Low-Grade Malignant Lymphoma, Hepatitis C Virus Infection, and Mixed Cryoglobulinemia

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Because a close relationship has been established between mixed cryoglobulinemia and hepatitis C virus (HCV) infection, the clinical, histologic, and virologic findings of 31 patients affected by mixed cryoglobulinemia have been determined. HCV infection was investigated by the presence of anti-HCV antibodies and by polymerase chain reaction (PCR) amplification of the 5' untranslated region (5'UTR), and the phenotype of HCV was also determined according to Okamoto et al. (J Gen Virol 73:673, 1992). A bone marrow (BM) biopsy was performed in all patients, and liver and kidney biopsies were performed when indicated. The prevalence of anti-HCV antibodies was high (83.9%); polymerase chain reaction amplification of the 5' untranslated region was positive in 26 subjects (83.9%), and Core region amplification in 26 of 27 subjects (96.2%). A high prevalence of genotype II was found (76.8%). Chronic liver disease was present in 15 (48%) patients. BM biopsy specimens showed the presence of low-grade non-Hodgkin's lymphomas in 12 cases (38.7%), whereas, in 11 patients (35.5%), the BM infiltration was not monoclonal (reactive). Mixed cryoglobulinemia is closely associated with HCV infection. Apparently, only 1 patient was not infected by the virus. Several HCV genotypes are involved in the pathogenesis of mixed cryoglobulinemia. The disease is associated with a high prevalence of low-grade non-Hodgkin's lymphomas.

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MIXED CRYOGLOBULINEMIA (MC) is considered a lymphoproliferative disorder characterized by arthralgias, weakness, purpura, and organ involvement such as glomerulonephritis, peripheral neuropathy, vasculitis and chronic liver disease. The development of malignant B-cell lymphoma has been reported in a variable fraction of these patients. Recently, evidence of hepatitis C virus (HCV) infection in the majority of these patients has been shown even without clinical or biochemical evidence of liver damage, suggesting that HCV could be the main etiologic factor of MC. Furthermore, it has been shown that HCV is a hepatotropic as well as a lymphotropic virus. Positive- and negative-stranded (replicative) forms of HCV have been observed in peripheral blood mononuclear cells of patients with chronic liver disease, and the presence of HCV infection in patients affected by IgM monoclonal gammapathies has been recently reported. On the basis of these observations, HCV infection could be responsible not only for chronic liver disease but also for lymphoproliferative disorders.

Recent reports showed that HCV presents a high degree of genomic diversity, and major differences have been observed between Japanese and American isolates. Both structural (Core, Envelope) and nonstructural (NS1 -5) regions show heterogeneity, whereas only the 5' untranslated region (5'UTR) seems to be conserved among all HCV genotypes so far isolated. Different classifications of HCV have been proposed on the basis of the entire genome or of limited regions. The classification of HCV is not solely a taxonomy problem but has important clinical significance, because different types have different geographical distributions and seem to determine liver disease of different severity.

To better define the role of HCV infection in the pathogenesis of MC and MC-related lymphoproliferative disorders, the clinical, histologic, and virologic findings of a group of 31 patients affected by MC, followed up over a period of 10 years, have been carefully examined. Moreover, the genotype of HCV was obtained by amplification of the Core region of HCV with type-specific primers according to Okamoto et al.

MATERIALS AND METHODS

Patients

A total of 31 patients were included in the study, selected among a group of 82 subjects affected by MC. In all cases, the diagnosis of MC was based on the presence of the typical syndrome (purpura, weakness, and arthralgias) associated with circulating cryoglobulins. All patients were Italian heterosexuals and had no history of drug or ethanol abuse. Only patients with a follow-up lasting at least 10 years and with available stored serum samples, collected at the time of diagnosis, were included in the study. A total of 31 patients were excluded for the following reasons: absence of stored serum samples (42 cases), too short a follow-up period (5 cases), refusal to undergo bone marrow (BM) biopsy (4 cases), and death before the availability of fluorescence-activated cell sorter (FACS) technology (3 cases). The study was approved by the ethical committee of the Pordenone General Hospital, and each patient gave informed consent before entry.

Purpura scoring system.

To assess the severity of the vasculitis, a simple clinical scoring system was used. A score of 0 indicated the absence of skin lesions; a score of 1, the presence of less than 10 purpuric spots on the lower limbs; a score of 2, the presence of more than 10 spots on the lower limbs; a score of 3, the extension of the spots to the trunk and/or upper limbs; and a score of 4, the presence of skin ulcers and/or gangrene.

The presence of visceral complications (ie, peripheral neuropathy, renal or liver involvement) were investigated following standard methods. Rheumatoid factor (RF) and C3 and C4 fractions of complement were measured by rate nephelometry. Routine liver function tests as well as hematologic parameters were determined by usual laboratory methods. Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) markers were detected by enzyme-linked immunosorbent assay (ELISA) using commercial kits. In each patient, the presence of the following autoantibodies was also determined: antismooth muscle, antimitochondrial, organ nonspecific antinuclear.

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anti–liver–kidney microsomal antibodies. Thyroid function tests were also determined with standard methods.

Cryoglobulin isolation. A total of 20 mL of blood was kept at 37°C for 2 hours in a glass tube. The serum was cleared by centrifugation at 4,000 rpm for 20 minutes at room temperature and was stored at 4°C for 7 days. The cryoprecipitate was separated by centrifugation at 4,000 rpm for 30 minutes at 4°C. Mixed cryoglobulins were classified as type II by the presence of monoclonal Igs with RF activity, complexed with polyclonal IgG.

Histology. BM examination was performed in all patients. BM smears were stained according to standard procedures. BM biopsy specimens, obtained with Jamshidi needles, were placed in B5 solution and, 2 hours later, in ethanol 70%. All samples were blindly reviewed by the same experienced pathologist (S.S.), who was not aware of any clinical data of the patients. When appropriate, surgical excision of lymph nodes was performed.

Immunohistochemistry (alkaline phosphatase antialkaline phosphatase [APAAP] method) was performed in both BM and lymph node specimens. On the basis of the histologic and immunologic findings, the diagnosis was determined according to the “Working Formulation.”

Liver biopsies were performed only in patients with clinical and biochemical signs of chronic liver disease. The biopsy specimens were obtained using a Menghini-type needle (Hepafix, Braun, Germany) with an internal diameter of 1.8 mm. The samples were fixed in 10% formalin and routinely stained with hematoxylin and eosin and with Gomori stain for reticulum.

Phenotyping. Mononuclear cells were separated from blood samples and from BM aspirate on an Emagel density gradient. Cells were stained with specific monoclonal antibodies, and, after incubation and washing, immunofluorescence was measured by FACSscan flow cytometry (Becton Dickinson, Mountain View, CA). Monoclonal antibodies against CD3, CD4, CD5, CD8, CD16, CD19, CD57, and IgM were used. Anti-CD3, anti-CD4, and anti-CD8 (OKT3, OKT4, and OKT8) were purchased from Ortho Diagnostic Systems (Raritan, NJ); anti-CD19 (B4-DR1) was added, and the sample was incubated with hepatosomy and eosin and with Gomori stain for reticulum.

The monoclonality of peripheral and/or BM lymphocytes was evaluated by FACS determination of the surface light-chain distribution. The summation curves of the histograms were analyzed with Kolmogorov-Smirnov statistics and a D value (the magnitude of difference between and A distribution) more than 10.0 was considered significant for monoclonality.

Anti–HCV antibodies. The presence of anti–HCV antibodies was assessed by the second generation immunoenzymatic screening test ORTHO-HCV (Ortho). In all positive and negative tests, an additional confirmatory test (RIBA; Chiron Corp, Emeryville, CA) was performed. This assay detects specific reactivity to four HCV antigens, including three nonstructural (C100-3, 5-1-1, C-33c) and one structural (C22) antigen. Sera showing two or more positive bands were considered “reactive”, whereas those with only one band (usually C22) were defined as “indeterminate”, and those without HCV antigen bands were considered “negative.”

HCV-RNA Detection

The presence of HCV-RNA in serum was assessed in all patients by amplification with the polymerase chain reaction (PCR) of the 5’UTR of HCV, which is considered the most conserved region of the virus. The amplification was performed in two steps, the so-called “nested PCR.” To minimize the risk of contamination as a source of false positive results, a negative control was included in each batch of serum samples. Moreover, an additional negative control was included in each step of the procedure, ie, RNA extraction, reverse transcription, and PCR amplification. All pipettes and containers were disposable.

cDNA synthesis. A total of 3 mL of serum (3 mL of diethylpyrocarbonate [DEPC] H2O in negative control) was added to a mixture containing 5.0 μL of 5× buffer, 1.0 μL Nonidet p-40 10%, 1.0 μL human placental RNase inhibitor (HPR1), 50 pmol antisense external primer, and H2O/DEPC for a total volume of 22.0 μL. The mixture was incubated in ice-cold water for 5 minutes, at 92.0°C for 30 seconds, and again in ice-cold water for 5 minutes. After centrifugation, 4 μL of reverse transcriptase (RAV-2; Amersham, Arlington Heights, IL) were added, and the sample was incubated for 60 minutes at 42°C.

Nested PCR. For the first step, 25.0 μL of the cDNA (25.0 μL of DEPC water in the negative control) was placed in a mixture containing 10.0 μL of 10× buffer, 8.0 μL deoxyribonucleotide triphosphate (dNTP; 2.5 mmol/L), 50 pmol external primers, 2U/μL Taq Polymerase, and water for a total volume of 75.0 μL. Amplification was performed in a DNA thermal cycler for 35 cycles. Each reaction cycle included denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 2 minutes.

For the second step, 3 μL of the product of the first amplification was subjected to a second PCR (at the same conditions) for 25 cycles using the internal primers. The products of the second PCR were analyzed by agarose gel electrophoresis, were stained with ethidium bromide, and were observed under UV light. A Southern blot hybridization was also performed using an internal probe (5’CAATTCCCGGTGTTACTCCGGTCCG-3’) labeled with [32P]phosphorus adenosine triphosphate (Amity, Milan, Italy).

Primers. The following primers (synthesized with Gene Assembler [Pharmacia, Uppsala, Sweden]) were used: external antisense: 5’-GATGCACGCTTCTACGAGACCTC-3’, nucleotides (nts) -257 to -21; internal sense: 5’-AACTACTGTCTTCTACGGCAAGA-3’, nts -289 to -269; internal antisense: 5’-GGCAGCCAACACTACTCTGGCT-3’, nts -70 to -90; and internal sense: 5’-ATGGCGTTAGTA TGAGTG-3’nts -257 to -240.

HCV Genotyping

The HCV genotype was studied in both HCV-RNA–positive and –negative sera by amplification of the Core region according to Okamoto et al. Each HCV type was characterized by a different nt length: 57 for type I, 144 for type II, 174 for type III, and 123 for type IV. When a double infection was suspected by the presence of several bands, a second PCR was separately repeated with each of four type-specific antisense primers.

Primers. The following primers were used. The universal primers were specific: 5’-CAGGGAGACTAGGAAGACCTC-3’, nts 139 to 158; and antisense: 5’-ATGTACCCCATGAGGTCGGC-3’, nts 410 to 391. The internal primers were used as follows: type I: 5’-TGCCTTGCGGGATAGCGTAC-3’, nts 204 to 185 (174 bp); type II: 5’-GAGCCATCTGGCCACCCCA-3’, nts 291 to 272 (144 bp); type III: 5’-CCAAGAGGAGGAGAAGCCCTC-3’, nts 321 to 302 (174 bp); and type IV: 5’-ACCCTGGTTTCGGATAG-3’, nts 270 to 251 (123 bp).

Statistical Analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed by using the statistical package “SPSS.” Partial association was studied by using the hierarchical log-linear analysis in a multiway cross-tabulation.

RESULTS

The main clinical and laboratory data of the patients are summarized in Table 1. The ages of the patients are the age
at the time of the first diagnosis (mean age, 61.7 ± 11.5). All patients had purpura (mean score, 1.8 ± 0.6), weakness and variable levels of RF (ranging from undetectable to 20,400 IU), and cryoglobulins (mean level, 9.3% ± 12.1%). A total of 4 patients showed mild proteinuria and elevated serum creatinine. In these cases, kidney biopsy specimens showed the presence of membranoproliferative glomerulonephritis. Liver biopsy was performed in 15 patients (48%); the findings are reported in Table 2. The histologic diagnoses were highly variable, ranging from minimal changes (microvascular steatosis and/or portal lymphoid aggregates) to chronic active hepatitis with or without superimposed cirrhosis.

The virologic findings of the patients are reported in Table 2. The majority of patients (77.4%) were positive for the presence of anti-HCV antibodies. Only 1 patient was positive for the presence of hepatitis B surface antigen (HBsAg), and 3 subjects showed the presence of anti-HBsAg antibodies. None of these patients were in the active phase of viral replication, because circulating HBV-DNA was absent.

PCR amplification of the 5'UTR region of HCV was positive in 26 of 31 stored sera (83.9%) at the time of diagnosis. PCR was again performed in all patients still alive (27 subjects), and HCV-RNA was detected in 22 cases. Two patients positive for HCV-RNA at the time of diagnosis were negative at the time of the study; these 2 subjects had been treated with a-interferon (α-IFN). Two patients who were sera-negative at the time of diagnosis were currently positive.

The determination of the HCV genotype was assayed in either stored or recently collected sera, both positive and negative for HCV-RNA. As shown in Table 2, all known genotypes of HCV were found, with a high prevalence of type II (76.6%). In the 4 cases of co-infection, type II was always present (type II + IV, 2 cases; type I + II + III, 2 cases). A total of 3 cases negative for anti-HCV antibodies and negative for HCV-RNA were positive by Core PCR amplification. In 6 cases (19%), the HCV genotype was unclassifiable (UC; ie, Core amplification was positive but multiple or unexpected bands were present on the agarose gel). In these cases, PCR amplification was repeatedly performed, always with the same results.

BM biopsy was normal in only 9 (29.0%) subjects. In most patients, paratrabecular foci of infiltration by small lymphoid elements with plasmacytoid features were present. As shown in Table 3, clinical, biochemical, histologic, and immunologic findings in 9 subjects (29.0%) suggested the diagnosis of non-Hodgkin Lymphoma (NHL) at the time of diagnosis.
of 9 subjects had a diagnosis. Diagnosis was supported by a more than 50% plasmacytoid appearance, but light-chain distribution did not show monoclonality (D value less than 10.0 at the Kolmogorov-Smirnov test).

Three patients (patients no. 7, 12, and 19) with reactive lymphocytosis at the time of diagnosis, developed lymph-node enlargement. In 2 patients with cirrhosis died from liver failure. Two patients with chronic liver disease treated with α-IFN (3,000,000 U subcutaneously three times a week for 1 year) recovered with disappearance of HCV-RNA and normalization of ALT. At present, these 2 patients have no detectable levels of cryoglobulins and are asymptomatic. The remaining patients are treated by periodic plasma-exchange, glucocorticoids, or hypallergenic diet.

**DISCUSSION**

The present study confirms previous observations on the striking association between MC and HCV infection. In our 31 patients, the presence of anti-HCV antibodies was high (24 of 31 subjects; 77.4%), and the 5'UTR of HCV was detected by PCR in 26 cases in the stored sera (83.9%). In 2 cases, 5'UTR amplification was negative in stored sera (positive for anti-HCV antibodies) but positive in freshly
collected samples, which may have been caused by an incorrect storage of the sera. Therefore, the level of HCV-RNA detection by PCR was 90.3%. The presence of the virus was associated with clinical and biochemical signs of chronic liver disease in only a fraction of patients (15 of 31; 48.4%). In 4 patients negative for the presence of anti-HCV antibodies, PCR amplification of the 5'UTR of HCV was positive in 1, whereas Core amplification was positive in 3. This observation indicates that the absence of detectable levels of anti-HCV antibodies does not exclude the presence of HCV infection. The absence of anti-HCV antibodies could be explained, in some cases, by the low level of normal Igs. In all monoclonal neoplasias (multiple myeloma, Waldenström's disease, light chain disease) the production of normal (nonmonoclonal) Igs is impaired. Therefore, because MC is also a condition characterized by abnormal Ig synthesis, the level of anti-HCV antibodies may be under the detection limits of the assay. Another possibility is the presence of unusual HCV genotypes. The absence of detectable levels of anti-HCV antibodies was associated with the presence of genotype III (2 cases), with a coinfection (II + IV), and with an unclassifiable genotype (1 case). Because usual commercial kits to detect anti-HCV antibodies have been developed on the basis of the sequence of structural and nonstructural regions of HCV-H (type I), which shows a good nt homology with type II, the commonly used kits detect very efficiently the presence of antibodies in the case of type I or II infection, whereas slight (or even absent) positivity can be found in the case of type III or IV infection. On the basis of these data, close attention should be given to slight positivity of anti-HCV tests in immunocompromised patients, and even negative sera should undergo PCR amplification if HCV infection is suspected.

Amplification of the Core region of HCV allowed higher detection of viral RNA. In fact, 3 samples negative for anti-HCV antibodies and negative for 5'UTR showed clear positivity by Core amplification. Thus, the percentage of HCV-infected subjects increased to 96.7%. On the basis of these findings, the reliability of amplification of 5'UTR as absolute proof for the absence or presence of HCV-RNA should be reevaluated. Although 5'UTR is considered a very conserved region of HCV, sequence analysis shows a rather high heterogeneity, often in the first 20 nts where our external sense primer was placed. Therefore, the lack of amplification may derive from primer/template mismatch. The presence of HCV-RNA in subjects lacking anti-HCV antibodies

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<th>BM Monoclonality</th>
<th>Peripheral Blood Monoclonality</th>
<th>IgM level (μg/L)</th>
<th>Disease Progression</th>
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Abbreviations: NHL, non-Hodgkin lymphomas, in parentheses is the classification according the Working Formulation; ND, not determined; UC, unclassifiable HCV genotype.
and the presence of 5'UTR open a new field in the study of HCV epidemiology and pathophysiology.

HCV genotyping showed that multiple genotypes are involved in pathogenesis of MC. The high prevalence of type II is not surprising, because this genotype seems to be the most diffuse worldwide. A large epidemiologic study on the prevalence of different HCV genotypes in Northeast Italy showed that type II is the most common (71%), whereas types I, III, and IV and coinfections are less represented. Therefore, the distribution of HCV types in MC in the current study reflects the prevalence of different genotypes in our geographic area.

The study of the HCV genotype at the time of diagnosis and many years later showed that the genotype did not change over time and that additional infections by different genotypes did not occur during follow-up. This finding does not exclude the possibility of single nt mutations in each viral region because of the high mutation rate of the virus (1.44 × 10⁻³ base substitutions per year).²¹

The presence of 6 cases with unclassifiable genotypes indicates that unknown new genotypes could be present in our area, as recently pointed out in Southeast Asia.³²

Our data on the prevalence of BM infiltration in the population of subjects affected by MC suggests that this disease is sustained by a monoclonal (or monotypic) proliferation of B lymphocytes. When diagnosis is early, this proliferation remains speculative. The question is important because genotypes did not occur during follow-up. This finding does not change over time and that additional infections by different HCV in the immune system could greatly expand clones of lymphocytes.

The mechanism, and a mutational event could eventually lead to activation of oncogenes resulting in a B-cell neoplasm. For example, chronic infection of the immune system could play a direct role in the development and control of viral disease. Hepatology 15:382, 1992

The development of NHL could be further promoted by therapy for MC based, in the past, on the use of glucocorticoids or other immunosuppressive drugs, thus increasing viral replication and the number of infected cells. Currently, α-IFN seems to be the drug of choice to treat MC, because it reduces symptoms and cryoglobulin production.²⁰ IFN shows powerful antiproliferative action, but also shows antiviral effects, which would be very useful in MC. IFN reduces lymphocyte proliferation and inhibits viral replication. In our 2 subjects treated with IFN, cryoglobulin production was undetectable; this fact further supports the pathogenetic role of HCV in MC.

On the basis of the present data MC seems to be correlated with HCV in the near totality of cases. The presence of the virus in the immune system is associated with BM lymphocyte infiltration and with a high prevalence of low-grade NHL. Long-term IFN therapy might be useful for control of viral replication and, thus, for prevention of low-grade NHL. Finally, HCV should be considered a potential oncogenic virus, although its mechanism of action has yet to be established.

REFERENCES


Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia

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