Infection of Peripheral Blood Mononuclear Cells by Hepatitis C Virus in Mixed Cryoglobulinemia

By Clodoveo Ferri, Monica Monti, Luca La Civita, Giovanni Longombardo, Francesco Greco, Giampiero Pasero, Paolo Gentilini, Stefano Bombardieri, and Anna Linda Zignego

A striking association between hepatitis C virus (HCV) infection and mixed cryoglobulinemia (MC) has been shown; thus, HCV seems to play an important etiopathogenetic role in this lymphoproliferative disorder. Because HCV is both a hepatotropic and lymphotropic virus, this study aimed to investigate the prevalence of HCV infection of peripheral blood mononuclear cells (PBMCs) in a series of 16 patients with type II (IgMk) MC. Antibodies against HCV were detected by commercially available kits (Second Generation Chiron enzyme-linked immunosorbent assay (ELISA) and recombinant-based immunoblot assay (RIBA)), and the presence of HCV RNA was evaluated in both sera and isolated PBMCs using the polymerase chain reaction technique. A previous exposure to HCV was shown by ELISA and confirmed by RIBA in all cases (100%). Moreover, HCV RNA was present in the sera of 8 of 16 patients (50%), whereas its frequency markedly increased (13 of 16 [81%]) when genomic sequences were detected in peripheral lymphocytes. HCV RNA was never detected in the PBMCs of 20 control subjects. These findings showed that HCV infection, alone or in combination with other factors, may be responsible for the clonal B-cell expansion underlying the systemic manifestations of MC, and may explain the appearance of a malignant non-Hodgkin’s lymphoma in some subjects.

© 1993 by The American Society of Hematology.

Mixed Cryoglobulinemia (MC) is a lymphoproliferative disorder characterized clinically by purpura, arthralgias, weakness, and various organ involvements such as peripheral neuropathy, chronic hepatitis, glomerulonephritis, and generalized vasculitis. The typical pathologic lesions involving small- and medium-sized vessels are due to deposition of circulating immune-complexes (CIC) and complement. In MC patients, a monoclonal rheumatoid factor in the cryoprecipitates, a clonal expansion of IgMk-bearing B cell in peripheral blood, lymphoid aggregates in bone marrow biopsies, and lymphocytic infiltration of liver, spleen, or kidneys have been reported. Together, these findings raised the possibility that a malignant lymphoproliferative disorder is responsible for the disease. However, B-cell lymphomas develop in only a small number of cases, generally after a long follow-up period. More recently, evidence of hepatitis C virus (HCV) infection in the majority of patients has suggested that this virus could be the main etiologic factor of MC. HCV is responsible for different chronic liver diseases, such as posttransfusion non-A, non-B chronic hepatitis and autoimmune chronic hepatitis; in this respect, liver involvement present in two-thirds of MC could be regarded as a clinical variant of HCV-related chronic hepatitis. However, in almost 30% of MC patients, HCV infection has also been found without any clinical evidence of liver damage. This observation suggests that MC is not simply an extrahepatic manifestation of HCV liver infection, but primarily a systemic lymphoproliferative disorder. More recently, it has been shown that HCV is both a hepatotropic and lymphotropic virus, on this basis, we can suppose that, in MC patients, chronic HCV infection could be responsible for the lymphoproliferative disorder underlying clinical manifestations of the disease. To better define the role of this virus in the pathogenesis of the MC, HCV infection of peripheral lymphocytes was investigated in a series of patients.

Patients and Methods

Sixteen consecutive MC patients (mean age, 59 ± 8 years; mean disease duration, 10 ± 5 years) were included in the study. In all cases, diagnosis of MC was based on the presence of the typical syndrome (purpura, arthralgias, and weakness) and of circulating mixed cryoglobulins, and on the exclusion of other neoplastic or systemic disorders. All patients were Italian-born heterosexuals and had no history of contact with hepatitis or parenteral exposure to blood products, or of drug or alcohol abuse. The presence of visceral complications, ie, peripheral neuropathy and liver and renal involvement, were evaluated as previously described. Routine blood chemistry studies were performed by standard methods. Cryocrit, ie, the percentage of packed cryoglobulins, hemolytic complement activity, and C3-C4 fractions were measured as previously described. Mixed cryoglobulins were classified as type II or type III according to the presence of monoclonal or polyclonal IgM rheumatoid factor (RF). The presence of HCV RNA in the sera of HCV RNA, anti-HCV, hepatitis B surface antigen (HBsAg), anti-HBV (anti-HBsAb, -HBc, -HBe), and anti-human immunodeficiency virus antibody (anti-HIV) was detected on serum clotted and centrifuged at 37°C and stored at −80°C. Anti-HCV were detected by enzyme-linked immunosorbent assays (Chiron ELISA HCV, Second Generation; Emeryville CA, and confirmed in all cases by recombinant-based immunoblot assay (Chiron RIBA HCV, Second Generation Assay). HBV and HIV markers were detected by ELISA using commercially available kits (Hepanostika, Vironostika anti-HTLV III; Organon Teknika, Boxtel, The Netherlands). In the same blood samples, the presence of HCV RNA in the serum and in peripheral blood mononuclear cells (PBMCs) was investigated. PBMCs were prepared from heparinized venous blood samples by centrifugation on Ficoll-Hypaque density gradient (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden).

From the Istituto Patologia Medica I, University of Pisa, Pisa; the Istituto di Clinica Medica II, University of Florence, Florence; and the Blood Center, Ospedale S. Chiara, Pisa, Italy.

Submitted May 4, 1993; accepted August 10, 1993.

Supported in part by the National Research Council (CNR) Targeted Project "Prevention and Control Disease Factors." Subproject "SP3," Contract No. 91.00168, and the Italian Liver Foundation.

Address reprint requests to Prof Clodoveo Ferri, MD, Istituto Patologia Medica I, University of Pisa, Via Roma 67, 56100 Pisa, Italy.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/8212-0008$3.00/0
and were tested for the presence of HCV RNA. Briefly, three to four aliquots of 2 x 10^6 cells were obtained from each patient and washed three to five times by centrifugation with RNase-free phosphate-buffered saline (PBS).

HCV RNA was also detected in both sera and PBMCs of the following control groups: (1) 10 healthy subjects, and (2) 10 anti-HCV-negative patients with type B or δ chronic active hepatitis (HBsAg, IgM anti-Hbc positive or IgM anti-HD, serum HDV RNA positive, respectively).

Total RNA was extracted according to a previously described technique. Briefly, sera were digested in 4 vol of a lysis buffer containing 10 mmol/L EDTA, 1 mmol/L Tris-HCl (pH 7.5), 1% sodium dodecyl sulfate (SDS), and 500 μg/mL proteinase K. Proteins were then extracted with phenol-chloroform and total RNA precipitated. Nucleic acids were recovered by centrifugation (14,000 rpm for 30 minutes at 4°C), washed in 70% ethanol (500 μL), evaporated to dryness, dissolved in 30 μL of sterile diethylpyrocarbonate-treated water (DEPC H2O), and stored at -80°C.

HCV RNA sequences were determined using a "one-tube nested" polymerase chain reaction (PCR) assay as previously described. HCV PCR was performed using primers (Table 1) corresponding to the well-conserved 5' noncoding region of the HCV genome as described.

Amplification products were detected by ethidium bromide staining. Fifteen microliters of each reaction mix was applied to a nylon membrane (Gene Screen Plus; New England Nuclear, Boston, MA) was washed twice with 0.2X SSC/I% SDS at 55°C for 20 minutes, and exposed to a Kodak X-AR film at -80°C for 24 and 72 hours using an intensifying screen (Eastman Kodak, Rochester, NY).

A series of technical precautions were used to assure specificity and sensitivity of HCV-RNA determination: (1) the various steps involved in the method (RNA purification, PCR, and product analysis) were physically separated; (2) all pipettes and containers were disposable; (3) only cell preparations whose last wash was HCV contamination caused by double amplification, "nested" HCV PCR was performed in the same reaction tube by separating the reaction mixtures for the first and second PCR with a layer of mineral oil.

RESULTS

The main clinico-epidemiologic data and the results of laboratory parameter investigations are reported in Table 2. Liver involvement and peripheral neuropathy were the most common manifestations of the disease. At the time of the study, a chronic active hepatitis, evaluated on the basis of histologic and/or liver enzyme alterations, was present in 11 of 16 (69%) cases. In 2 patients, a renal involvement was recorded: renal biopsy showed a membranoproliferative glomerulonephritis in 1, and a constant proteinuria was detected in the other. Long-term steroid therapy was used in the majority of cases; its dosage was graduated to control minor symptoms of the MC, ie, purpura episodes, weakness, and arthralgias. In two cases (no. 5 and 6), α-interferon treatment was started (9 x 10^6 IU/wk) 12 months before the study. Immunologic parameter investigations showed the presence of monoclonal rheumatoid factor (IgMk) in all cryoprecipitates. The cryocrit widely varied from 0.6% to 20% (mean ± SD, 4.2% ± 6.3%). Hemolytic complement activity was depressed in 11 of 16 cases (mean ± SD, 118 ± 43 hu/mL; normal range, 160 to 220), with constantly marked reduction of C4 component (mean ± SD, 12 ± 3; normal range, 20 to 60 mg/dL) and normal C3 levels.

A previous exposure to HCV was shown in all MC patients by ELISA; all seropositives were confirmed by RIBA II (Table 2). The presence of HCV genome was detected in the serum of 8 of 16 MC patients (50%), indicating the ongoing HCV replication in these subjects. The percentage of HCV RNA positivity markedly increased when it was evaluated in isolated PBMCs, in which genomic sequences were detected in 13 of 16 cases (81%). In 1 of 2 patients (case no. 6) undergoing long-term α-interferon treatment, HCV RNA was absent in both serum and isolated PBMCs; in this subject, the presence of HCV viremia had been observed before the treatment. HCV RNA was never found in both sera and PBMCs of the healthy controls and patients with B or δ chronic active hepatitis.

**DISCUSSION**

This study first showed that HCV infects the peripheral lymphocytes in a relevant number of MC patients. In a previous report, HCV RNA has been shown in the PBMCs of patients with HCV-related chronic hepatitis; in the same subjects, HCV-RNA–negative stranded molecules, indicative of viral replication, were found in both fresh and cultured PBMCs. Moreover, an immunogenic activity of HCV viral proteins on peripheral lymphocytes has been reported in patients with chronic hepatitis C by Botarelli et

---

**Table 1. Nucleotide Sequences of HCV Oligonucleotide Primers and Probe**

<table>
<thead>
<tr>
<th>Primers</th>
<th>5' Noncoding Region Sequence</th>
<th>Nucleotide Product</th>
<th>Size (bp)</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (+)</td>
<td>5' GCC ATG GCG TTA GTA GTC T3'</td>
<td>260</td>
<td>66–84</td>
<td></td>
</tr>
<tr>
<td>2 (−)</td>
<td>5' TGC ACG GTC TAC GAG ACC T3'</td>
<td>322–304</td>
<td>88–107</td>
<td></td>
</tr>
<tr>
<td>3 (+)</td>
<td>5' GTG CAG CCT CCA GGA CCC CC C3'</td>
<td>210</td>
<td>299–279</td>
<td></td>
</tr>
<tr>
<td>4 (−)</td>
<td>5' GGG CAC TCG CAA GCA CCC TAT T3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>5' ACA CCG GAA TTG TGG CCA GGA CGA CCG GGT CCTTTT TGG T3'</td>
<td>148–184</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide position according to Okamoto et al.
al.18 On the whole, these observations support our previous hypothesis19,20 that HCV infection in MC may produce a monoclonal or polyclonal B-cell expansion, already described by Perl et al.21; this phenomenon is responsible for the wide spectrum of autoantibodies observed in MC sera.20,22 Among these, the RF plays an important role in immune-complex formation, being the antibody of circulating mixed cryoglobulins.1,2 The antigens involved in the cryoglobulins can be autologous denatured IgG,1,2 HCV genomic sequences,6 or other unknown factors. Circulating cryoglobulins are responsible for the cutaneous and visceral vascular lesions of the disease, ie, purpura, skin ulcers, peripheral neuropathy, glomerulonephritis, and systemic vasculitis,1,2 whereas cryoglobulinemic hepatitis could be the result of concurrent factors, ie, viral cytolysis, autoreactive cytotoxic T-cell, and autoantibodies directed against liver cell.22 The pathogenetic relevance of HCV infection in MC is indirectly confirmed by the usefulness of α-interferon treatment in these subjects as reported in our previous controlled study.16

Taken together, the above considerations suggest that HCV infection may trigger and maintain MC through different mechanisms. Among these, HCV infection of PBMCs can represent the primary step in the pathogenesis of the clinical manifestations; besides, the HCV lymphotropism could explain some relationships observed between MC and other HCV-related diseases.20,22 First of all, the infected lymphocytes may select HCV mutants with different clinical consequences, and they could be the viral “reservoir” responsible for the disease chronicity. Moreover, HCV may trigger the clonal B-cell expansion responsible for various autoantibody production; among these, antinuclear, anti-smooth muscle, and anti-liver-kidney microsomal antibodies are considered the serologic hallmarks of different diseases.11,12,20,23 In our previous study comparing MC and autoimmune liver diseases, we showed that these disorders share various clinical and immunologic parameter alterations.20,22 These observations suggest that the same virus, alone or in combination with other factors, ie, genetic, infectious, and/or environmental, may produce a variable host response with a spectrum of clinical patterns, ie, from the autoimmune chronic hepatitis to systemic manifestations of MC. This is a lymphoproliferative disorder that has been hypothesized on the basis of clinical and laboratory investigations.3,4 The proliferative response of the PBMCs to viral antigens16 indicates that HCV could be the trigger factor of the chronic lymphoproliferation underlying MC. Moreover, the present study showing the presence of viral genome in peripheral lymphocytes suggests a direct involvement of HCV genome in the lymphoproliferative process leading to the lymphoma. In this respect, an interesting example of virus-induced human tumor is the Burkitt’s lymphoma. This B-cell lymphoma is secondary to a long-lasting infection by Epstein-Barr virus (EBV), which is a highly transforming agent for B lymphocytes. Through a complex multistep process, often in combination with chronic malaria, EBV can generate Burkitt’s lymphoma.24 Similarly, in some MC patients, after a long preneoplastic period characterized by a chronic B-cell stimulation, HCV genome could promote the expression of particular oncogenes.

As usual, new information raises many more questions than it answers. The next studies should include different

### Table 2. Clinico-Epidemiologic and Laboratory Findings of 16 MC Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Disease Duration (yr)</th>
<th>Histology</th>
<th>Liver Histology</th>
<th>Renal Involvement</th>
<th>Neuritis</th>
<th>Cryocrit (%)</th>
<th>Serum-Component</th>
<th>Monoclonal</th>
<th>Cryo-Component</th>
<th>Therapy</th>
<th>Anti-HCV RIBA II</th>
<th>Serum-HCV RNA</th>
<th>PBMC HCV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57/M</td>
<td></td>
<td>2</td>
<td>CAH</td>
<td>1.1</td>
<td>--</td>
<td>+</td>
<td>0.6</td>
<td>IgM</td>
<td>6-MP (20)</td>
<td>1/0/3/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>48/F</td>
<td></td>
<td>11</td>
<td>ND</td>
<td>0.9</td>
<td>--</td>
<td>+</td>
<td>1.8</td>
<td>IgM</td>
<td>6-MP (4)</td>
<td>4/2/3/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>65/F</td>
<td></td>
<td>2</td>
<td>CAH</td>
<td>0.9</td>
<td>--</td>
<td>--</td>
<td>2.7</td>
<td>IgM</td>
<td>6-MP (6)</td>
<td>2/2/3/3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>55/F</td>
<td></td>
<td>5</td>
<td>ND</td>
<td>2.1</td>
<td>--</td>
<td>--</td>
<td>0.7</td>
<td>IgM</td>
<td>6-MP (4)</td>
<td>2/1/3/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>66/M</td>
<td></td>
<td>9</td>
<td>CAH</td>
<td>4.5</td>
<td>--</td>
<td>--</td>
<td>0.6</td>
<td>IgM</td>
<td>α-IFN</td>
<td>0/1/4/3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>58/F</td>
<td></td>
<td>19</td>
<td>CAH</td>
<td>0.3</td>
<td>--</td>
<td>--</td>
<td>1.6</td>
<td>IgM</td>
<td>α-IFN</td>
<td>1/1/2/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>69/F</td>
<td></td>
<td>10</td>
<td>CAH</td>
<td>3.7</td>
<td>--</td>
<td>--</td>
<td>1.4</td>
<td>IgM</td>
<td>--</td>
<td>2/2/3/3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>54/M</td>
<td></td>
<td>13</td>
<td>ND</td>
<td>0.9</td>
<td>MPGN</td>
<td>+</td>
<td>0.7</td>
<td>IgM</td>
<td>4-MP (10)</td>
<td>0/0/2/4</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>64/F</td>
<td></td>
<td>11</td>
<td>CAH</td>
<td>3.3</td>
<td>--</td>
<td>+</td>
<td>17</td>
<td>IgM</td>
<td>6-MP (4)</td>
<td>0/2/3/3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>62/M</td>
<td></td>
<td>1</td>
<td>ND</td>
<td>0.6</td>
<td>--</td>
<td>--</td>
<td>1.4</td>
<td>IgM</td>
<td>--</td>
<td>2/3/4/4</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>58/M</td>
<td></td>
<td>10</td>
<td>ND</td>
<td>0.5</td>
<td>--</td>
<td>+</td>
<td>20</td>
<td>IgM</td>
<td>6-MP (4)</td>
<td>4/4/3/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>68/F</td>
<td></td>
<td>19</td>
<td>CAH</td>
<td>5.4</td>
<td>--</td>
<td>+</td>
<td>1.5</td>
<td>IgM</td>
<td>6-MP (6)</td>
<td>4/2/3/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>56/F</td>
<td></td>
<td>13</td>
<td>ND</td>
<td>1.1</td>
<td>--</td>
<td>--</td>
<td>0.6</td>
<td>IgM</td>
<td>6-MP (8)</td>
<td>1/3/3/3</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>47/M</td>
<td></td>
<td>11</td>
<td>CAH + C</td>
<td>1.9</td>
<td>++</td>
<td>+</td>
<td>13</td>
<td>IgM</td>
<td>6-MP (8)</td>
<td>2/2/4/4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>65/F</td>
<td></td>
<td>10</td>
<td>ND</td>
<td>0.7</td>
<td>--</td>
<td>+</td>
<td>2.1</td>
<td>IgM</td>
<td>6-MP (2)</td>
<td>4/2/4/3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>72/F</td>
<td></td>
<td>10</td>
<td>CAH</td>
<td>7.2</td>
<td>--</td>
<td>--</td>
<td>1.7</td>
<td>IgM</td>
<td>6-MP (4)</td>
<td>4/2/3/4</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALT (XULN), serum alanine aminotransferase (multiple of upper limit of normal); 6-MP, 6-methyl-prednisolone (mg/d); α-IFN, α-interferon (9 mega-IU/wk); CAH, chronic active hepatitis; C, cirrhosis; MPGN, membranoproliferative glomerulonephritis; ND, not done; 5.1.1, C100, C33c, C22, antibodies against recombinant HCV-related antigens used in RIBA II (score 0-4).

* Proteinuria.
patients' subsets, i.e., HCV-related chronic hepatitis, MC, and MC complicated by frank lymphoma. In addition, the detection of HCV RNA in lymphoid aggregates of the bone marrow and/or liver or lymphnodes and the isolation of specific HCV mutants, together with genetic and molecular biology investigations, should clarify this intriguing matter.

REFERENCES


Infection of peripheral blood mononuclear cells by hepatitis C virus in mixed cryoglobulinemia

C Ferri, M Monti, L La Civita, G Longombardo, F Greco, G Pasero, P Gentilini, S Bombardieri and AL Zignego