformed stage is seen in patients with MZ splenic lymphoma and leukemia-like lymphocytosis associated with genetic anomalies. Transformed cells are $IgM^{low/neg}IgD^-CD21^{low/neg}CD95^-CD27^{dim}CD19^{high}$, and can be either $CD5^{dim}$ or $CD5^-$.

that the $G6^+$ B cells expanded in cryo-II are related to murine MZ/B1 cells producing natural Ab (16). The splenic origin of these cells is also suggested by the fact that cryo-II may regress following splenectomy (41), and that the spleen is the major source of natural Ab-producing memory B cells (42).

Discussion

A central point in our study is the evidence that a monoclonal population of B cells, producing a polyreactive natural Ab bearing the G6 Id, undergoes an unconstrained expansion in patients with cryo-II secondary to chronic HCV infection. Exaggerated clonal expansion could be a general feature of B cell responses elicited by HCV, or could depend on the capacity of these particular cells to escape homeostatic control. The former mechanism is suggested by the fact that HCV, by binding CD81 through its E2 envelope glycoprotein, might nonspecifically enable B cells to respond to lower concentrations of Ag and enhance their proliferation (43). However, a compelling argument against this possibility is that, although HCV infection triggers multiple B cell clones (33, 44), only B cells expressing the G6 Id expand abnormally in cryo-II (9, 12–14).

The tendency of $G6^+$ B cells to outgrow might depend on their relatedness to murine B-1 and MZ B cells producing natural Abs (10). In fact, B-1 and MZ cells are known to be intrinsically more prone to clonal expansion and transformation than conventional B cells (45–47). Additional advantage may be provided to $G6^+$ B cells by their reactivity with an endogenous autoantigen, owing to the RF activity of the expressed Ab. Studies in transgenic mice have shown that autoreactive B cells are positively selected by self Ags (20). In mice transgenic for a $G6^+17.109^+$ RF, coexpression of human IgG increases the number of $G6^+$ B cells (19). Thus, the positive regulatory effect of endogenous IgG may contribute to the outgrowth of $G6^+$ B cells in cryo-II patients.

Several reports have shown that B cell expansion in cryo-II may completely regress following effective antiviral therapy, even when sensitive molecular assays are used to detect residual monoclonal B cells (7, 48, 49). Monoclonal B cells persist when treatment fails to eradicate HCV (48). Previous studies (22, 50) also reported the regression of splenic lymphoma associated with HCV infection; failure of antiviral treatment resulted also in this case in the persistence of monoclonal B cells (50). Taken together, these studies strongly support the concept that the growth and survival of monoclonal B cells in HCV-associated lymphoproliferative disorders depend strictly on the continued stimulation by HCV. Consistently with this, in our three patients with splenic lymphoma the absolute number of circulating B cells decreased one log or more after IFN therapy (from 9,170 to 612, from 24,097 to 190, and from 2,640 to 269 B cells, respectively). Nevertheless, after completion of treatment $G6^+$ cells continued to represent >90% of circulating B cells in these patients. Persistence of monoclonal B cells despite regression of splenic lymphoma was also reported by Hermine et al. (50). The fact that regression of monoclonal B cell expansion after eradication of HCV can be complete in cryo-II (7, 48, 49), but only partial in splenic lymphoma, may depend on a more profound disturbance of B cell homeostasis in the latter disorder, as suggested by Hermine et al. (50).

The ecology of lymphocyte subpopulations is tightly regulated (51). Cell transfer experiments in immunodeficient mice suggest that the homeostatic regulation of activated IgM-secreting and of resting B cells is autonomous (52). Thus, naive and memory B cells occupy different ecological niches, and their survival follows the first come, first served rule (52). Our results apparently contradict this concept, because clonally expanded B cells of cryo-II patients appear to compete with naive B cells and replace them in the circulating pool. In the murine model, however, when the transferred B cells have enhanced capacity to survive due to the enforced expression of bcl-2, there is a reduction of newly formed naive B cells in the peripheral B cell pool (52). At this regard, it is of interest that B cells expanded in cryo-II patients have been reported to translocate and overexpress bcl-2 (48). Elucidating the role of bcl-2 overexpression in the ecological success of monoclonal B cells in HCV-associated cryo-II deserves further investigation.

$CD5^+$ B cells have been reported to be increased in the peripheral blood of patients with chronic hepatitis C or HCV-associated cryoglobulinemia (53–56), but contrasting results have been reported (34, 57). At this regard, our finding that CD5 is expressed substantially only by $G6^+$ B cells at the early memory stage highlights the fact that the $CD5^+$ population is not representative of the whole B cell clone expanded in cryo-II. Functionally, CD5 has a negative regulatory action on BCR signaling that may serve to prevent the spreading of autoreactive cells (17). Thus, our findings suggest that its regulatory action may take place only in a limited window of differentation of $G6^+$ B cells.

A recent report described the phenotype of B cells in individuals with chronic HCV infection (57). Increased percentages of circulating B cells were observed in nearly 40% of patients and, surprisingly, mostly in those without detectable cryoglobulins. Even more intriguingly, expanded B cells had an $IgM^+IgD^+CD27^-$ naive phenotype, lacked activation markers, and had a reduced frequency of CD5 expression. It is difficult to reconcile these results with our results and with those of previous reports (53–56).

The findings presented here may help understanding the mechanisms for HCV-associated lymphomagenesis (4). The property of $G6^+$ B cells to escape the homeostatic mechanisms controlling Ag-driven clonal expansion provides a foreground for their evolution into overt lymphoid neoplasia. This outcome is caused by the accumulation of genetic anomalies, including trisomy 3q, during the phase of loosely controlled proliferation.

The Sjögren's syndrome represents another pathological model of the evolution from B lymphocyte activation to oligoclonal/monoclonal B cell expansion, which may culminate in the development of a malignant lymphoproliferative disease. Our finding of the identity of the CDR3 sequences from a cryo-II patient and from a Sjögren's syndrome patient supports the hypothesis of De Re et al. (35) that these two lymphoproliferative disorders share molecular characteristics and are possibly sustained by similar antigenic stimuli.

A pathogenetic mechanism similar to that of HCV-associated lymphoproliferative disorders appears to take place in gastric B cell lymphomas caused by *Helicobacter pylori*. In this condition, the protracted antigenic stimulation by *H. pylori* determines the evolution from benign, Ag-dependent clonal expansion of autoreactive B cells (58), to low- or high-grade lymphomas characterized by genetic anomalies including trisomy 3q (32). Thus, the capacity to escape mechanisms that control Ag-driven clonal expansion may be a general property of human B cells producing polyreactive natural Abs that facilitate their neoplastic transformation.

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Disclosures

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