different histotypes, 9 of them complicating the course of MC syndrome. The variable heavy (V_H) and variable light (V_L) IgR chains were sequenced, and the amino acid sequences deduced from the complementarity-determining regions (CDR3) were compared with those of antibodies with a known specificity, including anti-HCV antibodies. To ascertain the occurrence of an antigen-driven stimulus in the pathologic process, IgR sequences were compared with their germline counterparts, and the replacement-(R)-to-silent (S) mutation ratio was calculated. Intraclonal variation analysis was also performed to ascertain the persistence of antigenic stimulation after neoplastic transformation. Finally, structural differences in IGR genes between MC syndrome–related and MC syndrome–unrelated NHLs were determined to understand the molecular basis of the lymphoproliferative disorders in different clinical settings.

Materials and methods

B-cell non-Hodgkin lymphomas in individuals infected with hepatitis C virus

Seventeen selected patients (12 women and 5 men; mean age, 65 years; range, 49-76) with overt B-cell NHL of recent onset, all infected with HCV, were studied (Table 1). Informed consent was obtained from all patients according to the Helsinki declaration. All were seronegative for human immunodeficiency virus and heterosexual, with no history of intravenous drug abuse. Sera obtained at disease diagnosis were all positive for anti-HCV antibodies (by the enzyme-linked immunosorbent assay [HCV 3.0; Ortho Diagnostic Systems, Raritan, NJ] and the recombinant-based immunoblot assay [Chiron RIBA; Ortho Diagnostic Systems]) and for HCV RNA.[3] Overt lymphoma was revealed by the onset of clinical manifestations previously absent and suggesting a neoplastic evolution (B-cell–related symptoms, lymph node enlargement, organomegaly, organ nodular lesions, and others) detected on physical examination or imaging investigation. Bone marrow biopsy was performed in all patients at the time of NHL diagnosis, along with extensive staging procedures, as described previously.[3]

The association between B-cell NHL and previous MC syndrome in these patients was investigated by a clinical expert (S.D.). An accurate clinical history was obtained followed by a complete physical examination. All the laboratory and instrumental tests performed in the years preceding NHL onset were reviewed. Symptoms and signs of MC such as purpura, asthenia, arthralgia, renal involvement, and peripheral neuropathy were investigated, as well as serum cryoglobulin, C3 and C4, and rheumatoid factor (RF) levels. Symptoms and signs of MC were also investigated at the time of onset of NHL.

Nine patients with overt B-cell NHL had a previous history of open MC syndrome (all with type II serum cryoglobulins and positive serum RF); the remaining 8 patients definitely lacked clinical features of MC syndrome both in the previous clinical history and at the time of NHL onset. Serum cryoglobulins were detected in 7 of these latter 8 patients (type II in patient 13; type III in patients 10, 11, 14, 15, 16, 17; not detected in patient 12) and positive serum RF in 7 patients (negative in patient 17).

Histopathology

Tissues were fixed in Bouin solution or neutral buffered formalin. In a few cases, a portion of unfixed tissue was snap frozen in liquid nitrogen and stored at $-80°C$. Pathologic specimens were classified according to the revised European-American classification of lymphoid neoplasms.[20]

Deparaffinized and cryostat sections were used for immunophenotyping and lineage assignment of lymphoma cases with monoclonal antibodies (CD3, CD4, CD5, CD8, CD9, CD10, CD15, CD19, CD20, CD21, CD22, CD24, CD30, CD38, CD43, CD45, CD45RA, CD45R0, CD68, CD74, CDw75, LN3, MB2, DDBB42, DBA44, OPD4, DRC-1, Leu8, anti-κ and λ immunoglobulin (Ig) light chains, epithelial membrane antigen [EMA], vimentin, and cytokeratin [MNF116]). Immunohistochemistry was performed with the avidin-biotin-peroxidase complex (ABC-px) or alkaline phosphatase antialkaline phosphatase (APAAP) methods, as previously described.[21,22]

Polymerase chain reaction (PCR) amplification and sequencing of V_H and V_K gene regions

Total DNA was isolated from biopsy specimens by phenol-chloroform extraction according to standard protocols. DNA was analyzed for B-cell clonal expansion using the seminested third framework (FR3) protocol of amplification, where the upstream primer is directed to the FR3 variable (V) region and the downstream primer is directed to the joining (J) region of the IGH gene.[23] PCR products were analyzed on 10% polyacrylamide gels stained with ethidium bromide. The single-band PCR product from an amplified DNA sample was subjected to direct DNA sequencing on an ABI 310 Genetic Analyzer (Perkin Elmer, Foster City, CA) using the dye-terminator protocol.[16]

A clonospecific antisense oligoprimer was then synthesized based on the CDR3 DNA sequence and used as a downstream primer paired with each V_H family-specific framework 1 primer (FR1 protocol).[24] The amplified PCR fragments were purified from the gel and directly sequenced. All of the sequences were confirmed by sequencing twice in both directions.

Table 1. Histologic parameters in patients with NHL and HCV infection

Patient no., age, sex	Histologic diagnosis (REAL classification)	Site of pathologic diagnosis	Other sites of pathologic and/or clinical NHL involvement	Presence of overt MC syndrome
1. 60, F	Follicle center, follicular	Bone marrow	—	Yes
2. 58, F	Extranodal marginal zone	Submandibular gland, parotid gland	Bone marrow	Yes
3. 63, F	Small lymphocytic	Skin, liver, lymph node	Bone marrow	Yes
4. 73, M	Diffuse large cell	Lymph node	—	Yes
5. 70, F	Diffuse large cell	Lymph node, liver	Bone marrow	Yes
6. 66, F	Extranodal marginal zone	Stomach	—	Yes
7. 49, M	Small lymphocytic	Bone marrow	Liver	Yes
8. 76, F	Diffuse large cell	Parotid gland	—	Yes
9. 61, M	Lymphoplasmacytoid	Bone marrow	Spleen	Yes
10. 57, M	Diffuse large cell	Lymph node	—	No
11. 69, M	Diffuse large cell	Lymph node	Bone marrow	No
12. 72, F	Extranodal marginal zone	Stomach	—	No
13. 66, F	Diffuse large cell	Skin	Bone marrow	No
14. 69, F	Extranodal marginal zone	Submandibular gland	Bone marrow	No
15. 65, F	Diffuse large cell	Lymph node	—	No
16. 71, F	Extranodal marginal zone	Spleen	Bone marrow	No
17. 59, F	Mantle cell	Lymph node	Bone marrow, spleen	No

The VL gene region was amplified using V_K family-specific primers and a mixture of J_K primers.[14] PCR products were analyzed on 3% agarose gel stained with ethidium bromide. The single-band PCR product was then subjected to direct DNA sequencing.

The amino acid sequences deduced from the V_H, V_K, and CDR3 DNA sequences were analyzed by the NCBI Basic Local Alignment Search Tool (BLAST) program (see http://www.ncbi.nlm.nih.gov/blast/blast.cgi?form=0) for homology to multiple protein sequence databases. The protein sequences closely related to the given V_H, V_K, and CDR3 sequences were scored, starting from the most homologous one, with rigorous statistical analysis to assess the significance of the matches.

The identification of V_H, V_K, and CDR3 Ig germline sequences was performed by sequence comparison with the International Immunogenetics Database program on the Internet (http://imgt.cnusc.fr:8104/dnaplot/).

Analysis of mutations

Calculation of the number of expected R mutations in IGR gene CDRs or FRs was based on a computer algorithm developed by Chang and Casali.[25] This algorithm calculates the inherent susceptibility to amino acid replacement given any single nucleotide change. The number of expected R mutations was calculated as follows: $n \times$ (CDR Rf or FR Rf) $\times$ (CDRrel or FRrel), where n = total number of observed mutations, Rf = replacement frequency inherent to CDR or FR sequences, and CDRrel or FRrel = relative size of the CDRs or FRs.

The probability (P) that excess or scarcity of R mutations in the IGR gene CDRs or FRs, observed in the predominant clone, was due to chance only was calculated using the binomial distribution model, as reported by Chang and Casali[25] as follows: $P = \{n!/[k!(n-k)!]\} \times q^k \times (1 - q)^{n-k}$, where n = total number of observed mutations, k = number of observed R mutations in the CDRs or FRs, and q = probability that an R mutation localizes to CDRs or FRs (q = $CDR_{rel} \times$ CDR Rf or $FR_{rel} \times$ FR Rf).

Intraclonal variation analysis

Electrophoretically purified V_HDJ_H PCR fragment products were ligated into a pGem-T vector (Promega, Madison, WI) and transfected into *Escherichia coli* DH5α competent cells.[16] Clones were picked up at random and DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega). Sequence reactions were performed on the ABI 310 Genetic Analyzer (Perkin Elmer) and confirmed by sequencing twice in both directions.

Results

Tumor histopathology

According to the REAL classification, 7 of the 17 HCV-positive patients (41%) presented with diffuse large B-cell lymphoma; extranodal marginal zone lymphoma was observed in 5 patients (29%); small lymphocytic lymphoma occurred in 2 patients (12%); and lymphoplasmacytoid lymphoma/immunocytoma, follicle center lymphoma, and mantle cell lymphoma were seen in 1 patient each (Table 1).

Analysis of R/S mutation ratio in VH and VL genes

The complete nucleotide sequence of the V_HDJ_H rearrangements was obtained for all 17 patients (data not shown). The complete nucleotide sequence of the V_KJ_K rearrangements was obtained in 12 patients (data not shown), whereas the gene family only was determined in patients 8 and 15 due to inefficient sequencing. The inability of sequencing the V_KJ_K gene rearrangement in patients 6, 9, and 17 was due to scarcity of DNA from small biopsy samples.

In the FR regions, the R/S mutation ratio was significantly lower than expected by chance alone ($P = .05$) in most of the VH genes (13 of 17 patients) (Table 2) and VK genes (11 of 12 patients), indicating a selective pressure for maintenance of a functional IgR (Table 3).

In the CDR regions, all of the sequences (except the VK gene of patient 10) showed a lower R/S mutation ratio than expected by chance alone. However, the R/S mutation ratio values were not statistically significant (Tables 2 and 3).

Intraclonal variation in non-Hodgkin lymphomas

Intraclonal variation was analyzed in 8 NHLs, 5 of which were associated with MC syndrome (patients 1, 2, 6, 7, and 9) and 3 not so associated (patients 11, 12, and 16), by cloning the V_HDJ_H PCR-amplified product. A minimum of 9 randomly selected clones for each NHL were sequenced. Intraclonal variation was observed in all cases analyzed, although with low level of nucleotide substitution between clones (data not shown, except for patients 1 and 11). The number of distinct subclones ranged from 7 of 10 in patient 1 to 5 of 9 in patient 11 (Figure 1).

Table 2. Distribution of mutations in V_H genes of HCV-associated NHLs

Patient no.	V_H family\gene	R/S mutation ratio in FRs			R/S mutation ratio in CDRs		
		Observed (R:S)	Expected	P	Observed (R:S)	Expected	P
1	VH1\DP-88	4.000 (4:1)	3.297	**.04387**	∞ (3:0)	3.028	.19795
2	VH1\DP-88	0.833 (5:6)	3.297	**.00020**	0.750 (3:4)	3.028	.21336
3	VH1\DP-10	1.000 (5:5)	3.306	**.00074**	2.000 (4:2)	3.028	.20844
4	VH3\DP-47	0.667 (2:3)	3.264	**.00764**	0.500 (1:2)	3.792	.36656
5	VH3\DP-54	0.666 (4:6)	3.264	**.00433**	∞ (2:0)	5.091	.23696
6	VH3\DP-54	0.400 (2:5)	3.226	**.00061**	1.500 (3:2)	5.091	.24162
7	VH4\DP-71	∞ (1:0)	3.000	0.29193	∞ (1:0)	3.704	.34102
8	VH3\DP-51	3.000 (6:2)	3.265	**.00160**	3.000 (6:2)	4.875	.10878
9	VH1\DP-75	4.000 (4:1)	3.686	0.10068	∞ (1:0)	3.152	.31985
10	VH3\DP-54	1.154 (15:13)	3.198	**9.81×10^{-7}**	5.000 (5:1)	5.091	.16141
11	VH3\DP-47	2.400 (12:5)	3.548	**.000204**	∞ (10:0)	3.230	.06237
12	VH4\DP-71	1.200 (6:5)	2.870	**.01760**	∞ (4:0)	3.310	.19803
13	VH4\DP-63	1.143 (8:7)	3.129	**.00333**	∞ (2:0)	3.483	.13081
14	VH3\DP-49	1.000 (7:7)	3.171	**.00202**	∞ (3:0)	4.000	.19038
15	VH3\DP-54	∞ (4:0)	3.226	.07310	∞ (3:0)	5.091	.20099
16	VH1\DP-8	0.600 (3:5)	3.713	**.01920**	0.000 (0:0)	3.152	.13103
17	VH3\DP-51	1.000 (1:1)	3.265	.29014	0.000 (0:0)	4.875	.54760

P indicates probability that the observed R mutations occurred by chance; the value is reported in bold when statistically significant ($P = .05$).

Table 3. Distribution of mutations in VK genes of HCV-associated NHLs

Patient no.	VK family\gene	R/S mutation ratio in FRs			R/S mutation ration in CDRs		
		Observed (R:S)	Expected	P	Observed (R:S)	Expected	P
1	VKIII*humkv325*	0.500 (1:2)	3.407	**.01942**	∞ (2:0)	3.833	.13270
2	VKIII*humkv325*	0.428 (3:7)	3.404	**.00030**	0.000 (0:1)	3.647	.16295
3	VKIII*humkv325*	6.000 (6:1)	3.404	.08305	0.000 (0:0)	3.833	.17392
4	VKIII*humkv325*	2.000 (2:1)	3.370	**.04108**	∞ (2:0)	3.833	.14776
5	VKIII*humkv328*	0.500 (2:4)	3.280	**.00995**	0.000 (0:0)	3.091	.58344
7	VKIII*humkv325*	0.500 (1:2)	3.407	**.01942**	1.000 (1:1)	3.833	.38194
10	VKIII*humkv328*	0.900 (9:10)	3.288	**.00000118**	6.000 (6:1)	3.706	.09955
11	VKI*L12a*	2.666 (8:3)	3.264	**.00474**	∞ (2:0)	4.923	.28112
12	VKIII*humkv325*	2.000 (4:2)	3.370	**.03242**	∞ (1:0)	3.833	.37445
13	VKI*A30*	1.500 (3:2)	3.292	**.01618**	∞ (2:0)	3.158	.19169
14	VKIII*humkv328*	0.750 (3:4)	3.294	**.007609**	∞ (1:0)	3.706	.38134
16	VKIII*humkv325*	1.000 (4:4)	3.405	**.00808**	∞ (1:0)	3.833	.34521

P indicates the probability that the observed R mutations occurred by chance; the value is reported in bold when statistically significant (*P* = .05).

Characterization of gene segments involved in IgR rearrangements

Sequencing data showed that the assigned germline counterparts of the VH genes were closely related to the V_HIII family (DP 54, DP 51, DP 47, and DP 49) in 9 patients, the V_HI family (DP 10, DP 88, DP 8, and DP 75) in 5 patients, and the V_HIV family (DP 71 and DP 63) in 3 patients (Table 4). The V_HI gene family members were more frequently used in MC syndrome–related than in MC syndrome–unrelated NHLs (4 of 9 patients versus 1 of 7), whereas the V_HIII and V_HIV family genes were almost equally distributed in both groups (Table 4).

Concerning the D_H segment, 7 of 9 MC syndrome–associated NHLs used the *D21/9* or *D21/10* gene (*D21/9* and *D21/10* differ by only one amino acid) and the other 2 used the *D2* gene. This was also used by one NHL not associated with the MC syndrome but associated with another autoimmune disease (ie, Sjögren syndrome [SS]). The remaining NHLs that were not associated with autoimmune syndromes did not use the previously mentioned D_H segments, but each of them used a different D_H segment (Table 4).

No specificity in the use of J_H fragments was noted (J_H2 to J_H5 gene segments were used), although the J_H4 segment was the most represented one in both groups of NHLs (Table 4).

Data for the light-chain sequences were obtained in 14 patients only. Seven NHLs used a mutated *VKIII kv325* gene, 3 a segment similar to the *VKIII kv328h5* gene, and 2 each a *VKII* and a *VKI* gene (Table 5). The *VKIII kv325* gene was more frequently used in the MC syndrome–associated NHL group (5 of 7) than in the MC syndrome–unrelated group (2 of 7, 1 of them associated with SS) (Table 5).

The J_k1 or J_k2 gene fragments were used in both groups of NHLs, with a predominance of the J_k1 fragment in the MC syndrome-unrelated group (Table 5).

V_H-CDR3– and V_K-CDR3–deduced amino acid sequences and homology to antibodies with known specificity

The amino acid sequences deduced from the V_H-CDR3 and V_K-CDR3 sequences are reported in Tables 4 and 5, respectively. Because the antibody specificity is mainly based on the amino acid sequence of the CDR3 IgR region,[26] we compared by the BLAST program the deduced CDR3 amino acid sequences with those of antibodies with defined specificity. The significance of the rigorous statistical comparison is indicated by the E value, which is inversely proportional to the similarity between antibody and CDR3.

V_H-CDR3–deduced amino acid sequences showed the highest significant similarity (Table 4) to human antibodies with RF specificity. In particular, 4 NHLs had the highest homology with RF-WOL, 4 with RF-WA, 2 with RF-MR-20, and the remaining 7 with other RFs. Homology to RF-WOL was only associated with MC syndrome-related NHLs. Moreover, higher similarity values between V_H-CDR3 and antibodies with RF activity were found in the MC syndrome–associated NHL group (Table 4). Three NHL cases (patients 7, 12, and 15) also exhibited a significant homology to an antibody specific for an epitope expressed by HCV E2 protein (Table 4).

The V_K-CDR3–deduced amino acid sequences also showed the highest similarity to antibodies with RF specificity in 9 of 12 patients. The remaining 3 patients (1, 12, and 13) had similarity to an antibody with RF activity but lower than that to antibodies with other specificities (Table 5). Interestingly, 7 of 12 patients (5 MC syndrome–associated and 2 MC syndrome–unrelated, but 1 of them with SS; patients 1, 2, 3, 4, 7, 12, and 16) also exhibited a high homology (> 90%) to an antibody specific for HCV E2 protein (accession number AJ236551; data not shown) and all used the V_KIII *kv325* gene. Furthermore, all of the V_K-CDR3–deduced amino acid sequences presented a very similar motif (Table 6).
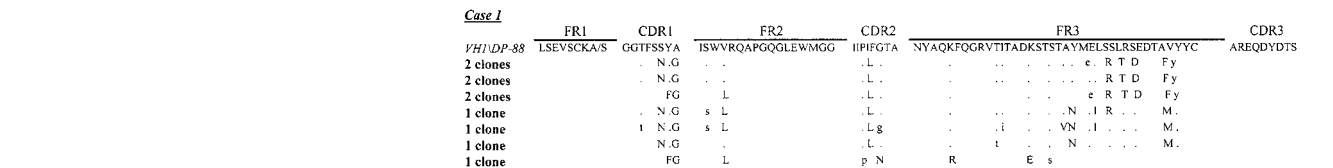

Figure 1. Deduced amino acid sequences of VH genes of a follicular (patient 1) and a diffuse large-cell lymphoma (patient 11). The amino acid sequences of the most homologous germline VH genes are shown above the lymphoma sequences. Amino acid R mutations are indicated by upper case letters and S mutations by lower case letters.

Table 4. V$_H$ gene segments and CDR3–deduced amino acid sequences of HCV-associated NHLs and homology to protein sequence databases

Patient no.	Ig$_H$ CDR3 deduced amino acid sequences	Length of CDR3 AA	Sequences producing the highest significant similarity	E value*	V$_H$ family/gene	D$_H$ segment	J$_H$ segment
1	CAREQDYDTSSYYYGYWGQGTLV	13	RF-WOL	6e-05	VHI/DP-88	D21/9	J4
2	CARGTDSSNYYYFYWGQGTLV	11	RF-WOL	0.002	VHI/DP-88	D21/9	J2
3	CARAFSSDTSGYYYYWGQGTLV	12	RF-WOL	0.005	VHI/DP-10	D21/9	J4
4	CARGPETSGYYYFYWGQGTLV	11	RF-WOL	0.005	VHIII/DP-47	D21/9	J4
5	CARGDYYDTSGYFSDAFDIWGQGTLV	16	RF-WA	1e-06	VHIII/DP-54	D21/9	J3
6	CARGDYDNSGDFVDAFDIWGQGTLV	15	RF-WA	1e-05	VHIII/DP-54	D21/10	J3
7	CARDRFCSGGSCFDWYFDLWGQGTLV	19	RF-MR-20/HCV	2e-07/1e-04	VHIV/DP-71	D2	J2
8	CARDYGGSSGYLNWFDPWGQGTLV	14	RF	4e-05	VHIII/DP-51	D21/9	J5
9	CARDLCSGGSCPALGAIDYWGEGTLV	16	RF-C93	0.017	VHI/DP-75	D2	J4
10	CARGDYFDDDGPFIDVFNVWGQGTLV	17	RF-WA	9e-06	VHIII/DP-54	D5-24	J3
11	CARGAYYNFFGDSYHYLDYWGQGTLV	16	RF-WA	0.030	VHIII/DP-47	DXP1	J4
12	CARDSFCTGGSCFDWYFDWGQG	15	RF-MR-20/HCV	7e-06/0.023	VHIV/DP-71	D2	J5/J2
13	CTSTGDRALVYSYDFWGQGTLV	19	RF-BOR	1700	VHIV/DP-63	D5-18/DK4	J4
14	CAQGGFSTSWYVPTGGYWGQGTLV	21	RF-10-2	1000	VHIII/DP-49	DN1	J4
15	CARAPTRMGPLWELGYYFDCWGQGTLV	17	RF/HCV	0.030/0.15	VHIII/DP-54	DN4	J4
16	CERSKYFDSWSGECVSARESHFDYWGQGTLV	21	RF	0.010	VHI/DP-8	DXP4	J4
17	CARANKEQWLVLEYNWFDPWGQGTLV	16	RF	2e-04	VHIII/DP-51	D6-19	J5

* 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 below 0.05.

Genbank accession numbers for RF-WOL: prf0707281c; RF-WA: U03400; RF-MR-20: U85234; anti E2 protein of HCV: AJ236544 for patient 7 and 12 and AJ236543 for patient 15; RF: AF021950 for patient 8, AF021978 for patient 15, AF021967 for patient 16 and AF022007 for patient 17; RF-C93: 573143; RF-BOR: prf1313976A; RF-10-2: S74542.

GenBank accession numbers for the mentioned antibodies with defined specificity are reported in Tables 4 and 5.

Discussion

Epidemiologic and experimental data strongly suggest an etiopathogenic role for HCV in the development of a subset of NHLs, particularly those complicating the course of some autoimmune diseases, mainly the MC syndrome.[3,5,6,11-13,16,19,27,28] At present, the most plausible pathogenic hypothesis supposes that specific B-cell clones proliferate, mainly in the bone marrow and liver tissue, as a consequence of a chronic antigenic stimulation exerted by HCV-associated antigen(s).[19,29,30] The continuous expansion of such chronically stimulated B-cells may then represent a risk for malignant transformation.

Our recent report[16] supports such a pathogenic hypothesis, demonstrating that premalignant and malignant lymphoproliferations in an HCV-infected patient with MC syndrome were sequential phases of an antigen (HCV E2 protein)-driven pathologic process. The aim of the present work was to ascertain whether such a pathogenic mechanism may involve a higher proportion of NHLs arising in HCV-infected patients with or without MC syndrome.

Sequencing of the IgR expressed by 17 HCV-associated NHLs and analysis of intraclonal variability in 8 of them suggest that the malignant lymphoproliferations derive from an antigen-driven

Table 5. V$_K$ gene segments and CDR3–deduced amino acid sequences of HCV-associated NHLs and homology to protein sequence databases

Patient no.	Ig$_K$ CDR3–deduced amino acid sequences	Length of CDR3 AA	Sequences producing the highest significant similarity	E value*	V$_k$ family/gene	J$_k$ segment
1	CQQYGSSRTFGQGTKLEIK	19	anti-HCV/RF-CUR	0.003/0.011	VKIII-kv325	JKI
2	CQQYGSSPYTFGQGTKLEIKR	21	RF-FLO/anti-HCV	4e-06/2e-04	VKIII-kv325	JK2
3	CQQYGSSPYTFGQGTKL	17	RF/anti-HCV	7e-04/0.006	VKIII-kv325	JK2
4	CQQYGSSPYTFGQGTKVEI	19	RF-FLO/anti-HCV	1e-04/2e-04	VKIII-kv325	JK2
5	CQHYNNWPPWTFGQGTKLEIK	21	RF-WA	6e-07	VKIII-kv328h5	JKI
6	ND	ND				
7	CQQYGSSPRTFGQGTKL	17	anti-HCV/RF-CUR	0.001/0.003	VKIII-kv325	JKI
8	ND	ND			VKII	
9	ND	ND				
10	CQQYNNWPPWTFGPGTKVKSNR	22	RF-4C9	5e-06	VKIII-kv328h5	JKI
11	CQQYNIYPYTFGQGTKLEIKREY	23	RF-FLO	5e-04	VKI-L12a	JK2
12	CQQYGSSPTFGQGTKL	16	anti-HBV/anti-HCV/RF-FLO	0.073/0.12/0.12	VKIII-kv325	JKI
13	CLQYNSYPRTFGQGTKVEIK	20	anti-GPIIb/anti-CMV	5e-04/7e-04	VKI-A30	JKI
14	CQQYNNWPPWTFGQGTKVEIK	21	RF-WA	7e-08	VKIII-kv328h5	JKI
15	ND	ND			VKII	
16	CQQYGSSPRTFGQGTKVEI	19	RF-CUR/anti-HCV	1e-04/2e-04	VKIII-kv325	JKI
17	ND	ND				

* 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 below 0.05.

ND indicates not determined.

Genbank accession numbers for anti E2 protein of HCV: AJ236554 for patients 1 and 12, AJ236552 for patients 2, 3, and 4, and AJ236551 for patients 7 and 16; RF-CUR: prf1206991A; RF-FLO: prf1714186A for patient 2 and prf1206991B for patients 4, 11, and 12; RF: prf1714186A; RF-WA: U03401; RF-4C9: L48238; anti-HBV: M88319; anti-GPIIb: S73911; anti-CMV: L26893.

Table 6. VKIII-CDR3 multiple sequence alignment

Patient no.	VK-CDR3 sequence from *kv325* gene
4	CQQYGSSPYTFGQGTKVEI–
12	CQQYGSSPRTFGQGTKVEIK-
6	CQQYGSSPRTFGQGTKL—-
2	CQQYGSSPYTFGQGTKLEIKR
3	CQQYGSSPYTFGQGTKL—-
1	CQQYGSS-RTFGQGTKLEIK
9	CQQYGSS-PTFGQGTKL—-
	******* *******.

Patient no.	VK-CDR3 sequence from *kv328* gene
5	CQHYNNWPPWTFGQGTKLEIK-
14	CQQYNNWPPWTFGQGTKVEIK-
10	CQQYNNWPPWTFGPGTKVKSNR
	.******** ***.: :

* Presence of the same amino acid in the sequences.
: Presence of amino acids with conservative change.

pathologic process. In fact, the NHLs presented a significantly lower R/S mutation ratio than expected by chance only in both the heavy-chain (14 of 17) and light-chain (11 of 12) FR sequences, consistent with a selective pressure to conserve such structure to provide the scaffolding for the antigen-contracting CDRs. Moreover, the neoplastic populations presented intraclonal variation, a phenomenon highly indicative of an ongoing antigen-dependent proliferation. The absence of significant R mutations in the CDRs argues against the selection for variants with higher antigen-binding specificity; alternatively, it may derive from elimination of variants too autoreactive and, therefore, dangerous for the host.[16,31-35]

The sequencing of IgR expressed by the HCV-associated NHLs showed a highly restricted use of gene segments. All VH chains used exclusively the VH3, VH1, and VH4 family genes. Seven of the 12 sequenced VK chains (58%) used the *kv325* gene and 3 (25%) the *kv328* gene, which present very similar deduced amino acid sequences (Table 6). Both these *VH* and *VK* genes are known to be preferably used by B cells secreting RF in patients with type II MC, and they are associated with WA cross-idiotype (XId), the major cross-idiotype among human monoclonal RF.[15,31,36-38] Such a restricted use of specific gene segments in many different lymphomas is unlikely to occur by chance only, thus suggesting that the antigen-binding specificity of the IgR must be causally associated with events predisposing B cells to neoplastic transformation.[39] The picture that emerges from these experimental data is, therefore, compatible with the pathogenic hypothesis that a chronic, and possibly common, antigenic stimulus drives the expansion of specific B-cell clones in a GC-like reaction where the vigorous expansion of the B cells makes them susceptible to mutation events responsible for neoplastic transformation.

Concerning the antigen responsible for the chronic B-cell stimulation, a clue derives from the comparison of the IgR CDR3–deduced amino acid sequences with those of antibodies with defined specificity. For almost all NHLs, both heavy- and light-chain CDR3 showed the highest similarity to antibodies with RF activity that have been found in MC syndrome.[15,28,31,36-38,40,41]

These data suggest, therefore, that a common antigenic stimulus is involved in MC syndrome and in HCV-associated lymphomagenesis also in patients without an overt autoimmune disease.

Because HCV is the recognized pathologic agent of MC and the CDR3 amino acid sequences of some HCV-associated NHLs also present a high homology for an antibody specific for the E2 protein of HCV, it may be reasonable to speculate that the HCV E2 protein is one of the chronic antigenic stimuli involved both in the lymphomagenetic process and in the production of antibody with RF activity. Further studies are, however, required to directly demonstrate such a possibility.

The 17 patients presented, at the time of NHL diagnosis, a dichotomic clinical behavior: 9 had a history of overt MC syndrome and 8 did not, although serum cryoglobulins were detected in 7 of them. Interestingly, the IgR-VH CDR3 sequencing data seem to offer a plausible molecular interpretation of such differential clinical behavior. The patients with MC syndrome used only the *D21* (7 of 9) or *D2* (2 of 9) gene segments to assemble the IgH chain, but none of the patients without overt MC syndrome used such segments, with the only exception being patient 12. Patient 12, however, had SS, an autoimmune syndrome, which may be complicated by NHL development in patients with consistent levels of serum cryoglobulin.[42,43] Such findings suggest that specific D segments may confer to the Ig expressed and possibly secreted by the lymphoma cells (and their precursors) high affinity for antigens involved in the MC syndrome or other cryoglobuline-mic-associated autoimmune syndromes. This is in agreement with previous reports suggesting that a discrete-sized D region in WA Xid-positive IgM[33] is strictly required for RF activity. Moreover, the *D21-9* gene segment is frequently expressed by B-cell clones from myoepithelial sialadenitis lesions occurring in patients with SS,[44] B-cell clones from patients with HCV-associated immunocytomas and MC syndrome,[15] and B-cell clones producing IgM with RF activity from healthy donors[45] than by the totality of VDJ rearrangements occurring in normal individuals.[46]

In conclusion, our data provide evidence that a large proportion of HCV-associated NHLs derive from B-cell clones chronically stimulated by a common agent. The high homology of the VH and VK IgR chains with antibodies with RF activity, which are mainly found in MC syndrome etiologically related to HCV infection, and with antibodies specific for E2 protein of HCV (particularly evident for the VK chain) suggests a possible involvement of viral antigen(s) (E2) in driving B-cell clonal expansion. Finally, the use of specific D segments in assembling IgR possibly confers on B-cell disorders the property to produce antibodies that may contribute to the development of an overt MC syndrome. In this context, HCV eradication may lead to reduction of the HCV-driven lymphoproliferative expansion, with mitigation of the MC syndrome, if present, and to a decreased probability of NHL occurrence.

Acknowledgment

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