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.
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 d 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 10 mmol/L EDTA, 1 mmol/L Tris-HCl (pH 7.5), 1% sodium dodecyl sulfate (SDS), and 500 μg/ml protease K. Proteins were then extracted with phenol-chloroform and total RNA was precipitated overnight at −20°C after the addition of sodium acetate 0.2 mol/L, transfert RNA, and 2 vol of ice-cold ethanol. The 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 1.9% agarose slab gel in a Tris-borate buffer (pH 8). Amplification products were also analyzed by Southern blot hybridization using 32P-labeled HCV Oligonucleotide probes corresponding to HCV sequences inside the primers used. The membrane (Gene Screen Plus; New England Nuclear, Boston, MA) was washed twice with 0.2X SSC/1% SDS at 20°C for 15 minutes, and twice with 0.2X SSC/1% 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 PCR negative were examined; (4) RNA extraction and PCR were performed at least three times for each sample (PBMCs, sera, last washes) and a result was considered definitive only when confirmed in different experiments; and (5) positive and negative controls were used, which included RNA and DNA extracted from PBMCs and sera of healthy subjects. RNA extracted from baker's yeast, the reaction mixture without the reverse transcriptase of RNA, distilled water, and RNA extracted from 10 PBMCs and serum samples known to contain HCV RNA sequences. In particular, PCR was able to detect HCV sequences in samples containing as little as 5 to 1 HCV-infected cells as shown by limit dilution experiments of T-cell lines obtained from HCV chronically infected patients (Zignego et al, in preparation), as well as in 10^4 to 10^7 diluted sera obtained from the same patients. Finally, to avoid the risk of 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; i.e., 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. Immunosuppressive parameter investigations showed the presence of monoclonal rheumatoid factor (lgMk) 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 d 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
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 al22; 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, 2 HCV genomic sequences, 5 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 cells.20-23 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.11,12,20-25 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 may evolve into a frank non-Hodgkin’s lymphoma.24,25,39 The possible relationship between MC and lymphomas had been hypothesized on the basis of clinical and laboratory investigations.34,35 The proliferative response of the PBMCs to viral antigens18 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)/Sex</th>
<th>Duration (yr)</th>
<th>Disease Cryo Histology (XULN)</th>
<th>Liver Involvement</th>
<th>Neutritis</th>
<th>Cryocrit</th>
<th>ALT (XULN)</th>
<th>Renal Involvement</th>
<th>Neuritis</th>
<th>C33c/C22</th>
<th>C100</th>
<th>HCV RNA</th>
<th>PBMC</th>
<th>Anti-HCV RIBA II</th>
<th>Serum-HCV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57/M</td>
<td>2</td>
<td>CAH</td>
<td>1.1</td>
<td>-</td>
<td>+</td>
<td>0.6</td>
<td>IgMk</td>
<td>6-MP (20)</td>
<td>1/0/3/4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>48/F</td>
<td>11</td>
<td>ND</td>
<td>0.9</td>
<td>+</td>
<td>-</td>
<td>1.8</td>
<td>IgMk</td>
<td>6-MP (4)</td>
<td>4/2/3/4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>65/F</td>
<td>2</td>
<td>CAH</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>2.7</td>
<td>IgMk</td>
<td>6-MP (6)</td>
<td>2/2/3/3</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>55/F</td>
<td>5</td>
<td>ND</td>
<td>2.1</td>
<td>-</td>
<td>-</td>
<td>0.7</td>
<td>IgMk</td>
<td>6-MP (4)</td>
<td>2/1/3/4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>48/M</td>
<td>9</td>
<td>CAH</td>
<td>4.5</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>IgMk α-IFN</td>
<td>0/1/4/3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>58/F</td>
<td>19</td>
<td>CAH</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>1.8</td>
<td>IgMk α-IFN</td>
<td>1/1/2/4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>69/F</td>
<td>10</td>
<td>CAH</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
<td>1.4</td>
<td>IgMk</td>
<td>-</td>
<td>2/2/3/3</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>54/M</td>
<td>13</td>
<td>ND</td>
<td>0.9</td>
<td>-</td>
<td>+</td>
<td>0.7</td>
<td>IgMk</td>
<td>4-MP (10)</td>
<td>0/0/2/4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>64/F</td>
<td>11</td>
<td>CAH</td>
<td>3.3</td>
<td>-</td>
<td>+</td>
<td>17</td>
<td>IgMk</td>
<td>6-MP (4)</td>
<td>0/2/3/3</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>62/M</td>
<td>1</td>
<td>ND</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>IgMk</td>
<td>6-MP (4)</td>
<td>4/4/3/4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>58/M</td>
<td>10</td>
<td>ND</td>
<td>0.5</td>
<td>+</td>
<td>-</td>
<td>1.4</td>
<td>IgMk</td>
<td>-</td>
<td>2/3/4/4</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>62/F</td>
<td>19</td>
<td>CAH</td>
<td>5.4</td>
<td>+</td>
<td>-</td>
<td>1.5</td>
<td>IgMk</td>
<td>6-MP (6)</td>
<td>4/2/3/4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>56/F</td>
<td>13</td>
<td>ND</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>IgMk</td>
<td>6-MP (8)</td>
<td>1/3/3/3</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>47/M</td>
<td>11</td>
<td>CAH + C</td>
<td>1.9</td>
<td>+</td>
<td>+</td>
<td>13</td>
<td>IgMk</td>
<td>6-MP (8)</td>
<td>2/2/4/4</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>65/F</td>
<td>19</td>
<td>ND</td>
<td>0.7</td>
<td>-</td>
<td>+</td>
<td>2.1</td>
<td>IgMk</td>
<td>6-MP (2)</td>
<td>4/2/4/3</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>72/F</td>
<td>10</td>
<td>CAH</td>
<td>7.2</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
<td>IgMk</td>
<td>6-MP (4)</td>
<td>4/2/4/3</td>
<td>-</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, ie, 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

Updated information and services can be found at:
http://www.bloodjournal.org/content/82/12/3701.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml