Detection and Distribution of Hepatitis C Virus-Related Proteins in Lymph Nodes of Patients With Type II Mixed Cryoglobulinemia and Neoplastic or Non-Neoplastic Lymphoproliferation

By Domenico Sansonno, Salvatore De Vita, Vito Cornacchiulo, Antonino Carbone, Mauro Boiocchi, and Franco Dammacco

The role of hepatitis C virus (HCV) in the pathogenesis of type II mixed cryoglobulinemia (MC) has been strongly emphasized in the last few years. Although MC is a benign lymphoproliferative disorder, the risk of overt B-cell malignancy greatly increases during its course. The occurrence of HCV infection in 10% to 30% of patients with non-Hodgkin's lymphoma (NHL) suggests that this virus may have a role in the development of MC-associated B-cell malignancies. We identified 2 patients with hyperplastic reactive lymphadenopathy (HRL) and 12 with NHL in two series of MC patients chronically infected with HCV collected over a 5-year period. Structural and nonstructural HCV-related proteins were investigated in lymph node sections by immunohistochemistry and their location and distribution were correlated with clinical and histologic findings, viremic state, and HCV genotypes. In HRL, HCV proteins were found in the cytoplasm of lymphoid cells, mainly in interfollicular areas. However, occasional positive cells were found in the mantle zone and in the germinal centers of follicles. In addition, strong reactivity was found in the circulating mononuclear cells of capsular blood vessels. HCV immunodeposits were found in 3 of 12 (25%) NHL cases. Positive cells were frequently restricted to the cortex; if not, they were randomly diffused in the neoplastic tissue. Positivity was related to the low-grade type of NHL; in the 2 composite cases, HCV immunodetection was found in the small cells, whereas large anaplastic cells were regularly negative. Other viruses previously involved in lymphoproliferation, i.e., human herpes virus-6 and Epstein-Barr virus, were absent in all tissues. These data emphasize that lymphoid organs may be a site of HCV infection. The demonstration of HCV-related proteins in a nonmalignant condition, namely HRL, indicates that HCV infection precedes the neoplastic transformation and possibly plays a major role in lymphomagenesis in MC.

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Materials and Methods

Samples were obtained after human protection committee validation and informed consent were received. An extensive review was made of cases of pathologically documented lymphadenopathies recorded at the Department of Biomedical Sciences and Clinical Oncology of the University of Bari and the Department of Experimental Oncology of Aviano Hospital from January 1991 to December 1995. We selected 12 Mc patients with NHL and 2 with HRL who represented part of a cohort of consecutive patients, all of whom were human immunodeficiency virus (HIV)-negative, who were under study because of concomitant HCV-infection and lymphoproliferative disease. They accounted for about 10% of all the new cases of lymphoproliferative disorders referred to the above institutions during the study period. These cases included 91 patients with B-cell lymphomas, 7 with T-cell lymphoma, 13 with atypical lymphoid proliferation, 4 with follicular hyperplasia, 2 with angiofollicular hyperplasia, and 16 with Hodgkin’s disease. Diagnoses were based on routinely stained sections prepared from formalin or Zenker’s fixed paraffin-embedded surgical biopsy and were carefully reviewed by one of the investigators (A.C.). NHLs were subclassified according to the International Working Formulation.14

Table 1. Clinical, Histologic, and Virologic Parameters in Patients With Self-Limited and Neoplastic Lymphoadenopathy and Type II MC

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age</th>
<th>Cell Phenotype</th>
<th>Duration (yr)</th>
<th>Histology</th>
<th>Duration (yr)</th>
<th>Antibody Pattern</th>
<th>HCV RNA</th>
<th>HCV Genotype</th>
<th>Cryocrit (%)</th>
<th>Duration (yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BM</td>
<td>F</td>
<td>65</td>
<td>B</td>
<td>0.2</td>
<td>CPH</td>
<td>1.9</td>
<td>0/0/4/3</td>
<td>+</td>
<td>+</td>
<td>1/1b</td>
<td>4</td>
</tr>
<tr>
<td>2. MS</td>
<td>M</td>
<td>58</td>
<td>B</td>
<td>0.6</td>
<td>CPH</td>
<td>2.0</td>
<td>0/1/3/3</td>
<td>+</td>
<td>+</td>
<td>1/1b</td>
<td>6</td>
</tr>
<tr>
<td>3. QE</td>
<td>M</td>
<td>67</td>
<td>B</td>
<td>2.4</td>
<td>CAH</td>
<td>6.2</td>
<td>0/0/0/4</td>
<td>+</td>
<td>+</td>
<td>III/2a</td>
<td>3</td>
</tr>
<tr>
<td>4. ZA</td>
<td>M</td>
<td>77</td>
<td>B</td>
<td>3.6</td>
<td>CAH</td>
<td>7.0</td>
<td>0/0/0/2</td>
<td>+</td>
<td>+</td>
<td>1/1b</td>
<td>5</td>
</tr>
<tr>
<td>5. MB</td>
<td>M</td>
<td>67</td>
<td>B</td>
<td>1.0</td>
<td>C</td>
<td>10</td>
<td>0/0/4/4</td>
<td>+</td>
<td>+</td>
<td>1/1b + 1/1a</td>
<td>3</td>
</tr>
<tr>
<td>6. IS</td>
<td>F</td>
<td>72</td>
<td>T</td>
<td>1.0</td>
<td>CAH</td>
<td>5</td>
<td>0/0/2/3</td>
<td>+</td>
<td>+</td>
<td>1/1b</td>
<td>12</td>
</tr>
<tr>
<td>7. GE</td>
<td>M</td>
<td>72</td>
<td>T</td>
<td>5.0</td>
<td>ND</td>
<td>11</td>
<td>0/1/2/4</td>
<td>+</td>
<td>+</td>
<td>III/2a</td>
<td>4</td>
</tr>
<tr>
<td>8. PZ</td>
<td>F</td>
<td>66</td>
<td>B</td>
<td>2.0</td>
<td>ND</td>
<td>15</td>
<td>1/1/2/4</td>
<td>+</td>
<td>+</td>
<td>1/1b + 1/1a</td>
<td>52</td>
</tr>
<tr>
<td>9. MM</td>
<td>F</td>
<td>59</td>
<td>B</td>
<td>2.0</td>
<td>ND</td>
<td>7</td>
<td>0/0/0/4</td>
<td>+</td>
<td>+</td>
<td>III/2a</td>
<td>3</td>
</tr>
<tr>
<td>10. DM</td>
<td>F</td>
<td>79</td>
<td>B</td>
<td>0.8</td>
<td>ND</td>
<td>16</td>
<td>0/1/2/4</td>
<td>+</td>
<td>+</td>
<td>1/1b</td>
<td>3</td>
</tr>
<tr>
<td>11. PL</td>
<td>F</td>
<td>65</td>
<td>B</td>
<td>4</td>
<td>ND</td>
<td>10</td>
<td>0/0/1/4</td>
<td>+</td>
<td>+</td>
<td>1/1b</td>
<td>10</td>
</tr>
<tr>
<td>12. CU</td>
<td>M</td>
<td>76</td>
<td>B</td>
<td>2</td>
<td>ND</td>
<td>14</td>
<td>0/0/0/0</td>
<td>+</td>
<td>+</td>
<td>1/1b + 1/2b</td>
<td>4</td>
</tr>
</tbody>
</table>

Non-neoplastic lymphoproliferation

1. SE F/69 Reactive hyperplasia 3 C 20 2/4/4/4 + + 1/1b 3 25
2. CP M/31 Reactive hyperplasia 1 CAH 6 4/4/4/4 + + 1/1b + III/2a 5 6

Abbreviations: CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; C, cirrhosis; ND, not determined.

HCV RNA assay. Sera and tissues were obtained from patients on the same day. Frozen lymph node tissue (weighing 40 to 50 mg) was homogenized in 450 mL 4 mol/L guanidium thiocyanate, 2 mmol/L sodium citrate, pH 7.0, 5% sarkosyl, and 0.1 mol/L mercaptoethanol. Sequentially, 45 mL of 2 mol/L sodium acetate, pH 4.0, 450 mL water-saturated phenol, and 300 mL chloroform-isooamyl alcohol mixture (49:1) were added and mixed. The suspension was placed on ice for 15 minutes and then centrifuged at 10,000g for 20 minutes. The aqueous layer was then extracted with an equal volume of chloroform and alcohol precipitated overnight. The RNA pellet was washed in 70% ethanol and resuspended in RNasin-treated autoclaved water (200 U/mL H2O). The amount of RNA extracted from lymph nodes was determined by spectrophotometry. RNA was extracted from 100-mL serum samples in guanidium thiocyanate, phenol-chloroform, as previously described.25 A two-stage PCR with nested primers was used to amplify complementary DNA synthesized on reverse transcription. Primers were selected from highly conserved 5′-untranslated region of the HCV genome. After specific reverse transcription, minus strand HCV replicative intermediates were also searched for in this region with suitable strand primers. HCV positivity was confirmed by a nested PCR amplification of the core region of HCV according to Okamoto et al26 to characterize the HCV genotypes. Each sample was tested in triplicate, and adequate positive and negative controls were always included.

Immunohistochemistry for HCV infection. In situ evidence of HCV infection was provided by indirect immunohistochemistry on consecutive fixed cryostat sections. Positive and negative results were confirmed by repeated experiments. Murine monoclonal antibody (MoAb) reactivities against HCV-insoluble recombinant antigens c23-3, c33c, and c100-3 have been reported in detail elsewhere. Briefly, the following anti-HCV MoAbs were used: (1) anti-c23-3 antibody (clone 4,6E7-7F6) recognizing amino acids (AA) 29-43 of the core protein (working concentration, 0.9 mg/mL); (2) anti-c33c antibody (clone 1,4G11-B4G10) recognizing a structural...
detected. For each antibody, protein concentration in working mixture was adjusted to 1 mg/mL.

All MoAbs were produced in mice by using recombinant antigens as immunogens (c22-3: yeast-expressed SOD fused HCV NS3 protein, AA 1192-1457; c100-3: 2C4G3, yeast-expressed determinant in the NS3-encoded protein (working concentration, 1 mg/mL); and (3) a mixture of three anti-c100-3 antibodies—clones 2C4G3 recognizing AA 1690-1696, clone 22A5B12 recognizing AA 1694-1711, and clone 20A6F3 recognizing a linker sequence at the junction of superoxide dismutase (SOD) and HCV sequences in the NS4-encoded protein. For each antibody, protein concentration in working mixture was adjusted to 1 mg/mL.

For 4 hours at RT. After further washings, excess PBS was removed and the slides were incubated with a sheep antidioglobin conjugated with colloidal gold particles (anti-DIG-gold; 4 mg/mL; code no. 1450.590; Boehringer Mannheim) for 3 hours at RT. The slides were washed once with PBS, followed by extensive washings (200 mL) with redistilled water to remove phosphate ions. Detection of colloidal gold particles in tissue was intensified by silver enhancement procedure (code no. 1465.350; Boehringer Mannheim). After incubation for 10 to 60 minutes, the slides were counterstained and mounted.

HCV antigen specificity of positive in situ signal was assessed before and after effective absorption with c22-3, c33c, and c100-3 recombinant proteins; an HCV-infected liver homogenate; and hepatitis B and A virus-associated proteins. In addition, the specificity of anti-HCV antibodies was examined on anti-HCV, HCV RNA-negative lymph nodes from 10 patients with NHL and 5 with HRL. Omission of anti-HCV antibodies and substitution with irrelevant murine MoAb (ie, antihuman chorionic gonadotropin) were also performed in all cases.

Serology. Antibodies against HCV were detected by a second-generation ELISA and confirmed by a second-generation recombinant-based immunoblot assay (Ortho Diagnostic Systems). HBV-associated antigens and antibodies and anti-HIV antibodies were detected with commercial kits (Abbott Laboratories, Abbott Park, North Chicago, IL). RESULTS

Clinical, histologic, and virologic features of the 14 patients are summarized in Table 1. All patients were hetero-

Table 2. Occurrence of Tissue HCV-Related Proteins in Relation to Lymph Node Histology

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>NHL Histology</th>
<th>Working Formulation</th>
<th>HCV-Related Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Follicular predominantly small cleaved cells</td>
<td>(B)</td>
<td>c100 + c33 - c22 -</td>
</tr>
<tr>
<td>2</td>
<td>Follicular predominantly small cleaved cells</td>
<td>(B)</td>
<td>+ + +</td>
</tr>
<tr>
<td>3</td>
<td>Small lymphocytic</td>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Diffuse large cells</td>
<td>(G)</td>
<td>+ + +</td>
</tr>
<tr>
<td>5</td>
<td>Diffuse large cells</td>
<td>(G)</td>
<td>+ + +</td>
</tr>
<tr>
<td>6</td>
<td>Mixed: Small lymphocytic; follicular predominantly</td>
<td>(A + D)</td>
<td>+ + +</td>
</tr>
<tr>
<td>7</td>
<td>Large-cell, immunoblastic</td>
<td>(H)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Diffuse, mixed small cleaved and large cells</td>
<td>(F)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Follicular predominantly small cleaved cells</td>
<td>(B)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Mixed: diffuse large cells and small lymphocytic</td>
<td>(G + A)</td>
<td>+ + +</td>
</tr>
<tr>
<td>11</td>
<td>Small lymphocytic</td>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Follicular, mixed small cleaved and large cells</td>
<td>(C)</td>
<td></td>
</tr>
</tbody>
</table>

Non-neoplastic lymphoproliferation

1. Reactive hyperplasia with intact follicular architecture + + +
2. Reactive hyperplasia with intact follicular architecture + + +

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Fig 1. (A) through (D) (original magnification × 100) show sequential reconstruction from the capsule to the hilar structures of a lymph node of a patient with HRL. HCV-related proteins are detected mainly in interfollicular areas, with almost the same distribution in subcapsular, cortical, and medullary zones. Occasional positive cells (arrows) are detected in the mantle (C) and in the germinal centers (D) of the follicles. In (E), higher magnification defines the cytoplasmic distribution of the immune deposits within lymphoid cells, whereas the nuclei are completely negative (original magnification × 250). In (F), HCV-specific immune reactants are detected as granular bead-like deposits within blood vessel lumen (arrows) of the hilar fibrous trabecula (original magnification × 100). In (G), higher magnification shows that the signal is found within circulating mononuclear cells (original magnification × 400). In (H), complete absence of the signal in HCV-unrelated HRL is shown (original magnification × 250). Immune reaction is referred to the core-derived protein and detected by mouse monoclonal anti-c22 antigen. Sections were counterstained with hematoxylin.
sexual and denied drug abuse. All were anti-HIV and hepatitis B surface antigen (HBsAg) negative. Anti-HBV antibodies (ie, anti-HBs and/or anti-HBc and/or anti-HBe) were present in 8 (57.1%). Eleven NHL patients had B-cell lymphoma, whereas patient G.E. had a Ki-1 T-cell anaplastic lymphoma. Five patients (41.7%) presented with extensive disease involving extranodal sites. Four (33.3%) also had marrow lymphomatous infiltration at diagnosis.

Neither EBV nor HHV-6 genomes were detected in biopsy specimens by sensitive PCR approaches, and neither LMP-
I staining nor in situ hybridization showed EBV infection in HRL and NHL tissues.

All patients were suffering from chronic active liver disease: 8 with a biopsy-proven and 6 with a clinical and laboratory diagnosis. Diagnosis or history of liver disease had a mean duration of 9.4 years and had always preceded that of NHL and HRL. Cryoglobulinemia preceded the onset of NHL in 9 (75%), was detected at the same time as NHL in 1, and followed its diagnosis in the other 2. Cryoglobulinemia preceded the diagnosis of HRL in both patients.

Results from the tissue studies are shown in Table 2. HCV proteins were difficult to quantify, owing to large areas of negativity; thus, they were arbitrarily considered as present (+) or absent (−). Features of HCV distribution were defined by the preservation of follicular structure in HRL and compared with those observed in frank neoplasia.

Strong and unequivocal reactivity in the two HRL patients was shown by an intense dot-like labeling pattern extending from the lymph node capsule to the hilar structures (Fig 1A through D). Positive cells were prevalently confined to interfollicular zones and distributed in the same way in the cortex, the paracortical, or the peri-hilar areas. Positive cells identified inside the germinal centers or in the mantle zone of the follicles were usually isolated and few in number, but the label was clearly evident in single cells of germinal centres (Fig 1D). Figure 1E illustrates the distribution of the signal over the infected cells and indicates that the location of HCV proteins is cytoplasmic, whereas the nuclei are completely negative.

A further frequent feature was the presence of intravascular positive mononuclear cells. As shown in Fig 1F and G, marked labeling was shown in mononuclear cells in the lumen of a capsular arteriole. No differences were found in the distribution of structural and nonstructural HCV-related proteins. The three anti-HCV antibodies always displayed the same HCV antigen distribution.

HCV proteins were found in only 3 (25%) NHL patients. Positive signal was irregularly distributed within neoplastic lymphoid tissues. It appeared to be confined to the cortex (Fig 2A and B) or randomly distributed within single cells (Fig 2C and D). Single elongated elements reminiscent of interdigitating reticulum cells were sometimes defined by positive signal (Fig 2E). Immunodetection of HCV proteins was related to the histologic pattern of NHL: the 3 positive patients had low-grade lymphoma, suggesting that lymphocyte differentiation may modulate viral protein expression. This suggestion was corroborated by the lack of HCV proteins in a biopsy from patient no. 2 taken 2 years later when NHL had progressed from grade B to grade G.

Specificity of HCV immunodetection was shown by the complete unreactivity of the probes on lymph node sections from anti-HCV, HCV RNA-negative controls (Fig 1H) and on selected positive samples before and after absorption of the probes with insoluble recombinant antigens derived from structural and nonstructural regions of HCV genome. Preincubation with c22-3, c33c, and c100-3 antigens completely abolished the equivalent positive signal (Fig 2F). Similar results were obtained after absorption of the probes with an autopit liver homogenate from a patient with HCV-related cirrhosis. Absorption with SOD protein, HBV-associated proteins (HBsAg and HBeAg) or HAV-Ag had no significant effect. In addition, absorption with normal human and mouse lymph node homogenates affected neither the intensity nor the pattern of the immunohistochemical pictures.

The absorption efficiency of probe reactivities was further documented on immunoblotting. The results showed the complete abolition of the signal after preincubation with HCV antigens, whereas it was unchanged with control proteins.

**DISCUSSION**

After the cloning and sequencing of the entire HCV genome, an increasing number of observations have focused on the peculiar lymphotropism of HCV, which has been thought to sustain B-cell clonal expansion in type II MC, a disorder postulated to be a low-grade lymphoid malignancy. This has provided new clues to the putative role played by HCV in development of the true B-cell neoplasms that occur in MC.

Our present results point to different situations of HCV distribution in the lymphoid tissue microenvironment. By using HCV-specific structural and nonstructural MoAbs, we showed a large number of HCV-positive lymphoid cells in hyperplastic reactive lymph nodes from chronically infected patients with type II MC. These cells were mainly detected in extrafollicular areas. However, their observation in isolated germinal centers indicates that they may participate in physiologic germinal center reactions that ensure their long survival.

The demonstration of HCV-infected mononuclear cells in the capsular vessels of lymph nodes is consistent with the highly dynamic traffic between infected compartments and suggests that the nodes may be major sites for establishment of an HCV reservoir.

The distribution of HCV-related proteins in HRL nodes is completely lost in NHL. HCV-infected cells are confined to limited areas, often the cortex, or randomly distributed in the neoplastic tissue. Examination of A + D and G + A NHL showed that the HCV-specific signal was confined to small cells. HCV proteins were found in only 3 of 12 (25%) NHL patients, all with low-grade disease, suggesting that expression of HCV gene products is closely related to certain stages of cell differentiation. These observations confirm and extend our preliminary report on the occurrence of HCV core protein and plus and minus HCV RNA strands in residual ductal and acinar salivary epithelial cells, but not in neoplastic lymphoid cells of parotid B-cell NHL, suggesting that malignant B cells are not permissive to HCV replication. However, HCV RNA was always detected by PCR assay in either the presence or the absence of HCV proteins in lymph node biopsy specimens from the NHL patients. HCV infection in the tumor mass may be undetectable by immunohistochemistry for technical reasons or due to latent infection in the malignant or nonmalignant accompanying cells. This point deserves further study for the obvious pathobiologic implications.
A point that has not been clarified in the present study is whether nonproliferating lymph nodes in patients with HCV infection also stain positively for HCV antigens. Although these biopsy samples were not available, the putative detection of the virus in normal lymph nodes should not lead to conclude that HCV has little to do with B-cell lymphomagenesis. HCV might play a pathogenic role only in the specific individual background. This has already been emphasized for other infectious agents related to certain lymphoma subtypes, such as EBV in Hodgkin’s disease or Helicobacter pylori in gastric lymphoma. For instance, H. pylori infection is quite common and may be detected even in gastric mucosa with minimal abnormalities. However, such an infection seems to be crucial for gastric B-cell lymphoma, which develops in indeed in only a small number of patients.

Similarly, the association between HCV infection and malignant and nonmalignant B-cell lesions does not prove a causative role of HCV. However, the direct demonstration of the infectious agent within the tissue where NHL may arise is one of the key steps to establish an association between infection and malignancy. In addition, the occurrence of HCV in nonmalignant conditions such as HRL with monoclonal gammopathy strongly suggests that virus infection precedes neoplastic transformation.

If the liver is the primary anatomical site of infection, lymphoid organs may act as reservoirs of the body burden of HCV in distinct phases of the chronic carriage state, because the virus may be differently compartmentalized. In this context, the demonstration of HCV-related proteins in elements resembling interdigitating reticulum cells supports the notion that HCV is trapped therein in the form of immune complexes regularly found in chronic HCV carriers.

Although a pathogenetic role of HCV in the development of a subset of B-cell lymphomas may be suspected, functional studies will be crucial to show whether HCV may sustain B-cell proliferation in a T-cell-independent or -dependent manner or may directly infect and deregulate lymphoid cells. However, low levels of virus-encoded proteins undetectable by the immunohistochemical technique employed cannot be ruled out. Further efforts are needed for the demonstration of HCV RNA genomic sequences by sensitive in situ hybridization procedures.

REFERENCES


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