Hepatitis G virus infection markers (RNA and anti-E2 antibodies) in a multicenter cohort of hemophiliacs

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ABSTRACT

Background and Objectives. To investigate the prevalence and evolution of hepatitis G virus (HGV) infection in hemophiliacs and to correlate evolution of HGV infection markers with immunologic parameters in those patients co-infected with HIV.

Design and Methods. HGV RNA and anti-E2 antibodies were studied in 124 patients. Serial samples were drawn every 4 months from 1992 to 1996. Lymphocyte subsets including T-helper lymphocytes, T-suppressor lymphocytes, T-cytotoxic lymphocytes, activated T-lymphocytes and natural killer cells were analyzed.

Results. Prevalences were 22.6% for HGV RNA and 18.5% for anti-E2. Four patients had both HGV RNA and anti-E2, so the overall prevalence of HGV infection in hemophiliacs was 37.9% (11.5% in 200 controls, p<0.0001). After a median follow-up of 36.6 months 20 patients remained HGV RNA positive, whereas HGV RNA had cleared in 8, with an actuarial probability of clearance at 36 months of 34.6%. Only 2 patients developed anti-E2 antibodies. Four patients cleared anti-E2, with an actuarial probability at 36 months of 24.8%. In patients with HIV infection, both lower CD4+ lymphocyte count (p=0.01) or higher CD8+ lymphocyte count (p=0.03) showed predictive value for probability of clearing HGV-RNA. CD4+/CD8+ ratio (p=0.002) was the only variable included in the best model for HGV-RNA disappearance.

Interpretation and Conclusions. A more accurate estimation of the prevalence of hepatitis G infection can be achieved with the determination of both HGV RNA and anti-E2. Anti-E2 response can be undetectable or transitory after disappearance of HGV-RNA, giving therefore rise to the possibility of underestimating HGV prevalence with currently diagnostic methods. In HIV-positive patients, cellular immune function seems to be involved in the resolution of HGV infection, following the significant correlation found between clearance of HGV-RNA and CD4+/CD8+ lymphocyte populations.

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Transfusion of plasma derivatives is a vital therapeutic intervention in hemophiliacs, but it carries the risk of transmitting viral infections. Many hemophilia A patients treated in the past with non-virus-inactivated factor VIII concentrates became infected with human immunodeficiency virus (HIV), and almost all have been infected with hepatitis C virus (HCV). Recently, a new parenterally transmitted agent potentially responsible for some cases of non-A-E hepatitis has been reported by two independent groups and named as the hepatitis GB virus C (GBV-C) and hepatitis G virus (HGV). Sequence analysis data have demonstrated that GBV-C and HGV are independent isolates of the same virus, showing 86% nucleotide and 96% amino acid similarity. The HGV genome is a positive single stranded RNA molecule containing approximately 9,400 nucleotides and has a genomic organization resembling that of Flaviviridae with 25% nucleotide homology to hepatitis C virus.

Studies of viral RNA have demonstrated a worldwide distribution of HGV. It has been reported to infect blood donors, recipients of either transfusions or organs, and populations with risk factors for blood-borne viral infections such as intravenous drug users, patients on hemodialysis, and hemophiliacs. Initially HGV was implicated in acute or chronic hepatitis of non A-E etiology, and in some cases of fulminant hepatitis, although there is growing evidence of the scarce pathogenic role of HGV in hepatitis. In addition, viral RNA has been detected in plasma pools, intravenous immunoglobulins, and non-virus-inactivated factor VIII concentrates. Parenteral transmission of HGV is widely documented and seems to be the most common route of infection, although vertical transmission of HGV has also been reported. On the other hand, the high frequency of HGV infection in patients co-infected with hepatitis B virus (HBV) or with HCV suggests that they might have common routes of transmission.

Analysis of HGV prevalence has been based to date on detection of viral genome by nucleic acid amplification techniques and, recently, a test for antibody detection to the E2 envelope region of HGV (anti-E2
Patients were categorized in CDC groups I to IV. Of the 124 patients, 117 had severe hemophilia (range: 5-60 years). Thirty-eight were children (<16 years). Informed consent was obtained from each of the participants. The median age of the patients was 22 years (range: 5-60 years). Of the 124 patients, 117 had severe hemophilia (factor VIII:C ≤2 U/dL), 4 moderate (factor VIII:C 2.5-5 U/dL) and 3 mild (factor VIII:C ≥5 U/dL). Patients were categorized in CDC groups I to IV according to the CDC classification. Prior to inclusion in the study the patients had been treated with non-viral-inactivated factor VIII concentrates until 1985 and with viral-inactivated concentrates thereafter. From inclusion in the study the patients were treated only with affinity chromatography-purified factor VIII (n=70), ion exchange chromatography-purified factor VIII (n=51), or intermediate purity factor VIII (n=3). The average units/year transfused of affinity chromatography-purified factor VIII were 79,278, of ion exchange chromatography-purified factor VIII 75,938, and of intermediate purity factor VIII 87,554.

A total of 200 consecutive non-renumerated healthy blood donors (114 men, 86 women, mean age 39 years, range 18-65 years) were also studied as the control group.

**Sampling**

Blood samples were obtained from patients every four months from 1992 to 1996, whereas a single sample from blood donors was evaluated. For viral studies, serum was separated from whole blood and immediately frozen at −70°C until tested. Samples were carefully handled and aliquoted to avoid contamination and degradation of viral nucleic acid. Samples were not thawed more than once. For determination of lymphocyte subsets, EDTA anticoagulated whole blood was obtained and immediately analyzed.

**HIV and HCV serology**

Anti-HIV and anti-HCV antibodies were detected in serum by third generation ELISA methods (Ortho HIV-1/HIV-2 Ab-Capture ELISA /Ortho HCV 3.0 ELISA, Ortho Diagnostic Systems Inc., Raritan, NJ, USA) and confirmed by Western Blot (Bioblot HIV-1 plus, Biokit SA, Barcelona, Spain) and RIBA (Chiron RIBA HCV 3.0 SIA, Emeryville, CA, USA), respectively.

**HGV RNA PCR**

RNA was extracted from 140 µL of serum using silica-gel-based membrane columns (QIAGEN® viral RNA, Qiagen, Hilden, Germany) and eluted in a final volume of 50 mL of RNase-free water. Complementary DNA was synthesized from 10 µL of RNA by 45 min incubation at 42°C with 25U of Expand™ reverse transcriptase, 50 nM of random primers, 1 U of ribonuclease inhibitor, and 200 µM of each deoxynucleotide in a final volume of 20 µL (all from Boehringer Mannheim, Mannheim, Germany). The primers selected for HGV cDNA amplification were deduced from the 5' non-coding region (5'NCR), due to the high conservation of the 5’NCR sequence postulated for HGV. The following primers were used: 5'-CCG CCA AAA GGT GGT GGA TG-3', sense primer, position 101-120 and 5'-CGA CGA GCC TGA CGT CGG G-3' antisense primer, position 285-267 and digoxigenin labeled deoxynucleotides (Boehringer Mannheim). PCR was performed in a Technegene thermal cycler (Technegene, Cambridge, UK) by 40 cycles at 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min, and a final extension cycle of 5 min at 72°C. Digoxigenin-labeled PCR products were detected by solution hybridization to a biotin-labeled 5’NCR specific capture probe (sequence 5’-GCT AGC CAC TAT AGG TGC G-3’, position 161-179) that is complementary to the inner part of the amplicon. Hybrids were immobilized to a streptavidin-coated microtiter plate surface. Unspecific amplification products do not hybridize to the capture probe and are removed during the following washing steps. The bound hybrid...
was detected by an anti-digoxigenin peroxidase conjugate (PCR ELISA DIG detection, Boehringer Mannheim). Absorbance was measured at 405 nm. The mean optical density (O.D.) of positive controls was 1.204 (range: 1.021-1.427) and the mean O.D. of negative controls was 0.046 (range: 0.038-0.057). Samples giving a signal five times above the mean O.D. of negative controls were considered positive. Positive results were accepted upon agreement on repeated testing. The sensitivity of the assay has been reported to be of 8×10⁶ genome equivalents per mL.28 The recommendations from Kwok and Higuchi29 were followed strictly in order to avoid false positive results. Appropriate positive and negative controls were used for extraction, retrotranscription, amplification, and detection steps. Positive controls included serum samples known to contain HGV RNA in high and low concentrations, and amplification and detection of a control human gene (tissue plasminogen activator gene). Negative controls included sera negative for HGV RNA, as well as reagents without template.

Detection of antibodies to the E2 protein of HGV
Antibodies to the E2 envelope protein of HGV were determined by an ELISA method (mPlate anti-H Genv, Boehringer Mannheim) as described elsewhere.30

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The mean optical density (O.D.) of the positive controls was 1.015 (range: 0.896-1.188) and the mean O.D. of the negative controls was 0.061 (range: 0.053-0.069). The cut-off value was calculated as recommended by the manufacturer. A sample was considered negative if the absorbance was lower than the cut-off value. A sample was considered positive if the absorbance was higher than the cut-off value. Positive samples were considered positive if the absorbance was higher than the cut-off value and confirmed by repeated testing.

Lymphocyte subsets
Mononuclear cells were first isolated from whole blood by standard Ficoll-Hypaque density gradient centrifugation. The cells were washed twice in phosphate-buffered saline (pH 7.2, Bio-Mérieux, Marcyl’Etoile, France) containing bovine serum albumin (Sigma, St. Louis, MO, USA) and human decomplemented AB group serum. Lymphocyte subsets were analyzed by double direct immunofluorescence flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA, USA) using Lysis II software. Monoclonal antibodies conjugated with phycoerythrin or fluorescein (all from Becton Dickinson) were used to analyze the following cell subsets: total T-lymphocytes (CD2⁺/CD3⁺), total B-lymphocytes (CD19⁺), T-helper lymphocytes (CD4⁺), T-suppressor lymphocytes (CD8⁺), natural killer cells (including CD57⁺/CD8⁻; CD16 (Leu-11c); CD56⁺/CD3⁻), T-cytotoxic lymphocytes (CD3⁺/CD56⁻), and activated T-lymphocytes (CD3⁺/CD25⁻; CD3⁺/CD71⁻; CD3⁺/HLA-DR⁻). A total of 10,000 lymphocytes were acquired for each sample. Positivity for each monoclonal antibody was established using isotype controls with the same fluorochrome.

Results
The median follow-up of the cohort was 36.6 months (range: 6.1 to 44.3). Four patients died at 6.1, 7.2, 8.2 and 26.0 months of follow-up. Antibodies to HCV were detected in 114 patients (92.0% CI: 87.2-96.8%) and antibodies to HIV in 105 patients (84.6% CI: 78.2-91.0%). Fifty-seven patients were classified in CDC group II, 7 patients in CDC group III, and 41 patients in CDC group IV.

Lymphocyte subsets counts in HIV-positive patients and in controls are depicted in Table 1. Statistically significant differences were observed between patients and controls for almost all the lymphocyte subsets analyzed. However, no significant differences were found in total T-lymphocytes, total B-lymphocytes, T-helper lymphocytes, T-suppressor lymphocytes, natural killer cells, T-cytotoxic lymphocytes or activated T-lymphocytes counts at the end of follow up in comparison to the recruitment values (Table 1). CD4⁺ lymphocyte count decay slopes ranged from -0.91 to -0.80 with a median of -0.01.

HGV infection on recruitment
At the time of inclusion in the study, HGV RNA was detected in 28 patients (22.6%, CI: 15.2-30.0%) and anti-E2 antibodies were detected in 23 patients (18.5%, CI: 11.7-25.3%). Only 4 patients had HGV RNA and anti-E2 antibody simultaneously (Table 2). The whole prevalence of HGV infection in hemophiliacs was, therefore, 37.9% (CI: 29.3-46.5%). In the control group HGV RNA was detected in 3% (CI: 0.6-5.4%) and anti-E2 antibodies in 8.5% (CI: 4.6-12.4%). The total prevalence in the control group was thus 11.5% (CI: 7.1%-15.9%) (p < 0.0001 compared
with hemophiliacs). No significant differences were found in serum aminotransferase levels between patients with or without HGV infection (AST mean ±SD: 76.1±134.2 U/L vs 70.1±163.4 U/L; ALT mean ±SD: 72.8±143.4 U/L vs 63.2±102.1 U/L).

In HIV positive patients, no differences were found in the lymphocyte subsets analyzed among patients with or without HGV infection. We did not find significant differences in the O.D. of the anti-E2 test in anti-E2 positive and anti-E2 negative samples between patients infected or not by HIV.

The prevalence of HGV infection increased with age in hemophiliacs. Pediatric patients had a significantly lower prevalence of HGV infection than adults (23.6% vs. 44.2% p <0.05). The prevalences of HIV and HCV infection were also lower in the children (65.8% vs. 93% for HIV, p <0.001 and 73.6% vs. 98.8% for HCV, p <0.001).

HGV infection through follow-up

Of 28 initially viremic patients, 20 (71.4%) remained HGV RNA positive throughout the study. In 8 patients (28.6%) HGV RNA became undetectable at 5.5, 5.7, 10.6, 11.1, 17.5, 26.0, 32.0, and 36.0 months of follow-up. The actuarial probability for HGV RNA clearance at 36 months was 34.6% (CI: 30.6-38.6%) (Figure 1). Two of these patients showed an anti-E2 antibody response while 6 remained anti-E2 antibody negative and HGV RNA negative in repeated samples (median follow-up after HGV RNA clearance: 27.6 months, range: 14.3-36.4 months). Of the 4 patients with simultaneous positivity for HGV RNA and anti-E2 antibody at recruitment, 3 remained positive for both markers at 40.4, 41.3 and 44.3 months of follow-up, while one patient cleared HGV RNA at 26.6 months (Table 2). In patients with HIV infection, lower CD4+ lymphocyte counts (p=0.01), and higher CD8+ lymphocyte counts (p=0.03) showed predictive value for the probability of clearing HGV-RNA. No relationship was found between HGV-RNA clearance and the remaining lymphocyte subsets or CD4+/CD8+ lymphocyte count decay. In the multivariate analysis the predictive model for HGV-RNA clearance included both CD4+ lymphocyte counts (considering <300×10^6/L=1, 300-600×10^6/L=2, >600×10^6/L=3; p=0.01, coefficient=1.6695, standard error=0.8055, RR=5.31) and CD8+ lymphocyte counts (considering <700×10^6/L=1, 700-1,000×10^6/L=2, >1,000×10^6/L=3; p=0.03, coefficient=0.9030, standard error=0.5119, RR=0.41) (Figure 2). When CD4+/CD8+ ratio was considered in the analysis, CD4+/CD8+ ratio was the only variable included in the best model for HGV-RNA clearance (considering <0.25=1, 0.25-0.50=2, >0.50=3; p=0.002, coefficient=1.5764, standard error=0.5917, RR=4.84) (Figure 2).

Of 19 patients anti-E2 antibody positive at recruitment, 15 (78.9%) remained positive while in 4 patients (21%) the anti-E2 antibody became undetectable and persisted negative for a median follow-up of 15.8 months (range: 8.7-21.5 months). The actuarial probability of becoming anti-E2 antibody negative at 36 months was 24.8% (CI: 19.6-30.0%) (Figure 1). No significant relationship was found between the studied variables and anti-E2 clearance.

In an 11-year old HIV and HCV positive patient, HGV RNA became positive at 18.2 months of follow-up. Anti-E2 antibody response was observed following HGV RNA positivity and HGV RNA became undetectable 25.5 months later. He had not received packed red cells, plasma, platelet concentrates or cryoprecipitates during the study.

Discussion

The prevalence of HGV viremia found in our series (22.6%) is consistent with previous reports on hemophiliacs from other geographical areas, ranging from 15% to 24% (5,16,35). The prevalence of anti-E2 antibodies was 18.5%, indicating that overall exposure to HGV in hemophiliacs is higher than expected from the
sole determination of HGV RNA. This prevalence is significantly higher in hemophiliacs than in the healthy population with no known risk for blood-borne viral infections; overall prevalence of HGV in our blood donor population (11.5%) is similar to that reported for German blood donors (10%). In our series, prevalence of HGV infection increased with age; the group of patients under 16 years had a significantly lower prevalence of HGV infection than adults. This observation could be related to the lesser exposure to non-virus-inactivated factor VIII concentrates in the pediatric group and mirrors the lower prevalence of HIV and HCV infection in pediatric hemophiliacs than adult ones.

We found that most of the initially viremic hemophiliacs remained viremic during follow-up with an actuarial probability of clearing HGV RNA of only 34.6% at three years. Long-term HGV viremia has been reported to occur in hemodialysis patients and in a high proportion of non-immunosuppressed patients infected with HGV. It is noteworthy that, in a proportion of our patients (6/28), HGV RNA became undetectable during follow-up without an anti-E2 antibody response being observed, and, in addition, of 19 patients initially anti-E2 antibody positive, 4 (21%) became anti-E2 negative during follow-up, indicating that anti-E2 antibody response may be transitory. Although the development of anti-E2 antibodies has generally been associated with clearance of the HGV RNA and resolution of infection, the coexistence of both markers has also been observed in some patients for a limited time. In a recent publication, failure to clear HGV RNA despite the presence of specific antibodies was reported to occur in patients co-infected with HIV and/or HCV and this fact has been attributed to the immunosuppressive effects of HIV infection. In our series, simultaneous positivity for both markers was observed at recruitment in 4 patients (3.2%) but we found that HGV RNA and anti-E2 antibodies can overlap for a prolonged period. Furthermore, in two patients HGV RNA cleared after many months of overlapping of
both markers, suggesting that, despite HIV related immunosuppression, some patients are finally able to clear the virus. The exact mechanisms of persistence of or recovery from HGV infection are, however, still not well known. In this context, it has been recently reported that anti-E2 antibodies protect against de novo HGV infection in liver transplant recipients, suggesting that these antibodies have a neutralizing action. Nevertheless, the development of anti-E2 may not be the only factor implicated in HGV infection resolution, since we observed, in agreement with a recent report from Prati et al., that some patients seem to clear their infection without developing anti-E2. Thus, there is the possibility of underestimating the true incidence of HGV infection (and also the true incidence of reinfection). However, we did not identify any reappearance of HGV RNA, suggesting HGV reinfection, during the follow up.

In contrast to HCV, the ability of HGV to evade the immune system does not appear to rely on hyper-variable regions within E2, suggesting that other mechanisms involving interference of HGV with cellular T-cell response may be implicated. An additional finding of our study is that in HIV positive patients CD4+ and CD8+ lymphocyte counts have predictive value for the disappearance of HGV-RNA whereas natural killer cell counts did not show a significant relationship. This indicates that in HIV-infected hemophiliacs mechanisms associated with the cellular immune response may be involved in the resolution of HGV infection, independently of the development of the anti-E2 antibody. In HIV-infected patients, natural killer activity can be reduced despite a normal cell number. This effect, in addition to CD4+ T-lymphocytes depletion and disturbances in cytokine balance, may represent a key pathogenic factor which facilitates the persistence of HGV infection in these patients. However, it must be noted that in these patients the possibility of co-infection with known or unknown viruses could influence the persistence of infections.

Finally, one patient became HGV RNA positive during the study at 18.2 months of follow-up. Most transfusion recipients convert to HGV RNA positivity 7 days after transfusion but conversion times of 21 to 140 days have been reported in hemodialysis patients. In this patient, acquisition of HGV prior to inclusion in the study is the most plausible explanation, since he was only treated with high purity factor VIII concentrates and current methods of inactivating HCV are thought to be equally effective against HGV. However, reactivation of a low-level infection in which serum levels of the virus were below the limit of detection, or HGV infection acquired by another route cannot be excluded.

In conclusion, overall prevalence of HGV infection (HGV-RNA and anti-E2) in hemophiliacs is higher than would be deduced from the sole determination of HGV-RNA. In our series HGV-RNA tends to persist in a high proportion of patients, and the disappearance of HGV-RNA is not necessarily associated with anti-E2 response but is correlated to a lower impairment of CD4/CD8 lymphocyte subsets in HIV co-infected patients.

Contributions and Acknowledgments

DT and JM were responsible for the conception of the study. Its design and wrote the paper. JM was the coordinator of the multicentric study and was also responsible for day-to-day contact with participants. JCR, JM and DT were responsible for data handling and statistical analysis and interpretation. DT was responsible for the genetic studies. MM, MQ, MFU, CRP, JT, CA, JCR and JM followed the patients clinically and were responsible for the clinical management and clinical data acquisition. RM, AO and DT were responsible for the studies performed in the control group. The order of appearance of the authors (except for DT and JM) is based on the hospital list and does not reflect differences in the amount of work done.

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Disclosures

Conflict of interest: none. Redundant publications: no substantial overlapping with previous papers.

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