Hepatitis G Virus RNA and Hepatitis G Virus-E2 Antibodies in Dutch Hemophilia Patients in Relation to Transfusion History


The prevalence of hepatitis G virus (HGV)-RNA and HGV-E2 antibodies was studied in a cohort of Dutch hemophilia patients in relation to clotting products used, age, and coinfection with hepatitis C. Between 1991 and 1995, blood samples were taken from 294 patients with hemophilia A, B, or von Willebrand disease. From each patient one fresh frozen sample was tested for HGV cDNA polymerase chain reaction (PCR) and HCV cDNA PCR. Alanine aminotransferase (ALT) tests were performed on plasma samples of all patients. The presence of HGV-E2 antibodies was tested on plasma samples from a subset of 169 patients representing all age groups. Based on the origin and viral safety of the products used, three subgroups of patients were distinguished: Group A: patients who used viral noninactivated factors derived from small and large donor pools; group B: patients who used factors prepared with ineffective viral inactivation techniques derived from small and large donor pools; and group C: patients treated only with optimally viral inactivated large pool clotting factor or recombinant clotting factor concentrate. The prevalence of HGV-RNA was 18%. In group A patients the prevalence was 71%, in group B 50%, and in group C 6%. When related to age, the highest prevalence of HGV-RNA (35%) was seen in patients born between 1980 and 1989. The prevalence of HGV-E2 antibodies increased with age. Of HGV-RNA-negative patients born before 1950, 96% tested positive. HGV viremia did not affect ALT levels, neither in HCV-RNA positive nor in HCV-RNA negative patients. HGV infection is frequently seen in patients with hemophilia. In older age groups a lower rate of HGV-RNA positivity is seen coinciding with a higher rate of antievelope antibodies.

IN THE PAST, HEMOPHILIA patients have been infected with hepatitis C virus (HCV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV) after treatment with nonviral inactivated clotting factor products derived from human plasma. Since the introduction of dry-heat-treated clotting products in the mid 80s, HIV transmission has no longer been observed. Introduction of wet heating or solvent detergent inactivation steps in the manufacturing process of clotting products has shown them to be effective in preventing HCV transmission. These inactivation steps are relatively ineffective in reducing the risk of transmission of nonenveloped viruses such as hepatitis A virus (HAV) and parvo B19 virus.

Recently, the hepatitis G virus ("HGV", identical to "GBV-C") has been characterized as an agent potentially causing non-A-E hepatitis. HGV is a widely distributed RNA virus related to HCV and other flaviviridae. In several studies, it has been shown that 1% to 3% of blood donors test HGV-RNA positive, while approximately 10% of blood donors have detectable antibodies against the putative envelope protein (E2 antibodies). HGV is also highly prevalent among intravenous (IV) drug users, of whom approximately 40% test positive for E2 antibodies and another 40% are found to be HGV-RNA positive. HGV is shown to be transmitted parenterally through blood and blood products, as well as vertically from mother to child. Therefore, recipients of blood products, such as hemophilia and thalassaemia patients, have been shown to be at risk for HGV infection.

Most HGV infections appear to be asymptomatic. A few cases of fulminant hepatitis have been reported, but it remains unclear whether the hepatitis was actually caused by HGV. Anti-E2 seroconversion seems to be associated with viral clearance.

A coinfecion of HGV with HCV, often seen in patients with hemophilia, seems not to affect the course of HCV infection or the response to interferon-alpha therapy.

In the present study, we determined from among Dutch hemophilia patients (1) the prevalence of HGV-RNA, HCV-RNA, and E2 antibody reactivity, (2) the effect of viral inactivation of blood products on the prevalence of HGV-RNA and E2 antibody, and (3) the effect of HGV viremia on the alanine transaminase (ALT) levels in HCV-RNA positive and HCV-RNA negative patients.

MATERIALS AND METHODS

Patients

Blood samples were taken from 294 patients. Fresh frozen plasma samples from a total of 252 hemophilia A, 34 hemophilia B, and 8 von Willebrand disease patients, born between 1919 and 1993, were tested for HGV-RNA. The plasma samples originally had been drawn for HCV polymerase chain reaction (PCR) testing in patients visiting the hemophilia center for annual check-ups. At the time of plasma sampling (1991 to 1995), none of the patients was receiving antiviral therapy for HCV infection.

For the anti-E2 assay, an equal number of patients from various age groups was selected (30 patients born before 1950, 30 patients born, respectively, in the period between 1950 and 1959, between 1960 and 1969, between 1970 and 1979, and between 1980 and 1989, and 19 patients born after 1990). From each age group, we tested the available samples from HGV-RNA positive patients and we added samples from HGV-RNA negative patients to complete the group of 30 patients. We had therefore selected plasma samples from 49 HGV-RNA positive and 120 HGV-RNA negative patients for detection of E2 antibodies.
**Transfusion History**

Based on the viral safety and the origin of the clotting factor concentrates, three viral categories of products can be distinguished. (1) Group A, nonviral inactivated small pool cryoprecipitate or nonviral inactivated large pool clotting factor concentrate (used until 1985); (2) group B, small pool cryoprecipitate (1985 to 1992) or large pool clotting factor concentrate (1985 to 1990), which were suboptimal viral inactivated (dry heating of lyophilized products at 60°C for 72 hours); (3) group C, large pool optimal viral inactivated clotting factor concentrate (SD treated, pasteurized) (1990 to present) or recombinant DNA-derived clotting factor concentrate (1992 to present).

**Plasma Samples**

From all 294 patients, a cell-free fresh frozen plasma sample had been obtained for HCV-RNA detection. Within 1 hour after venipuncture, the EDTA plasma samples had been frozen and stored at −70°C before HCV-RNA testing. Subsequently, the samples had been stored at −20°C until HGV-RNA and E2 antibody testing was performed.

**Assays**

**HGV cDNA-PCR.** HGV-RNA detection by cDNA-PCR was performed with two sets of primers, one pair from the NS3 region and a second pair from the 5′-UTR. Nucleic acid was extracted from 0.1 mL plasma with proteinase K digestion and organic extraction, and subsequently reverse transcribed with random prime labelling, and amplified by PCR, as described previously.7 The amplification product from the NS3 region (primer set 77F/211R) was detected with oligonucleotide detection with probe 152F, as described by Linnen et al.6 For amplification in the 5′ UTR primers NC1 (5′-GGG.CCA.AAA.GGT.G-GTG.GGA.TG-3′) and NC4 (5′-CCA.ACA.CCT.GTG.GAC.CGT.GC-3′) were used and the product was detected with oligomer hybridization with probe NC3 (5′-GGT.AGC.CAC.TAT.AGG.TGG.G-3′).

All samples were analyzed with the NS3-cDNA PCR in duplicate. Samples were considered positive when the duplicate tests were both reactive. Samples were considered negative if both duplicate tests were nonreactive. Results were considered indeterminate if one reactive and one nonreactive result was obtained. All negative and indeterminate samples were further analyzed with the 5′ UTR PCR on the reverse transcribed template in duplicate. Two NS-3 PCR indeterminate samples were considered HGV-RNA positive because the 5′ UTR PCR was reactive in duplicate.

**E2 antibody ELISA.** An experimental E2 enzyme-linked immunosorbent assay (ELISA; Abbott Diagnostics Division, Chicago, IL) was used as previously described.12 Purified GBV-C E2 was coated onto polystyrene beads. Plasma samples were diluted 1:300 and analyzed at Abbott Laboratories.

**HCV cDNA PCR.** HCV-RNA detection was performed with an in house developed cDNA-PCR assay.19,31

**ALT.** ALT values presented in this report were tested in the same serum samples as the samples used for PCR testing. Serum samples were tested for ALT by a standard automated method. Because for this method, the upper limit of normal varied with time, the results were expressed as an ALT index, calculated as the ALT level measured divided by the upper limit of normal. Hence an ALT index of <1 was considered to be normal.

**Data Management and Statistical Analysis**

Clinical and virological data were collected in separate data bases and were coupled after acquiring anonymity. The study protocol was approved by the Medical Ethical Committee of the University Hospital Utrecht, the Netherlands.

For statistical analysis, the Fisher’s exact test and the χ² Test for Trend were used.

**RESULTS**

**HGV-RNA, HCV-RNA positivity, and E2 antibody reactivity in relation to age.** The prevalence of HGV-RNA was 18%. No difference was seen between patients with hemophilia A (46 of 252, 18%) and hemophilia B (8 of 34, 24%). Seven of 49 (14%) HGV-RNA positive patients and 74 of 120 (62%) HGV-RNA negative patients were E2 antibody reactive.

Table 1 shows the proportion of HGV-RNA and HCV-RNA positive patients in relation to age. The number of HGV-RNA positive patients was significantly higher in the group born between 1980 and 1989 than in the older age groups (P = .026), whereas the proportion of HCV-RNA positive patients was significantly lower in this particular group of relatively young patients (P < .0001). Two patients born after 1990 tested HGV-RNA positive.

Table 2 shows the results of HGV-E2 antibody testing in patients with and without HGV viremia in relation to age. The prevalence of HGV-E2 antibodies increased with age. A total of 96% (CI, 80% to 100%) of patients born before 1950 tested positive.

**HCV-RNA and HGV-RNA positivity and HGV-E2 reactivity in relation to viral safety of clotting products.** Table 3 shows the rates of HCV-RNA and HGV-RNA tests in relation to the clotting products used. Patients ever treated with nonviral inactivated plasma-derived clotting product (group A) had a higher prevalence of HCV viremia (71%) than HGV viremia (17%). However, in the group treated with suboptimal viral inactivated product (group B), a higher prevalence of HGV viremia was found (50%) as compared with HCV viremia (27%). No HCV infection was seen in patients exclusively treated with optimal viral inactivated or recombinant clotting

**Table 1. Prevalence of HGV-RNA and HCV-RNA in Relation to Age**

<table>
<thead>
<tr>
<th>Year of Birth</th>
<th>No.</th>
<th>HGV-RNA Positive [95% CI]*</th>
<th>HCV-RNA Positive [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 1950</td>
<td>35</td>
<td>51 (14%) [5-30%]</td>
<td>274 (77%) [60-90%]</td>
</tr>
<tr>
<td>1950-1959</td>
<td>52</td>
<td>6 (12%) [4-23%]</td>
<td>401 (77%) [63-87%]</td>
</tr>
<tr>
<td>1960-1969</td>
<td>65</td>
<td>11 (17%) [9-28%]</td>
<td>491 (75%) [63-85%]</td>
</tr>
<tr>
<td>1970-1979</td>
<td>68</td>
<td>11 (16%) [8-27%]</td>
<td>466 (68%) [55-78%]</td>
</tr>
<tr>
<td>1980-1989</td>
<td>55</td>
<td>19 (35%) [22-49%]</td>
<td>131 (24%) [13-37%]</td>
</tr>
<tr>
<td>1990-1993</td>
<td>19</td>
<td>2 (11%) [1-30%]</td>
<td>0 (0%) [0-18%]</td>
</tr>
<tr>
<td>Total</td>
<td>294</td>
<td>54 (18%) [14-23%]</td>
<td>175 (60%) [54-65%]</td>
</tr>
</tbody>
</table>

*95% CI = 95% confidence interval.

**Table 2. Prevalence of E2 Antibody Reactivity in Relation to Age in Patients With and Without HGV Viremia**

<table>
<thead>
<tr>
<th>Year of Birth</th>
<th>No.</th>
<th>HGV-RNA Positive [95% CI]*</th>
<th>HGV-RNA Negative [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 1950</td>
<td>30</td>
<td>2/4 (50%) [7-93%]</td>
<td>25/26 (96%) [80-100%]</td>
</tr>
<tr>
<td>1950-1959</td>
<td>30</td>
<td>0/6 (0%) [0-46%]</td>
<td>19/24 (79%) [58-93%]</td>
</tr>
<tr>
<td>1960-1969</td>
<td>30</td>
<td>1/11 (9%) [0-41%]</td>
<td>14/19 (74%) [49-91%]</td>
</tr>
<tr>
<td>1970-1979</td>
<td>30</td>
<td>3/10 (30%) [7-65%]</td>
<td>10/20 (50%) [27-73%]</td>
</tr>
<tr>
<td>1980-1989</td>
<td>30</td>
<td>1/6 (16%) [0-30%]</td>
<td>6/14 (43%) [8-71%]</td>
</tr>
<tr>
<td>1990-1993</td>
<td>19</td>
<td>0/2 (0%) [0-84%]</td>
<td>0/17 (0%) [0-19%)</td>
</tr>
<tr>
<td>Total</td>
<td>169</td>
<td>7/49 (14%) [6-27%]</td>
<td>74/120 (62%) [52-70%]</td>
</tr>
</tbody>
</table>

Abbreviation: ab, antibody.
Table 3. Prevalence of HGV-RNA and HCV-RNA in Relation to Viral Safety of the Clotting Products Used

<table>
<thead>
<tr>
<th>Clotting Product Used</th>
<th>HGV-RNA Positive No. (% 95% CI)</th>
<th>HCV-RNA Positive No. (% 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non- or suboptimal viral</td>
<td>236</td>
<td>39 (17%) (12-22%)</td>
</tr>
<tr>
<td>Inactivated product</td>
<td></td>
<td>168 (71%) (65-77%)</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suboptimal and optimal</td>
<td>26</td>
<td>13 (50%) (30-70%)</td>
</tr>
<tr>
<td>viral inactivated product</td>
<td></td>
<td>7 (27%) (12-48%)</td>
</tr>
<tr>
<td>Group C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal viral inactivated</td>
<td>32</td>
<td>2 (6%) (1-21%)</td>
</tr>
<tr>
<td>product, recombinant product</td>
<td></td>
<td>0 (0%) (0-11%)</td>
</tr>
</tbody>
</table>

*95% CI = 95% confidence interval.
†Chi-squared test for Trend, not significant.
‡Chi-squared test for Trend, P < .00001.

Table 4. HGV-RNA Status in Relation to ALT Index and HCV-RNA Status

<table>
<thead>
<tr>
<th>ALT Index*</th>
<th>No.</th>
<th>HGV /HCV</th>
<th>HGV /HCV</th>
<th>HGV /HCV</th>
<th>HGV /HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>110</td>
<td>82 (74%)</td>
<td>12 (11%)</td>
<td>13 (12%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>1-2</td>
<td>89</td>
<td>16 (18%)</td>
<td>1 (1%)</td>
<td>55 (62%)</td>
<td>17 (19%)</td>
</tr>
<tr>
<td>2-4</td>
<td>52</td>
<td>3 (6%)</td>
<td>—</td>
<td>37 (71%)</td>
<td>12 (23%)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>38</td>
<td>2 (5%)</td>
<td>—</td>
<td>29 (76%)</td>
<td>7 (18%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

*ALT index <1 is normal.
†Fisher's Exact Test: ALT <1 versus ALT >1 P = .46 = NS.
‡Fisher's Exact Test: ALT <1 versus ALT >1 P = 1.00 = NS.

DISCUSSION

This study shows that 18% of our group of Dutch hemophilia patients is HGV-RNA positive. Fourteen percent HGV-RNA positive patients and 60% HGV-RNA negative patients were E2 antibody reactive. These results confirm the results of other studies showing that multitransfused hemophilia patients have often been exposed to HGV.14,21,23,24

Intriguingly, patients born between 1980 to 1989 were significantly more often HGV-RNA positive (35%) than the patients in the older age groups (12% to 17%). By contrast, in HGV-RNA negative patients, the proportion of anti-E2 antibodies increased with age from 0% in patients born after 1990 to 96% in patients born before 1950. These findings might be explained by the fact that clearance of HGV may only occur after a long period of viremia.25 Hence, patients who were exposed to HGV after 1980 may not yet have completely recovered. This theory is supported by the findings of Tacke et al10 and Dille et al12 who found that in patients with a posttransfusion hepatitis, the development of E2-specific HGV antibodies was associated with the loss of HGV viremia. Another possible explanation might be that patients born in the 1980s have more often initially been infected through intermediate or highly purified, non-, or suboptimal viral inactivated clotting factor concentrates. Plasma pools used for production of these concentrates might have been contaminated with a relatively high HGV load and through the purification process, the titer of neutralizing anti-E2 antibodies was reduced, but infectious viral particles persisted. Perhaps these patients have been at a higher risk of becoming chronic HGV carriers than have patients who were infected with less pure products, theoretically containing HGV complexed by neutralizing antibodies. Future follow-up studies on HGV-RNA and E2 antibody testing are needed to show the natural history of HGV infection in hemophilia patients and to exclude new infections. This is particularly important because it is still not clear whether the current optimally viral inactivated plasma-derived clotting products are HGV “safe.” HGV-RNA was found in various batches of plasma-derived clotting factor concentrate, which were optimal viral inactivated by dry heat treatment at 80°C for 72 hours or by pasteurization. Only products that were chemically viral inactivated by a solvent and detergent were HGV-RNA negative.14,16 Our study shows that the current methods of viral inactivation and the use of recombinant products strongly diminishes the evidence of HGV infection in recipients. In only two of 32 (6%) patients exclusively treated with this type of product was HGV viremia found, whereas none of the 23 patients tested was E2 antibody positive. By contrast, 52 of 262 (20%) of the patients ever treated with suboptimal or nonviral inactivated product were HGV viremic. We cannot exclude the possibility that the two young HGV-RNA positive patients were infected by the use of optimally viral inactivated (SD) plasma clotting factors, although vertical or intrafamilial transmission, or infection through other routes, is also possible and needs to be studied. It is shown that vertical transmission of HGV is more common than in hepatitis C.18,19 Currently, no data are available on the prevalence of HGV-RNA and E2 antibodies in the population under the age of 18 years. From donor testing we know that the prevalence of HGV infection is higher than HCV.10,11,33

The pathogenicity of HGV seems to be negligible. We did not find an increase of ALT levels in patients who were HGV-RNA positive and HCV-RNA negative. These results are in concordance with the studies published by Wang et al15 and Tanaka et al27 who also found no signs of chronic liver disease in HGV carriers or a negative effect of a superinfection with HGV on the severity of HCV infection.
CONCLUSIONS

HGV infection is frequently seen in patients with hemophilia. This might be a result of the past use of non- or suboptimal virus inactivated plasma derived clotting factor concentrates. However, as age-matched control studies for comparison are missing, it is not clear how many infections are the result of the use of plasma-derived clotting factors, but current viral inactivation methods and recombinant products seem to reduce the risk of transmission significantly.

In older age groups, a lower rate HGV-RNA positivity is seen coinciding with a higher rate of antienvelope antibodies, suggesting the development of neutralizing antibodies.

In our study, we did not find a correlation between the ALT levels and HGV infection.

ACKNOWLEDGMENT

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