TT Virus Infection in Patients with Primary Hypogammaglobulinaemia: Natural History and Relationship to Liver Disease in the Immunocompromised Host

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In 1997, the search for a causative agent of cryptogenic hepatitis led to the identification of a novel human DNA virus, named TT virus (TTV) (1). Studies on TTV characteristics revealed that the viral genome is circular, single-stranded DNA comprising 3,852 nucleotides, with no identity to other viruses, thus suggesting that TTV is a member of a new virus family that infects humans (2, 3). Initially, TTV DNA was amplified from serum samples from three patients with post-transfusion hepatitis of unknown aetiology (1). Based on these findings and on the high prevalence of TTV infection in Japanese patients with fulminant hepatitis and chronic liver disease of unknown aetiology, TTV was suggested as the causative agent for some cryptogenic liver diseases (1, 4). However, TT viraemia was detected in a large proportion of healthy subjects and in patients with no biochemical or histological evidence of hepatitis and it was proposed that TTV does not cause liver diseases (5). Subsequent studies demonstrated that TTV infection has a worldwide distribution with a high prevalence in healthy people such as blood donors—in the USA between 7.5% and 13% (6, 7); in Japan 35%–37% (8, 9); in Germany and Spain 7% and 14% (10, 11); as well as in rural populations in tropical countries—74% in Papua New Guinea and 83% in the Gambia (12). The pathogenic role and clinical significance of TTV infection remain unclear, as these investigations found no clear correlation with hepatitis or liver damage.

The role of TTV in patients with primary hypogammaglobulinaemia is of particular interest because hepatitis C virus infection (HCV) has been demonstrated to be both prevalent and to run a very severe clinical course in these patients (13, 14). Liver disease is a frequent complication in a substantial proportion of cases of unknown aetiology (15). Non-A, non-B hepatitis has been reported after intravenous
administration of immunoglobulins (13, 16–18) and hepatitis C virus (HCV) has been detected in some immunoglobulin preparations (19).

The aim of the current study was to test the hypothesis that TTV is responsible for cryptogenic liver disease in patients with primary hypogammaglobulinaemia. In a large group of well-characterized patients we determined the presence of TTV viraemia and investigated the clinical course of TTV infection and liver disease in a longitudinal study of TTV-positive patients. In addition, we assessed the presence of TTV in currently used immunoglobulin preparations.

### Materials and Methods

#### Selection of patients and serum samples

Eighty-three patients with PHG, followed regularly (median 10.2 years, range 2.9–30 years) at the Section of Clinical Immunology and Infectious Diseases, Dept. of Medicine, Rikshospitalet, National Hospital, Oslo were included in the study. These patients fulfilled the following inclusion criteria: 1) at least one serum sample available for analysis; 2) known history of substitution therapy with immunoglobulin in any form; 3) documented follow-up with clinical and laboratory investigations for the presence of liver disease. All patients included in the study were Caucasians (50 men and 33 women). The median age at the last visit was 44 years (range 14–76). Depending on the presence of liver disease, patients with PHG formed 3 subgroups: 20 patients with HCV-related liver diseases; 24 patients with non-B, non-C liver disease; and 39 patients with no liver disease (Table I).

The diagnosis of PHG was based on established criteria (20). The type of antibody deficiency was classified as described previously (22): 1) common variable immunodeficiency \( n = 52 \); 2) congenital hypogammaglobulinaemia \( n = 22 \); 3) X-linked agammaglobulinaemia (Bruton’s type) \( n = 7 \); 4) hyper-IgM syndrome \( n = 2 \) (Table I). All 83 patients had serum IgG levels below 2.0 g/L before the substitution therapy was initiated. The median duration of immunoglobulin therapy was 12.4 years (range 1.6–43 years). Thirty-one of 83 patients have received one or more different commercial preparations of intravenous immunoglobulins (IVIG). For the last 8–10 years the great majority of patients have been substituted by immunoglobulin preparations administered subcutaneously (23). Only a minority of the patients studied had other risk factors for parenterally acquired infections—a history of intravenous drug use \( (n = 2) \); tattoos \( (n = 3) \); transfusions of erythrocytes or platelets \( (n = 5) \).

The monitoring for liver disease was performed in all patients at least twice per year. This comprised clinical examination, liver function tests (serum alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, albumin) and coagulation. Patients were classified as having liver disease if one or more of the parameters tested were abnormal (>1.5 times upper limit of normal) in at least three subsequent samples obtained over a period of 6 months. Patients with PHG who had persistently normal LFTs during the entire follow-up period and no abnormality on the imaging investigations were classified as patients with no liver disease. Ultrasound examination of the liver and spleen was performed regularly in all patients and most patients had a CT-scan of the abdomen. Liver biopsy or endoscopic retrograde cholangio-pancreatography were performed when clinically indicated.

The first available serum sample from the 83 patients included in the study was tested for TTV DNA. In all cases, these samples were obtained after starting the substitution therapy with immunoglobulins. Serial serum samples from 25 patients who were found to be TTV positive (between 2 and 5 samples per patient, obtained over a period of 2–6 years) were also tested for TTV DNA. In addition, serial samples, collected over a period of 2–6 years, from 12 patients, who were initially TTV negative were also analysed for newly acquired TTV infection. All samples used in this study were stored at \(-70^\circ\text{C}\). As a control group we investigated the presence of TTV infection in 20 Norwegian patients with autoimmune chronic liver disease—autoimmune hepatitis \( (n = 10) \); primary biliary cirrhosis \( (n = 6) \) and primary sclerosing cholangitis \( (n = 4) \). Only 2 of these 20 patients had received blood transfusions and none had a history of intravenous drug use or therapy with immunoglobulins.

### Immunoglobulin preparations

Three different preparations currently used for substitution therapy were available to test for TTV DNA. Gammanorm (Pharmacia-Upjohn, Stockholm, Sweden) has been used for subcutaneous administration from 1996 and is produced from Scandinavian blood donors. The immunoglobulins were fractionated in accordance with Cohn et al. (24). The

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**Table I. Distribution of patients with primary hypogammaglobulinaemia according to the presence of liver disease**

<table>
<thead>
<tr>
<th>Type of primary hypogammaglobulinaemia</th>
<th>HCV RNA+ liver disease</th>
<th>Non-B, non-C liver disease</th>
<th>Non-B, non-C no liver disease</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Common variable immunodeficiency</td>
<td>7</td>
<td>22</td>
<td>23</td>
<td>52</td>
</tr>
<tr>
<td>2. Congenital hypogammaglobulinaemia</td>
<td>5</td>
<td>2</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>3. X-linked agammaglobulinaemia (Bruton’s type)</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>4. Hyper-IgM syndrome</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TOTAL</td>
<td>20</td>
<td>24</td>
<td>39</td>
<td>83</td>
</tr>
</tbody>
</table>
preparation was treated with Tris-n-butyl-phosphate and Tween 80, as virus inactivation. *Gammaglobulin* (Pharma-
cia-Upjohn, Stockholm, Sweden) has been used for intramus-
cular and subcutaneous administration since 1968. The preparation was obtained from European blood donors. Fractionation and virus inactivation were as described above. *Octagam* (Octapharma, Vienna, Austria) was obtained from Norwegian blood donors and has been used intravenously since 1994. In addition to the fractionation and virus inactivation steps described for the other two preparations, Octagam production includes incubation at pH 4.0.

Detection and analysis of TTV DNA

All samples were tested for TTV DNA in duplicate, which was performed blindly with respect to any information regarding the sample or the patient. TTV DNA was sought by semi-nested PCR, as previously described (4, 5). Briefly, total DNA was extracted from 100 μL serum using QIAamp Blood kit (QIAGEN Ltd, Crawley, UK) and resuspended in 50 μL elution buffer. For the first round PCR, a sense primer NG059 (5'-ACA GAC AGA GGA GAA GGC AAC ATG-3') and an antisense primer NG063 (5'-CTG GCA TTT CAT TTC CAA AGT T-3') were used. The amplification was for 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec, followed by 7 min at 72°C after the last cycle. The second round was performed with a sense primer NG061 (5'-GGC AAC ATG TTA TGG ATA GAC TGG-3') and NG063 as an antisense primer for 35 cycles at the same conditions. The amplicons were electrophoresed in 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

The serum level of TTV DNA was quantitated in selected PHG patients with and without liver diseases and compared with TTV viraemia in randomly selected healthy subjects. The level of TTV DNA was assessed by amplification of serial end-point dilutions of a standard amount of DNA extracted from the serum. Serial dilutions of a known TTV DNA positive sample were used as a positive control. The results were expressed as DNA copies/mL, as previously described (25).

To gain information about the changes in the TTV population over time in patients with PHG, serial samples from four TTV-positive patients were analysed by direct DNA sequencing. In addition, DNA sequencing analysis was performed for TTV DNA amplified from the immunoglobulin preparations. The amplicons were purified with the QIAquick PCR purification kit (QIAGEN Ltd) and sequenced on an ABI 377 automated sequencer (PE Applied Biosystems) with NG061 as a sequencing primer. Phylogenetic analysis was carried out using the PHYLIP package (26). A distance tree was constructed using the DNADIST and NEIGHBOR programs. Distances were calculated according to the two-
parameter method of Kimura (27), assuming a transition/ transversion ratio of 10, and the phylogenetic tree constructed using the UPGMA algorithm.

Serological assays

All serum samples were tested for markers of HBV (HBsAg), HCV (anti-HCV and HCV RNA) and HGV (HGV RNA), as previously reported (13, 17).

Statistical analyses

Comparisons between groups were performed using the Wilcoxon non-parametric test or chi-squared test with Yates’ correction, where appropriate.

Results

Prevalence of TTV in primary hypogammaglobulinaemia

TTV DNA was detected in the earliest serum sample tested from 27 of 83 (32.5%) patients. The presence of TTV infection was not associated with any particular type of hypogammaglobulinaemia. Of the 27 TTV-positive patients, 15 had common variable immunodeficiency, 6 congenital hypogammaglobulinaemia, 4 had X-linked agammaglobuli-
aemia and 2 hyper IgM syndrome. The comparison between TTV DNA-positive and -negative patients revealed that seropositivity for TTV DNA is associated with intravenous administration of immunoglobulins, the duration of substitution treatment and patient’s age (Table II). Eighteen of 27 TTV positive patients have received immunoglobulins intravenously, in contrast to only 10 of 58 TTV negative patients (P = 0.002). Among all patients who were negative for HCV and HBV markers with and without liver disease (n = 63), the TTV-positive cases had significantly longer duration of immunoglobulin therapy than TTV-negative cases—median 16 years (range 6–45) versus 11 years (2–31), respectively (P = 0.009; Table II). Within the subgroup of patients with non-B, non-C liver disease (n = 24), those with TTV infection were significantly older (median 58 years, range 29–81) than TTV-negative cases (median 33 years, range 23–55) (Table II; P = 0.004). There was no significant age difference between TTV-positive and -negative cases in the other two subgroups, although the number of TTV DNA-positive cases amongst 39 patients with no liver disease was too small (Table II). In the control group, 3 of 20 (15%) patients with autoimmune liver diseases were TTV DNA-positive.

Liver disease in patients with primary hypogammaglobulinaemia

TTV infection was found in 11 of 18 (61%) HCV RNA-positive patients; in 12 of 24 (50%) patients with non-B, non-C liver disease and in 4 of 39 (10%) patients with no liver disease (Table II). The HCV RNA-positive patients con-
tracted the infection in 1984–86 through HCV-contaminated immunoglobulin (Gammonativ) (12). Five of 11 patients who were HCV RNA and TTV DNA-positive died of end-stage cirrhosis and liver failure within 12 years of acquiring HCV infection. The other six patients have liver disease of varying severity, with cirrhosis in two. All nine HCV RNA-positive patients were female.
patients with no TTV infection also had liver disease; three of these died with end-stage cirrhosis and three from unrelated causes. Among 12 patients with non-B, non-C liver disease and TTV infection, none had a history of acute hepatitis. Serum ALT and alkaline phosphatase were moderately elevated in these patients, while the synthetic liver function was normal with no evidence of cirrhosis. One patient had granulomatous hepatitis on liver biopsy, which progressed to cirrhosis during the last 8 years.

TTV DNA titres did not differ between PHG patients with or without liver disease or healthy controls.

**Longitudinal follow-up of TTV infection and liver disease**

Between 2 and 5 serial serum samples were available from 25 of 27 TTV DNA-positive patients. Seventeen of 25 patients were TTV DNA-positive in all samples, while in 8 patients TTV DNA was not detectable in the follow-up samples (Fig. 1). Among 12 patients who were TTV-negative in the first sample, 2 became positive in a later sample (case nos. 16 and 17; Fig. 1A), while the other 10 remained TTV-negative. One of these two cases (no. 17; Fig. 1A) had established cirrhosis in 1993 prior to becoming TTV DNA-positive. During the follow-up, the liver damage progressed to decompensated cirrhosis with low albumin, prothrombin time and increased bilirubin. Five patients with PHG showed no evidence of liver diseases despite being TTV DNA-positive (Fig. 1A, case nos. 4, 7, 9, 14 and 16). The liver function tests in patients with non-B, non-C liver disease and TTV infection over time showed no significant changes with mild to moderate (<3 × ULN) increase of serum ALT and/or alkaline phosphatase. During the follow-up, three patients with non-B, non-C liver disease (Fig. 1A) and three HCV RNA-positive patients (Fig. 1B) lost TTV DNA from the serum. The clearance of TTV in these patients was not associated with normalization or improvement in the liver function tests.

To analyse the changes of TTV population over time in patients with PHG, TTV DNA was amplified and sequenced from serial serum samples of patients with the longest follow-up—patient nos. 1, 5 and 8 (Fig. 1A) and patient no. 20 (=3 in Fig. 1B). There was no nucleotide variation in the 200 base region of the TTV genome in all 4 samples, collected over a 6-year period, from case no. 8. This patient had common variable immunodeficiency and severe granulomatous hepatitis which progressed to cirrhosis during the observation period. The TTV population also remained unchanged in case no. 20, as TTV DNA in one of the samples differed by only two nucleotides from the other three samples which had identical sequences. Phylogenetic analysis revealed a significant evolutionary distance between TTV DNA in the sera taken at different time points from the other two cases (Fig. 1).
2). The first two samples from patient no. 1 contained almost identical viral population, which was related to the originally described TTV genotype 1 (4). The serum taken 6 years later showed predominance of TTV population with >30% nucleotide differences and close to genotype 2. Importantly, the new TTV strain in this patient is similar to the virus present in patient no. 8, who has severe liver disease (Fig. 2). The predominance of this new TTV strain was not associated with changes in the liver function tests. Significant differences were also found in the TTV population in serum samples taken 5 years apart from patient no. 5 (Fig. 3). The changes in TTV population from one to another genotype over time in the last two cases were not associated with changes in the degree of liver damage.

**TTV DNA in immunoglobulin preparations**

Three commercial preparations of immunoglobulins were tested for TTV DNA: three different batches of Octagam (Octapharma), two batches of Gammaglobulin (Pharmacia Upjohn) and two batches of Gammanorm (Pharmacia Upjohn). TTV DNA was detected in all batches of Octagam and Gammanorm, while both batches of Gammaglobulin were negative. DNA sequencing analysis of the amplicons showed no variations within the amplified region (Fig. 2). The TTV type in the immunoglobulins belonged to genotype 2, showing 11% nucleotide variability in comparison to genotype 2a (4). The same TTV genotype was found in the majority of samples tested (Fig. 2).

**Discussion**

TTV is a recently discovered human DNA virus with no clear disease association. In the present study we investigated a possible role of TTV in the development of liver disease in a large group of patients with primary hypogammaglobulinaemia followed for a median period of 10.2 years. The results show that TTV infection is highly prevalent in these patients and that substitution treatment with immunoglobulins, especially intravenous administration, represents an important route for acquiring TTV in this group. The longitudinal analysis revealed that one-third of patients with TT viraemia can clear the virus spontaneously over a period of 2–6 years, while TTV DNA remains detectable in the serum in the majority of patients. Despite viral persistence, we found no strong evidence that TTV is responsible for cryptogenic liver disease in patients with primary hypogammaglobulinaemia.

Viral infections in immunocompromised host are frequently associated with significant morbidity. Chronic HCV infection in patients with primary hypogammaglobulinaemia or in HIV-infected patients has a severe course with rapid progression to cirrhosis (14, 28). Although TT viraemia was more frequent in PHG patients with non-B, non-C hepatitis than in patients without liver disease, a careful analysis shows no causative relationship. Firstly, we found no specific viral characteristics (high level of viraemia or a particular TTV strain) in PHG patients with non-B, non-C hepatitis; secondly, patients who cleared TTV during the follow-up showed no
resolution of liver disease; thirdly, there was no difference in the severity of liver disease in patients co-infected with HCV and TTV and those with HCV alone; finally, the higher prevalence of TTV infection in patients with non-B, non-C hepatitis was associated with differences in age, intravenous use of immunoglobulins and a longer duration of substitution therapy. These findings in immunosuppressed patients extend a large body of evidence in patients with cryptogenic hepatitis with no immunodeficiencies suggesting that chronic TTV infection has no pathogenic role for liver disease (5, 8, 10, 11).

Our data provide further evidence that TTV is transmitted parenterally. The viral genome was detected in several batches of human immunoglobulin preparations, treated with ‘state of the art’ methods for viral inactivation, and the prevalence of TTV infection correlated with the use of immunoglobulins intravenously and the duration of treatment.

A strong association between the volume of transfused blood or blood products and TTV infection was shown in healthy subjects and in patients with haemophilia (6, 25). In addition, our study shows that TT viraemia persists for years in the majority of patients with primary hypogammaglobulinaemia. A similarly high rate of TTV persistence was also observed during a follow-up of multi-transfused patients (28). We observed spontaneous TTV clearance in 32% of patients, which is lower than the rate of TTV clearance (67%) reported in normal subjects with newly acquired TTV infection (6).

The great diversity of viral population is a characteristic feature of TTV and co-existence of several TTV genotypes has been shown in healthy subjects and in multi-transfused patients (2, 30). On this basis, it is difficult to establish whether the marked changes in TTV population over time in two of our patients reflected the co-existence of more than one TTV genotype or repeated infections; however, as the differences in DNA sequence are considerable, these changes are not due to simple mutations.

In summary, the present study demonstrates that TTV infection is very common in patients with primary hypogammaglobulinaemia. The substitution therapy with immunoglobulins, especially intravenous administration, has a significant role for transmitting TTV and the virus persists in the majority of patients. In this large cohort of well-characterized and prospectively followed patients with primary hypogammaglobulinaemia we found no strong evidence that TTV can cause liver disease in immunocompromised patients.

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References


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