RAPID COMMUNICATION

The Incidence and Natural Course of Transfusion-Associated GB Virus C/Hepatitis G Virus Infection in a Cohort of Thalassemic Patients

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To evaluate the risk of transmitting blood-borne GB virus C/hepatitis G virus (GBV-C/HGV) and to define the natural course of infection, we performed a prospective study in a cohort of multiply transfused β-thalassemics during a 6-year follow-up period. We analyzed serum samples of 150 patients collected at 3-year intervals from 1990 to 1996. GBV-C/HGV RNA was determined by reverse transcriptase-polymerase chain reaction and antibodies to E2-protein by an enzyme immunoassay. At baseline, 14.5% of patients had viremia and 18.5% anti-E2. None of the patients with anti-E2 in 1990 subsequently became viremic. Of the 100 GBV-C/HGV RNA-positive, anti-E2-negative patients, 10 acquired infection during follow-up, as indicated by positivity of GBV-C/HGV RNA (n = 2), anti-E2 RNA by reverse transcription-polymerase chain reaction (RT-PCR), which could identify subjects with active viral replication, but not those with past exposure to the virus.

GB VIRUS C (GBV-C) is a recently discovered RNA virus belonging to the Flaviviridae family. Its genomic sequence is very similar to that of another newly cloned viral agent, hepatitis G virus (HGV); hence, the two are currently considered different isolates of the same virus. Specific viral RNA has been detected in patients affected from hepatic and hematologic diseases, including fulminating, acute, and chronic non-A-C hepatitis, and aplastic anemia. The evidence that GBV-C/HGV is a transfusion-transmissible virus and that viremia is persistently present in 1% to 2% of repeat blood donors worldwide contributed to some concern about the safety of the current blood supply. Therefore, longitudinal surveys are deemed necessary to evaluate the risk of acquiring blood-borne GBV-C/HGV and to better define the natural history of transfusion-associated infection. Thus far, prospective epidemiological and clinical investigations on GBV-C/HGV in blood recipients have been hampered by two main difficulties. First, large numbers of patients need to be repeatedly tested to reach reliable conclusions. Second, the only available assay was the determination of serum GBV-C/HGV RNA by reverse transcription-polymerase chain reaction (RT-PCR), which could identify subjects with active viral replication, but not those with past exposure to the virus.

In this paper we report the current risk of acquiring blood-borne GBV-C/HGV infection among transfusion-dependent β-thalassemics of our country. Moreover, the virological, serological, and biochemical features of transfusion-transmitted GBV-C/HGV infection are prospectively described.

MATERIALS AND METHODS

Patients. In 1989, centers of the Cooleycare program were invited to participate in a prospective longitudinal survey aimed at assessing the risk of blood-borne infections. Thirty-six centers agreed to participate, and all 1,384 patients regularly receiving transfusions at these centers were enrolled (716 males, 688 females; median age, 16 years; range, 0 to 45). For each patient, a serum sample collected in December 1989-March 1990 (baseline sample) and a record including demographic data and information on the transfusion regimen were sent to Milan, where the reference laboratory of the Cooley-care group is located. Additional samples of the same patients were collected at the centers during the subsequent 6 years of follow-up, at 3-year intervals (in 1992-1993 and in 1995-1996).

At the time of the present study, the samples of 1,001 subjects completing the follow-up program were available in the repository. For the purpose of this investigation, we decided to study a cohort of 150 patients attending 3 centers of the 26 which completed the program. The 3 participating centers were chosen according to the following criteria: (1) being in charge of at least 30 patients each; (2) being located in three
different regions of the country (one each from Northern, Central-Insular, and Southern Italy). The patients included in the study were 81 males and 69 females, randomly selected, with a median age at enrollment of 14 years (range, 1 to 30). Of them, 133 (88.7%) were positive for antibodies to hepatitis C virus (HCV) at enrollment, and 1 (0.7%) acquired HCV infection during the study period, as indicated by seroconversion; the remaining 16 (10.6%) were persistently anti-HCV+. All patients remained negative for antibodies to human immunodeficiency virus throughout the follow-up. The number of blood units transfused to the patient group was measured on the basis of the Cooleycare database.11

Patients’ sera were screened as follows: GBV-C/HGV RNA and anti-E2 were initially determined on samples collected in 1989-1990 and in 1995-1996. If a discrepancy of results was observed between the two specimens, the sample collected in 1992-1993 was also examined. Liver function tests, including levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (GGT), were determined in the three specimens.

Methods. Enzyme immunoassays (EIAs) were used for anti-HCV (EIA-3; Ortho Diagnostic Systems, Raritan, NJ) and anti-HIV (Murex, Dartford, UK) determinations. A recombinant immunoblot assay (RIBA-3; Ortho Diagnostic Systems) was used to confirm anti-HCV reactivity. Liver function enzymes were determined using an Olympus AU510 analyzer (Eppendorf-Netheler, Hamburg, Germany). The patterns of ALT, AST, and GGT were considered altered when the result was greater than the upper reference limit (for ALT, 40 U/L in males, 30 U/L in females; for AST, 20 U/L in males, 18 U/L in females; for GGT, 40 U/L in males, 21 U/L in females) in at least one of the three determinations.

Serum GBV-C/HGV-RNA was determined by RT-PCR using primers derived from the 5' noncoding region (5'-NCR) of the viral genome.10,15 Positive results were confirmed with primers specific for the nonstructural region 5a (NS5a). Only concordant results were considered. In detail, RNA was extracted from 100 µL serum using the Purescript RNA Isolation Kit (Gentra Systems Inc, Minneapolis, MN) and resuspended in 20 µL of diethyl pyrocarbonate–treated water. Complementary DNA (cDNA) synthesis was performed on 10 µL purified RNA using 10 U of Moloney murine leukemia virus RT (BRL, Gaithersburg, MD), 1 U of ribonuclease inhibitor (Promega, Madison, WI), 0.2 mmol/L deoxyribonucleoside triphosphates (Promega), and 1 µmol/L random hexamers (Pharmacia, Uppsala, Sweden) in commercial buffer for 30 minutes at 42°C. For the PCR, the Hepatitis G Virus-Primers and Capture Probe Set and the PCR DIG Labeling Mix (Boehringer, Mannheim, Germany), were used to obtain digoxigenin-labeled amplified products. Briefly, 10 µL of cDNA was amplified in a 9600 thermal cycler (Perkin-Elmer, Emeryville, CA) according to the following protocol: 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. The reaction buffer contained 10 pmol of each primer (NCR primer 1 and 2 for 5'-NCR or NS5a primer 1 and 2 for NS5a region), 0.2 mmol/L Labeling Mix; 2.5 U of Expand High Fidelity DNA polymerase [Boehringer] in commercial buffer (50 µL reaction volume). The labeled PCR products were analyzed by hybridization to 5'-NCR or NS5a Capture Probes (Boehringer), using an EIA system (PCR ELSIA; Boehringer). Probes are biotin-labeled to allow immobilization of hybrids to a streptavidin-coated microtiter plate surface. The bound hybrids were detected by anti-digoxigenin peroxidase conjugate and a colorimetric substrate. Results were interpreted according to the manufacturer’s instructions.

Antibodies to the E2-protein of GBV-C/HGV were detected by a two-step sandwich enzyme immunoassay, the µPLATE Anti-HGEnv (Boehringer).13 Ten-microliter serum samples or controls were diluted in 200 µL of sample buffer. Twenty microliters of the diluted sample was added to streptavidin-coated microtiter wells, together with 80 µL of incubation solution, consisting of biotinylated mouse antibody linked to GBV-C/HGV-E2 antigen. The microplate was incubated at room temperature for 2 hours, and 100 µL of a solution containing peroxidase-conjugated anti-human IgG antibody was added. After incubation for 1 hour at room temperature and the addition of 100 µL of ABTS chromogen substrate, the optical density was measured at 405 nm within 10 minutes. All the incubation steps were performed using a shaker. Results were interpreted according to the manufacturer’s instruction. As recommended, reactive samples were submitted to a confirmatory test procedure, in which the incubation solution did not contain the GBV-C/HGV-E2 antigen.

Statistical analysis. The incidence of infection was expressed as the number of new infections per 100 person-years. The risk of acquiring infection was computed by the ratio between the number of seroconverting patients and the total number of red blood cell (RBC) units transfused to the patient cohort during the study period. The upper bound of 95% confidence interval (CI) of incidence and risk was calculated. The chi-squared test was used to compare proportions. The Student’s t-test was used when appropriate. A P value of less than .05 was considered statistically significant.

RESULTS

The results of GBV-C/HGV RNA and anti-E2 determination observed at the baseline and at the end of the 6-year follow-up period are reported in Table 1. In 1989-1990, 22 patients (14.5%) were GBV-C/HGV RNA+, and 28 (18.5%) had detectable anti-E2 reactivity, accounting for an overall prevalence of infection of 33%.

Of the 100 GBV-C/HGV RNA and anti-E2 patients, 10 acquired GBV-C/HGV infection during the 6 years of follow-up, as documented by either conversion to viremia (2 subjects; age at enrollment, 3 and 19 years), to anti-E2 (7 subjects; median age, 15; range, 6 to 21 years), or to both markers (1 subject, age 17 years) in the sample collected in 1995-1996. The analysis of the samples collected in 1992-1993 showed a concomitant positivity for GBV-C/HGV RNA and anti-E2 in one of the seroconverting patients. The incidence of GBV-C/HGV infection was 1.7 per 100 person-years (95% CI, 0.8 to 3).

Since approximately 19,000 RBC units were administered to the GBV-C/HGV RNA and anti-E2+ patients during the study

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Table 1. Results of GBV-C/HGV RNA and Anti-E2 Determination

<table>
<thead>
<tr>
<th>GBV-C/HGV RNA</th>
<th>Anti-E2</th>
<th>No. of Subjects</th>
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<tbody>
<tr>
<td>At Baseline (1989-1990)</td>
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<tr>
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<td>neg</td>
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<td>12</td>
</tr>
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</table>

*Including 5 patients testing GBV-C/HGV RNA- using primers from NS5a region, and GBV-C/HGV RNA+ with those from 5'-NCR. They were all negative with both primer pairs in the sample collected at the end of follow-up (1995-1996). Anti-E2 determination was persistently negative.
period, the risk of blood-borne GBV-C/HGV infection was 5.3 in 10,000 units (95% CI, 2 to 8.5).

Sixteen of the 22 patients with active infection at the baseline (73%) remained viremic throughout the study period. Two of them developed concomitant anti-E2 reactivity between 1993 and 1996. The remaining 6 GBV-C/HGV RNA+ patients (27%) became nonviremic during follow-up; in 4 of them, the appearance of anti-E2 accompanied the clearance of viremia.

Of the 28 patients with anti-E2 reactivity at the baseline evaluation, none developed GBV-C/HGV infection during follow-up; 12 (43%) became anti-E2 negative. Six of them lost reactivity between 1990 and 1993, the remaining 6 between 1993 and 1996.

Patients with GBV-C/HGV viremia were younger (age, 10 ± 5 years) than the patients with anti-E2 reactivity (16 ± 6 years, \( P < .005 \)) and than those negative for both markers (15 ± 6 years, \( P < .005 \)). The prevalence of HCV infection was 76% in patients with GBV-C/HGV viremia, and 92% in those GBV-C/HGV RNA and anti E2− (\( P = .056 \)). The patterns of ALT, AST, and GGT were altered in 20%, 68%, and 28% of the GBV-C/HGV RNA+ patients, as compared with 25%, 61%, and 35% of those GBV-C/HGV RNA−, without significant associations.

During the study period, 7 of the 150 patients received interferon-α treatment for chronic HCV infection, at a dose of 3 to 6 MU thrice weekly for 3 to 12 months. Five were persistently GBV-C/HGV RNA and anti-E2−, and 2 remained anti-E2− throughout the follow-up.

DISCUSSION

GBV-C/HGV is a transfusion transmissible agent, which is endemic among the blood donor population worldwide.2,8-10

In this article we report the results of a multicenter, prospective study performed in a large cohort of transfusion-dependent \( \beta \)-thalassemic patients, to evaluate the risk of acquiring blood-borne GBV-C/HGV in 1990-1996, and to describe the natural course of infection. At the baseline evaluation in 1989-1990, the frequency of GBV-C/HGV viremia was 14.5%, a figure not dissimilar to that reported by others in transfusion recipients who underwent cardiac surgery.5 Considering also the subjects with anti-E2 reactivity, the prevalence of infection increased to 33%, which is remarkably lower than the 90% observed for HCV in the same population of \( \beta \)-thalassemic patients. This finding was unexpected, because the prevalence rate of GBV-C/HGV viremia in the blood donors of our area is higher than that of HCV (1.5% v 0.7%).10,16 Moreover, during the 6-year follow-up period we observed that the incidence of GBV-C/HGV infection was approximately 1.7 per 100 person-years, which accounts for a risk of infection of 5.3 per 10,000 transfused blood units. The latter is about 10-fold lower than that recently estimated, using GBV-C/HGV RNA as the only marker of infection, among first-time blood recipients from Taiwan.8 As discussed below, the natural course of GBV-C/HGV infection provides a useful background for the interpretation of these data.

Whether the presence of specific anti-E2 antibodies has a protective role against GBV-C/HGV infection is still an open question, as pointed out by Tacke et al13 in a recent report describing the immunoassay used in this study. At the baseline investigation, we found that the presence of viral RNA and anti-E2 antibodies was mutually exclusive, as already described by others.13,17 Moreover, we observed that none of the 28 patients with detectable anti-E2 reactivity became GBV-C/HGV RNA positive in the following 6 years, although during this time they received several thousands of blood units unscreened for GBV-C/HGV. These results apparently suggest that anti-E2+ patients are protected against GBV-C/HGV infection. However, definitive conclusions on this issue can not be drawn given the low conversion to viremia rate of patients lacking antibody. In addition, it remains to be elucidated whether the protective immunity observed in our series is actually caused by the anti-E2 antibodies, or by other humoral and/or cellular factors. More than 25% of the GBV-C/HGV RNA+ subjects cleared the virus during a 6-year period, which indicates that, at least in some individuals, GBV-C/HGV infection may have a natural progression toward recovery. This is also supported by the evidence that viremic patients were younger than those with anti-E2 reactivity. Moreover, one patient showed a concomitant positivity of anti-E2 and GBV-C/HGV RNA before achieving a complete suppression of viral replication. This provides the experimental evidence that a time overlap may exist between anti-E2 seroconversion and seronegativity for viral RNA, as recently hypothesized by others.13,14,17

Interestingly, we found that two of the six patients who cleared the virus during the study period did not develop detectable humoral response to the E2 region. In these cases, GBV-C/HGV RNA and/or anti-E2 antibodies could be present at titers that are below the limit of detection of the tests used for this investigation. Alternatively, neutralizing antibodies might recognize viral antigens not included in the current anti-E2 assay. According to the latter hypothesis, the sequence studies performed so far indicate that significant genetic diversity exists between different isolates of GBV-C/HGV.18

Another striking finding of our study was that more than one third of the patients who were anti-E2+ at the baseline evaluation in 1989-1990 lost anti-E2 reactivity during the study period. Moreover, we observed that the age of GBV-C/HGV RNA and anti-E2− thalassemics was similar to that of anti-E2+ subjects, and that the former patients were older than those with active infection.

Taken together, these data seem to indicate that a proportion of patients with past, resolved GBV-C/HGV infection test negative for both markers. The relatively low incidence rate observed in our investigation could be justified considering that these subjects might still have a protective immunity against reinfection. In the light of these considerations, our estimate of the current risk of transfusion-associated GBV-C/HGV infection applies only to chronic transfusion-dependent patients, and not necessarily to first-time recipients of blood transfusion.

We found that the presence of active GBV-C/HGV infection does not significantly contribute to hepatocellular injury in patients affected from homozygous \( \beta \)-thalassemia. This adds more data to the growing literature indicating that GBV-C/HGV does not cause classic hepatitis in most cases.3,8-10,19 In our opinion, the evidence that GBV-C/HGV is not a major cause of chronic liver disease should not lead to exclude a potential pathogenetic role of this agent in inducing clinical disease in transfusion recipients. In fact, it should be taken into account.
that GBV-C/HGV sequences have been identified in patients with life-threatening conditions.\textsuperscript{1,4,6,7} In addition, some viral agents which generally cause mild, self-limiting infections in healthy individuals, may sometimes induce serious sequelae in blood recipients with compromised immune response or abnormal hematopoiesis.\textsuperscript{20-22}

In conclusion, GBV-C/HGV infection is epidemic among transfusion-dependent thalassemic patients of our country. In some individuals, viral replication may be spontaneously suppressed, even after several years of chronic infection. The clearance of viremia generally parallels the appearance of anti-E2 antibodies, which seem to have a protective role against reinfection. However, anti-E2 reactivity may decrease with time. Further studies are needed to better define the possible interplay between viral and host factors in determining the outcome of infection, and to explore the potential role of GBV-C/HGV in inducing extrahepatic manifestations in infected blood recipients.

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REFERENCES
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