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Original Articles

Monoclonal B Cell Lymphocytosis in Hepatitis C Virus Infected Individuals

Claudia Fazi,[1] Antonis Dagklis,[1] Francesca Cottini,[1] Lydia Scarfò,[1] Maria Teresa Sabrina Bertilaccio,[2] Renato Finazzi,[3] Massimo Memoli,[4] and Paolo Ghia[1,5,6*]

[1]Laboratory of B cell Neoplasia, Division of Molecular Oncology, Istituto Scientifico San Raffaele, Milan, Italy

[2]Laboratory of Lymphoid Malignancies, Division of Molecular Oncology, Istituto Scientifico San Raffaele, Milan, Italy

[3]Surgical Liver Unit, Istituto Scientifico San Raffaele, Milan, Italy

[4]Medical Liver Unit, Istituto Scientifico San Raffaele, Milan, Italy

[5]Unit of Lymphoid Malignancies, Department of Clinical Oncology, Istituto Scientifico San Raffaele, Milan, Italy

[6]Università Vita-Salute San Raffaele, Milano, Italy

Background: Monoclonal B cell lymphocytosis (MBL) is a preclinical condition characterized by an expansion of clonal B cells in the absence of B lymphocytosis (BALC < 5 × 10^9/L) in the peripheral blood, without clinical signs, suggestive of a lymphoproliferative disorder. B cell clonal expansions are also associated with hepatitis C virus (HCV) infection and they can evolve into lymphoproliferative disorders such as mixed cryoglobulinemia and non-Hodgkin lymphomas (NHL). The relationship between MBL and HCV infection has not been established yet.

Methods: By five-colour flow cytometry, we analyzed 123 HCV positive subjects with diagnosis of chronic hepatitis (94) or cirrhosis (29); 16 of those with cirrhosis had a diagnosis of hepatocellular carcinoma.

Results: MBL were identified in 35/123 (28.5%), at significantly higher frequency than in the general population. Sixteen/thirty-five were atypical-chronic lymphocytic leukemia (CLL) MBL ($CD5^+$, $CD20^{bright}$), 13/35 were CLL-like MBL ($CD5^{bright}$, $CD20^{dim}$), and 6/35 were $CD5^-$ MBL. Twenty-four/ninety-four (25.5%) patients affected by chronic hepatitis had MBL, whereas 11/29 (37.9%) patients with cirrhosis showed a B cell clone. A biased usage of IGHVgenes similar to HCV-associated NHL was evident.

Conclusions: All three types of MBL can be identified in HCV-infected individuals at a higher frequency than in the general population, and their presence appears to correlate with a more advanced disease stage. The phenotypic heterogeneity is reminiscent of the diversity of NHL arising in the context of HCV infection. The persistence of HCV may be responsible for the dysregulation of the immune system and in particular of the B cell compartment. © 2010 International Clinical Cytometry Society

Key terms: MBL; CLL; HCV

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Hepatitis C virus (HCV) is a well-known causative agent of chronic hepatitis C, liver cirrhosis, and hepatocellular carcinoma (HCC), and it is estimated that more than 170 million people worldwide are infected by this virus (1,2). In Italy the age-adjusted prevalence is 4.4% and follows a North–South gradient with a prevalence of 1.6% in the North and 7.3% in the South. Besides being a hepatotropic virus, HCV is also lymphotropic and its infection affects the B lymphocytes compartment, with the occurrence of B cell proliferative disorders. Accord-

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*Correspondence to: Paolo Ghia, Università Vita-Salute San Raffaele, c/o DIBIT 4A3, Via Olgettina 58, 20132, Milan, Italy.

E-mail: ghia.paolo@hsr.it

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ingly, HCV infection is strongly associated with mixed cryoglobulinemia (MC) (3), a benign disorder characterized by the proliferation of B lymphocytes producing polyclonal IgG or monoclonal IgM with rheumatoid factor (RF) activity (4) that characteristically may precipitate at low temperatures. This condition evolves in around 10% of the patients into an overt lymphoma (5,6). In addition, HCV has been suggested to play a role in the pathogenesis of B cell non-Hodgkin lymphomas (NHL) also outside the context of MC (7–9) as several distinct types of NHL can be associated to HCV infection, including diffuse large B cell-lymphoma (DLBCL) (10), marginal zone lymphoma (splenic —SMZL—, nodal and extranodal) (11–13), chronic lymphocytic leukemia (CLL) (14,15), and lymphoplasmacytic lymphoma.

Clinical data support this relationship between HCV and B cell proliferative disorders (16,17) as also demonstrated by the fact that antiviral therapy may lead to regression of at least a portion of HCV-related lymphomas (18).

In addition to overt clinical manifestations and frank B cell malignancy, HCV affects more generally the B cell compartment in infected individuals, leading to the appearance of monoclonal B lymphocytes in the blood, bone marrow, and liver as revealed by the detection of monoclonal immunoglobulin heavy chain (IGH) gene rearrangements in all these tissues (19–23).

The characterization of the preclinical B cell expansions has been limited, and contrasting results are present in the literature. Some studies showed that in the course of HCV infection, B cell clones were mainly $CD5^+$ (4,24), although this has not been confirmed by others (25,26). $CD5^-$ B cells have been reported to produce monoclonal IgM in MC (13,27,28).

Recently it has been reported that monoclonal B lymphocytes can be frequently found (3.5–12% depending on the technique used) circulating in the peripheral blood of otherwise healthy individuals, increasing with age and irrespective of HCV infection (29–31). This condition is named monoclonal B cell lymphocytosis (MBL) and is associated with an elevated risk of developing a clinically overt disorder, mainly CLL (32). However, because of the high prevalence and the association with a more advanced age, the possibility exists that MBL may be a mere manifestation of senescence of the immune system. This may be associated with a persistent antigenic stimulation as it may occur following the prolonged exposure to autoantigens or to infectious agents as it has been demonstrated for the T cell monoclonal expansions, frequently appearing in the elderly (33). Such expansions have been associated with persistent viral infections such as CMV and HCV, in the case of $CD8^+$ and $CD4^+CD8^+$ T cells, respectively (34–36).

MBL are rather heterogeneous in terms of phenotype (30,37) and they are classified on the basis of the presence or absence of the CD5 antigen and the expression levels of CD20 on the cell surface. Cases that do not express CD5 are named $CD5^-$ MBL, those expressing CD5 are subdivided into atypical-CLL (with bright CD20 expression) and CLL-like (with low levels of CD20) MBL (30).

In this work, we investigated the presence and analyzed the phenotype of MBL clones circulating in the peripheral blood of 123 HCV-infected individuals presenting with different degrees of hepatic disease (chronic hepatitis, liver cirrhosis, HCC). The results presented here will help to further define the relationship between HCV infection and B cell expansions and the cellular basis of the risk to develop clinically relevant B cell lymphoproliferations (MC and B-NHL).

METHODS

Study Population

From January 2006 to June 2009, we enrolled 125 patients positive for antibodies to HCV and serum HCV-RNA from the Medical Liver Unit and Surgical Liver Unit of San Raffaele Hospital, Milano, but we excluded two subjects as they were affected with NHL, leading to a total of 123 patients analyzed for the present study.

The research protocol was approved by the Institutional Ethics Committee at our institution, and all participants gave written informed consent in accordance with the Declaration of Helsinki.

Subjects were Caucasians except for 13 patients of various origins. Patients' group was composed of 76 males and 47 female with a mean age of 53 years (range 22–87). Of the 123 subjects, 94 had chronic hepatitis (median age 46 years) and 29 had diagnosis of cirrhosis (median age 70 years) based on clinical, histological, or imaging data. Among cirrhotic patients, 16 had a diagnosis of HCC (median age 71 years). HCV genotypes were available for 90 patients and reflected the regional distribution, with genotypes 1 and 2 mainly expressed by Caucasians and genotype 4 present mainly in North Africans. All patients tested positive for antibodies to HCV and serum HCV-RNA.

All patients were screened also for HBV infection, and two resulted positive. One of them was positive also for HIV and presented an MBL clone.

Leukocyte cell counts in the entire population had a mean value of 6.532×10^9/L (range $1.9–12.4 \times 10^9$/L) and the mean absolute lymphocyte count (ALC) was 2.137×10^9/L, ranging from 0.248 to 5.630×10^9/L, with four cases of lymphocytosis ($>4.0 \times 10^9$/L).

As controls, we considered a group of 1,725 healthy individuals previously described (38), analyzed with the same protocol and during the same time period, composed of 967 women and 758 men with a mean age of 55.2 years (range, 18–102 years) and a mean lymphocyte cell count of 2.2×10^9/L.

Blood Samples, Cell Preparation, Staining, and FACS Analysis

EDTA (ethylenediaminetetraacetic acid) peripheral blood (PB) samples obtained from all individuals enrolled were processed within 24 hours after blood withdrawal and incubated 30 minutes with phosphate-buffered saline (PBS) plus 10% fetal bovine serum to remove nonspecific binding. Samples were then

incubated with the proper antibodies, followed by NH_4Cl (8.3 g/L in distilled water) and washed with PBS. The following antibody mix was used: fluorescein isothiocyanate-conjugated F(ab)2-anti-κ, phycoerythrin (PE)-anti-λ light chain (Dako Cytomation, Carpinteria, CA), PE-cyanin7 (Cy7)-labeled anti-CD20 (Beckman Coulter, Miami, FL), PE-cyanin5 (Cy5)-conjugated anti-CD5 (Beckman Coulter), and PE-Texas Red (ECD)-conjugated anti-CD19 (Beckman Coulter). For each sample, up to 500,000 events were acquired on a FC500 (Beckman Coulter) equipped with 488 argon ion laser and 635 red HeNe laser and analyzed with the CXP software system (Beckman Coulter) according to the following gating strategy: low forward and side scatter (FSC/SSC) $CD19^+$ cells were gated and further divided into $CD5^-$ and $CD5^+$ subsets, and the κ/λ ratio was evaluated in both populations (indicating the presence of atypical-CLL or $CD5^-$ MBL) (30). The κ/λ ratio was considered abnormal when it was more than 3:1 or less than 1:3. Concomitantly, we used a dot plot showing CD5 versus CD20 expression, gated on $CD19^+$ cells, to identify CLL-like MBL (e.g. $CD19^+$cells, $CD20^{dim}$, $CD5^{bright}$) (38).

For quality control purposes, we daily used 0.4 mL flow-check fluorospheres (Beckman Coulter) mixed with 0.2 mL flow-check 770 (Beckman Coulter PC7 (770/488) Setup Kit) to assess flow cytometer optical alignment and fluidics system. In addition, we daily controlled light scatter intensity, fluorescence intensity, and hydrodynamics using 0.4 mL flow-set fluorospheres (Beckman Coulter) mixed with 0.2 mL flow-set 770 (Beckman Coulter PC7 (770/488) Setup Kit), to assess optimal conditions for quantitative analysis of human leukocytes.

PCR Amplification of IGHV-D-J Rearrangements and Sequence Analysis

DNA was extracted from 35 whole blood samples using the QIAmp blood kit (Qiagen, Hilden, Germany). Two different forward primers and two different PCR protocols were followed to confirm monoclonality. In particular, the upstream primer was complementary to IGHV framework region 1 (FR1) or IGHV framework region 2 (FR2), whereas the downstream primers were reverse complementary to the 3′ untranslated region (3′ UTR) of the corresponding genes. To increase sensitivity, we performed a seminested PCR approach using consensus IGHJ primers and ran the samples on a polyacrylamide gel (PAGE 6%). PCR products were excised from 3% low melting agarose gel and purified using the Gel extraction kit (Qiagen). Finally, PCR products were subjected to sequencing on an automated ABI sequencer with the same primers used in the PCR amplifications.

Sequence data were analyzed using the ImMunoGeneTics information system (IMGT) or NCBI immunoglobulin database and tools (39). The sequences with identity <100% were considered mutated, whereas sequences with 100% identity were considered unmutated.

RESULTS

All Types of MBL Are Frequent Among HCV Infected Patients

We observed the presence of a B cell clone in 35 patients (35/123, 28.5% of the entire HCV positive population) belonging to one of the three types of MBL, differentiated by CD5 and CD20 expression (Table 1 and Fig. 1). In HCV infected patients, all three kinds of MBL were significantly more frequent ($P < 0.001$) than in the control group of healthy subjects (38), and atypical-CLL MBL accounted for the plurality of cases (Table 1 and Fig. 2). In particular, 16 patients carried atypical-CLL MBL ($CD5^+CD20^{bright}$, 13.0% of all individuals, 45.7% of all MBL), 13 patients showed CLL-like MBL ($CD5^{bright}CD20^{dim}$, 10.6% of all individuals, 37.1% of all MBL), and six patients (4.9% of all individuals, 17.1% of all MBL) presented $CD5^-$ MBL (Table 1 and Fig. 2).

In the control group of healthy subjects, previously described by Dagklis et al (38), MBL clones were present in 128/1725 individuals (7.4%) of which 19 were atypical-CLL MBL cases (1.1% of all individuals, 14.8% of all MBL), 89 were CLL-like MBL cases (5.2% of all individuals, 69.5% of all MBL), and 20 were $CD5^-$ MBL cases (1.1% of all individuals, 15.6% of all MBL) (Fig. 2).

The median age of all individuals with MBL in HCV patients group was 55 years (range, 24–82 years) and the frequency was the same in males and females (14.6% males and 13.8% females of all individuals, 51.4% and 48.6%, respectively of all MBL).

MBL Frequency Increases with Age and the Severity of Disease

MBL clones in HCV positive population were present in 24.4% of subjects below 65 years of age, and the frequency increased to 37.8% in the individuals >65 years. Interestingly, while in healthy individuals <40 years MBL was virtually absent (1.75%) (38), it was found in 9.7% of HCV-infected subjects of the same age group. In the group of individuals aged 40–60, the controls presented MBL in 4.5% of cases whereas the percentage in HCV patients was 29.8%. Finally, among subjects older than 60 years, MBL was present in 12.5% of controls and in 38.6% of HCV samples (Fig. 3).

All 35 HCV-infected patients with MBL had a normal ALC [mean value 1.96×10^9/L (1,967/μL); range, 0.2–3.9×10^9/L (248–3,900/μL)] except for one case whose lymphocyte count was slightly above the 4.0×10^9/L value (4.151×10^9/L). The mean absolute B lymphocyte count (BALC) was 0.21×10^9/L (206/μL); range, 0.006–0.8×10^9/L (6–858/μL).

Monoclonal B cells represented a variable proportion of total $CD19^+$ B cells with a mean of 24.4% (0.3–100%), and it reached virtually 100% of all B lymphocytes in four $CD5^-$ MBL cases. $CD5^+CD19^+$ B lymphocytes had a mean value of 0.032×10^9/L (range 0.64–270/μL), representing 14.4% of all B cells (range 1.8–52.7%), which was not different from the control

Table 1
MBL Characteristics in HCV-Infected Patients

Data	$CD5^-$ MBL ($n = 6$)	Atypical-CLL MBL ($n = 16$)	CLL-like MBL ($n = 13$)	Non-MBL ($n = 88$)
Age, in years, median (range)	51 (44–71)	57.5 (24–82)	64 (36–77)	50 (22–87)
Gender (M:F ratio)	2 M and 4 F	9 M and 7 F	7 M and 6 F	58 M and 30 F
Clinical Status	5 chronic hepatitis	11 chronic hepatitis	8 chronic hepatitis	73 chronic hepatitis
	1 cirrhosis with HCC	5 cirrhosis, 4 with HCC	5 cirrhosis, 4 with HCC	8 cirrhosis, 7 with HCC
WBC (/μL)	6,733 ± 4,957	6,744 ± 2,839	6,360 ± 2,361	6,511 ± 2,268
Lymphocytes (/μL)	962 ± 696	2,190 ± 1,111	2,130 ± 966	2,255 ± 1,076
Lymphocytosis (>4.0 × 10^9/L)	None	1 case (4.151 × 10^9/L)	None	3 cases (4.598 × 10^9/L; 5,630 × 10^9/L; 4,536 × 10^9/L)
$CD19^+$B lymphocyte count (/μL)	104 ± 76	248 ± 250	169 ± 240	146 ± 159
$CD19^+$B lymphocytes (%)	9.4 ± 2.4	11.0 ± 6.8	9.9 ± 11.6	6.3 ± 4.8
$CD5^+$ $CD19^+$ B lymphocytes (/μL)	16 ± 17	27 ± 25	46 ± 81	286 ± 199
$CD5^+CD19^+$ B lymphocytes (%)	10.8 ± 5.6	13.7 ± 11.8	16.8 ± 11.6	12.5 ± 7.18
Monoclonal B cell count (/μL)	97 ± 82	31 ± 33	62 ± 192	
Monoclonal B cells (%)	91.8 ± 15	13.4 ± 11.7	6.9 ± 19.8	
Light chain expression	5 κ,1 λ	15 κ,1 λ	5 κ,5 λ, 3 policlonal	

population where the mean value was 0.030 × 10^9/L (range 0.42–341/μL), representing 17.2% (range 3.7–84.9%) of all B lymphocytes (38).

Neither the ALC nor BALC of all MBL samples in the HCV infected patients were significantly different than those of MBL samples in the control population, considering all MBL cases or the different MBL types separately (data not shown and (38)).

When we considered the presence of MBL clones based on the clinical presentation, among the individuals with chronic hepatitis 24 out of 94 (25.5%) carried an MBL clone whereas 11 of 29 (37.9%) cirrhotic patients, with or without HCC, had MBL cells circulating in the peripheral blood. This difference, though not statistically significant ($P = 0.374$), suggests a potential association with the severity of the disease.

Finally, 12 MBL cases presented a monoclonal gammopathy in the serum with or without a concomitant hypergammaglobulinemia. This finding was more common in cases showing atypical-CLL MBL than in other types of MBL as it was demonstrated in 7/16 atypical-CLL MBL, 4/13 CLL-like MB,L and 1/6 $CD5^-$ MBL.

IGHV Repertoire, Somatic Mutations, and HCDR3 Analysis in HCV-Infected MBL

Since previous studies have shown that an IGHV gene repertoire restriction in B cell lymphomas arises in the context of HCV infection (40,41), we used a PCR approach to study the IGHV-D-J rearrangements in the HCV-infected MBL cases (Table 2). We were able to amplify a monoclonal rearrangement in 10/35 samples (28.5%), as in the remaining cases the clonal rearrangement was admixed to a polyclonal IG background because of the small size of the clone. In one out of 10 positive cases, two in-frame rearrangements were obtained for a total of 11 sequences to be analyzed.

IGHV3 and IGHV4 subgroup genes were equally represented (5/10). At individual gene level, we identified IGHV genes that have been found in previous studies of HCV like IGHV3-48, IGHV3-23, IGHV3-7, and IGHV4-59, indicating that the IGHV gene repertoire in HCV^+ patients is restricted (2,21,41,42).

Based on the mutational load, 9/11 (81.8%) sequences analyzed harbored IGHV somatic mutations with a sequence identity to the closest germline gene ranging between 90.18–99.39%. Most of these cases (7/9) were heavily mutated and had a <97% identity with the closest germ line gene. The remaining two cases showed 100% identity and were thereby considered unmutated.

We then compared the IGH sequences obtained from our MBL to a series of sequences previously reported by De Re et al. (41), identified in HCV positive subjects with a diagnosis of NHL. Interestingly, in our case #HCV64 (Table 2), a 65-year-old woman affected by chronic hepatitis and an hypergammaglobulinemia with a monoclonal component in β2 region presented an atypical-CLL MBL (MBL cell count: 0.011 × 10^9/L) and expressed an IGH rearrangement utilizing the same

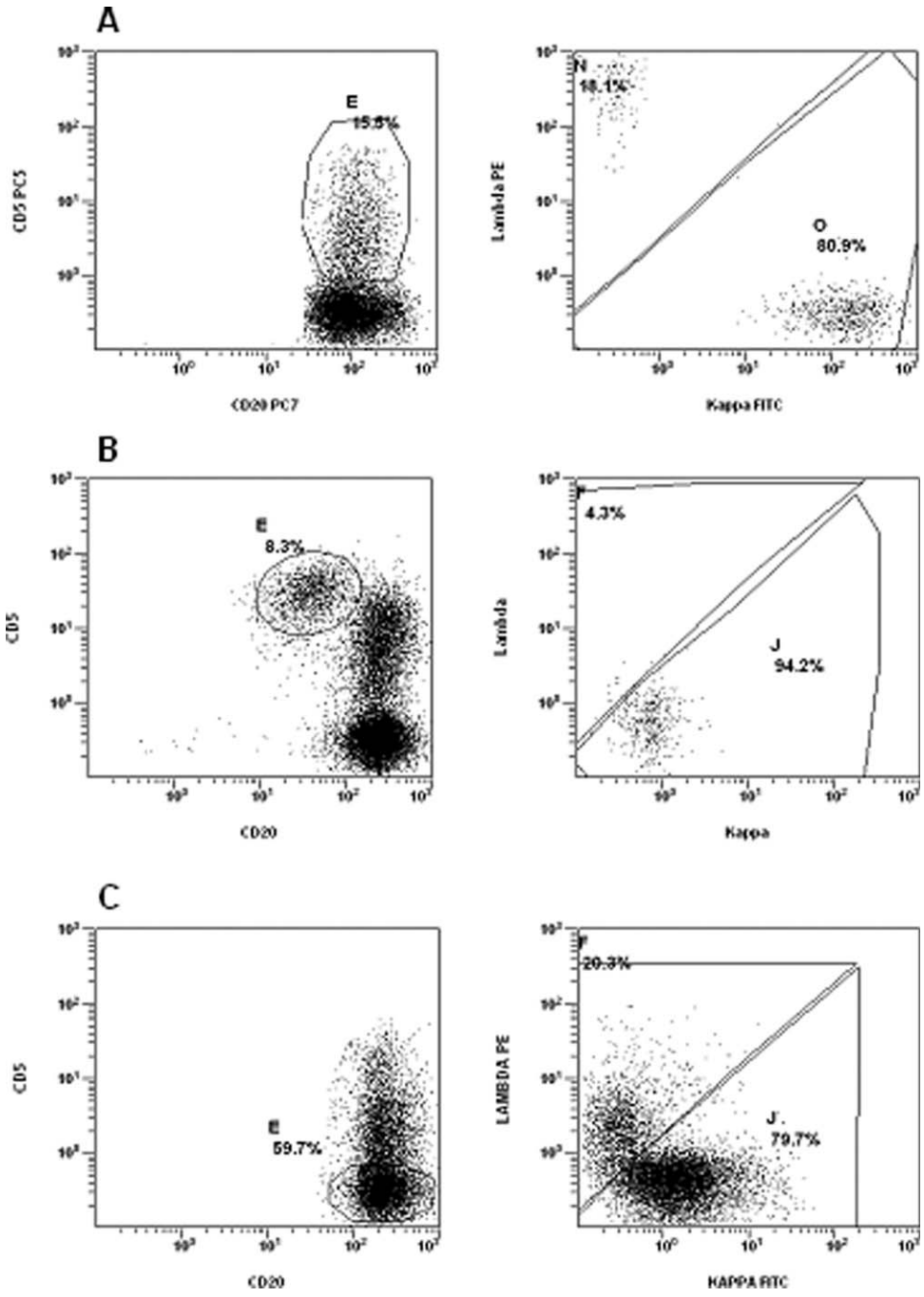

FIG. 1. All types of MBL could be detected in HCV infected individuals. **A**) Atypical-CLL; **B**) CLL-like; and **C**) $CD5^-$ MBL. For each of them, a representative CD5 versus CD20 dot plot is shown on the left, while the κ versus λ dot plots on the right (all gated through E, in the corresponding left panel) show an imbalance κ/λ ratio suggestive of monoclonality.

IGHV (IGHV3-7), IGHD (IGHD3-22), and IGHJ (IGHJ3) genes. In addition, this subject showed a 70% identity in the HCDR3 region by comparison with the immunoglobulin previously described in a DLBCL diagnosed in a 70-year-old woman affected by a HCV^+ cryoglobulinemia syndrome (41). This last rearrangement had a high sequence homology (63%) with an antibody known to have RF activity.

DISCUSSION

Using flow cytometric analysis, we investigated the presence and analyzed the phenotype of MBL clones circulating in the peripheral blood of 123 HCV-infected individuals presenting with different degrees of hepatic disease (chronic hepatitis, liver cirrhosis, HCC). We demonstrate that in HCV positive patients monoclonal B cells are present in around 30% of infected individuals

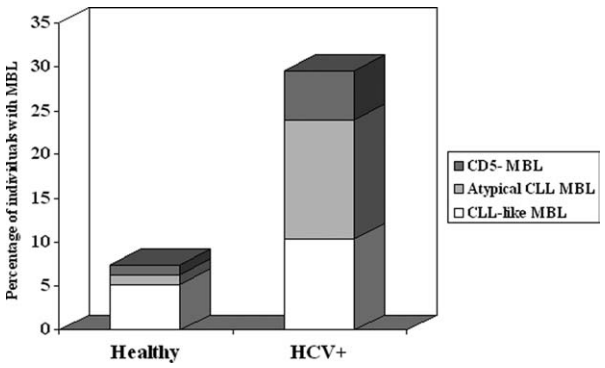

FIG. 2. All types of MBL are more frequent among HCV-infected patients as compared to healthy individuals. The columns represent the cumulative frequency of all three types of MBL (distinguished with different colors) in the general population (left column) and among HCV-infected individuals (right column), respectively. The increase in the frequency of atypical CLL (light grey) is particularly evident.

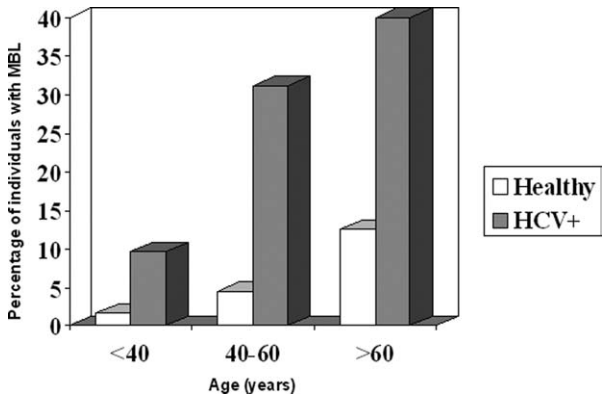

FIG. 3. MBL frequency increases with age in HCV-infected individuals. The columns represent the cumulative frequency of all three types of MBL (considered together regardless of the specific phenotype) in the general population (white bars) and among HCV infected individuals (grey bars), in different age groups.

and all three kinds of MBL (atypical-CLL, CLL-like, and $CD5^-$ MBL) are present at higher frequencies as compared with the control group. Interestingly, HCV infection appears to be associated with the presence of MBL irrespective of age, as even in young individuals (<40 years) the frequency is around 10%, while it is virtually irrelevant in healthy people.

Over 80% of all MBL cases were expressing CD5 antigen on the cell surface but, unlike healthy individuals where CLL-like MBL are by far the most common entity, in HCV-infected patients atypical-CLL MBL cases account for almost 50% of all cases (Table 1 and Fig. 2). That notwithstanding, also CLL-like MBL were significantly more frequent as compared with healthy individuals, though only two times the expected frequency and the increase in atypical-CLL MBL is definitely more striking being more than ten times higher.

This heterogeneity may help to explain the discrepancies present in the literature regarding the phenotype of B cells clones in HCV infected individuals (4,24–28). Accordingly, also a sizeable fraction of $CD5^-$ MBL do exist in HCV infected patients. In addition, this heterogeneity would also explain the wide spectrum of the lymphoproliferative disorders arising in association with HCV infection, ranging from typical $CD5^-$ lymphomas, as DLBCL to MZL, which may or may not express CD5 on the cell surface, to characteristic $CD5^+$ diseases such as CLL and its association with MC.

The MBL frequency appeared to correlate with advanced hepatic disease as it was higher in individuals with cirrhosis as compared with those with chronic hepatitis, thereby suggesting that the persistency of the viral infection may be critical for the onset of single B cell clones. We performed our study in the peripheral blood, but the phenomenon is expected to be even more frequent in the involved tissues, like the liver, as previously indicated (21).

Some investigators have suggested that the crosslinking between the HCV envelope protein E2 and the B cell surface molecule CD81 (43) may be crucial for the B cell stimulation. Experimental ligation of HCV particles to CD81, a fairly ubiquitary tetraspanin present on both hepatocyte and B-cell surface, lowers the threshold for B-cell activation and proliferation and induces hypermutation of IGHV genes in antigen reactive B-cells (44,45). Accordingly, virtually all IGH rearrangements obtained from our HCV-infected MBL were mutated and most had a high mutational load.

Table 2
IGH Rearrangements in HCV-Infected MBL

	MBL type	IGHV	IGHD	IGHJ	Homology	HCDR3 a.a.
HCV 2	Atypical-CLL	IGHV4-30-2	IGHD5-5	IGHJ4	100%	CATGGRLWLDYW
HCV 2	Atypical-CLL	IGHV3-23	IGHD5-5	IGHJ4	100%	CASLQLWLNYFDYW
HCV 5	Atypical-CLL	IGHV4-39	IGHD3-10	IGHJ5	98.2%	CAISPGGAAHWFDPW
HCV 20	Atypical-CLL	IGHV4-34	IGHD4-4	IGHJ2	96.9%	CARGRYDYSNYQYWYFDLW
HCV 23	Atypical-CLL	IGHV4-59	IGHD4-17	IGHJ5	94.3%	CACEVTPGSNWLDPW
HCV 64	Atypical-CLL	IGHV3-7	IGHD3-22	IGHJ3	96.9%	CARGDYSDSSGYYIEAFDVW
HCV 14	CLL-like	IGHV4-59	IGHD2-21	IGHJ4	95.0%	CARSQGVLTAIDYW
HCV 41	CLL-like	IGHV3-7	IGHD3-10	IGHJ3	90.2%	CARGDYHDGGNSFIDAFDIW
HCV 6	Non-CLL	IGHV3-74	IGHD5-5	IGHJ5	92.0%	CVRELSINGYFERGWFDSW
HCV 17	Non-CLL	IGHV4-39	IGHD2-21	IGHJ4	91.2%	CARHGGGMASAFDYW
HCV 53	Non-CLL	IGHV3-48	IGHD2-21	IGHJ6	97.5%	CARDTCGGDCSLYTVYYYYMDVW

The latter features suggest that monoclonal B cell expansions in HCV^+ patients are derived from mature antigen experienced B-cells, which had participated in a germinal center reaction and have undergone antigenic selection. Along the same line, a restriction in IGHV repertoire in patients with HCV-related benign or malign lymphoproliferative disorders has been shown, including a preferential usage of the IGHV1-69 gene in type II MC, with RF activity as well as a biased usage of distinct IGHV genes in HCV related-lymphomas: IGHV3-30 and IGHV3-23 in SMZL (40), IGHV3-07, IGHV3-23, or IGHV4-59 in DLBCL and MZL (41). Similarly, a κ dominance (25/35 overall; 20/22 in the $CD5^-$ and atypical CLL types of MBL) was also evident, as previously demonstrated in other MBL studies (30,46,47), further supporting the hypothesis of a selective Ag-driven pressure.

In our present work we demonstrate a similar usage of IGHV genes also at the MBL level, confirming a potential ontogenic relationship between the appearance of MBL and the occurrence of NHL and the potential identity of the antigenic elements responsible for the onset of both conditions. The presence of an IG rearrangement, very similar to another one observed in a frank lymphoma (41), is also very suggestive of this scenario. The identity of the antigen binding to the surface immunoglobulin involved in the expansion of monoclonal B lymphocytes is still a matter of research (4). Though theoretically this might be of viral origin, it is likely that it may derive from the microenvironment (e.g. autoantigens) as suggested by the MC findings.

Finally, the appearance of monoclonal B lymphocytes in HCV infected patients closely recalls the monoclonal expansions of $CD4^+CD8^+$ T lymphocytes occurring in the context of HCV infection, as shown in primates (36). These cells represent a negligible subset among circulating T cells but they are hypothesized to take part in the adaptive immune response against infectious pathogens especially those persistent.

No similar function has been reported for $CD5^+$ B cells, but it is tempting to speculate that also the occurrence of MBL in HCV-infected individuals may be expression of a similar physiological antiviral response that can expand progressively with time, likely because of the persistency of the virus.

Further molecular dissection of MBL in the blood of HCV positive patients will serve to elucidate the biological mechanisms, leading to a persistent expansion and, eventually, to neoplastic transformation. This may help to devise better tools to redirect the immune response toward the viral clearance and/or the prevention of the progression into overt lymphomas.

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