Prevalence and Prognostic Significance of Infection with TT Virus in Patients Infected with Human Immunodeficiency Virus

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No clear association between human disease and TT virus (TTV) has been documented. A possible pathogenic role of TTV was investigated in patients infected with human immunodeficiency virus (HIV). TTV serum concentrations were estimated in 185 HIV-infected patients by dilution polymerase chain reaction. Of these, 149 (76%) were TTV-positive, compared with 18 (7%) of 252 Danish blood donors ($P<.001$). Of the HIV-infected patients who were TTV-positive, 72 (51%) had high TTV viremia ($>5$ times the highest concentration observed among blood donors, i.e., $>3.5 \times 10^4$ TTV/mL of serum). High TTV viremia was associated with decreased survival ($P<.001$; relative hazard [RH], 2.0). There was a correlation between lower CD4+ T cell counts and higher TTV titers ($P<.01$). In a Cox regression model, CD4+ T cell count ($P<.001$), age ($P<.001$), HIV viral load ($P<.001$), $\beta_2$ microglobulin ($P<.02$), and high TTV viremia ($P<.01$; RH, 1.9) were independent predictors of survival. TTV is suspected to be an opportunistic pathogen with an independent influence on HIV progression.

**Materials and Methods**

**Patients.** The CHIC is composed of 347 HIV-infected patients from the Department of Infectious Diseases at Rigshospitalet, Copenhagen, Denmark, from September 1991 through October 1992; this group has been described elsewhere [11]. At enrollment, blood was drawn for determination of HIV viral load, CD4+ T cell counts, and other immunological parameters related to HIV progression. Sera drawn at inclusion from 185 patients were available for the present study. All parameters included in this study (TTV viremia, CD4+ T cell counts, HIV viral load, $\beta_2$ microglobulin, and age) were baseline values. The cohort was followed up from inclusion until June 1997, but the present survival analysis is based upon follow-up until 1 May 1996, when highly active antiretroviral therapy (HAART) was introduced.

In addition, 252 blood donors from the 4 major blood banks in Denmark—Aarhus ($n=60$), Aalborg ($n=77$), Odense ($n=90$), and Copenhagen ($n=25$)—were included for comparison. These serum samples were collected in 1999. All patient and donor serum samples were stored at $-80^\circ$C.

**Detection of TTV by polymerase chain reaction (PCR).** Viral DNA was purified from 200 $\mu$L of serum by use of the High Pure Viral Nucleic Acid Kit (Böehringer Mannheim, Indianapolis, IN) and eluted from filters with 50 $\mu$L of elution buffer. PCR reactions were performed with 10 $\mu$L of eluate in 50 $\mu$L reactions by use of Taq PCR Core Kit (Qiagen, Hilden, Germany) with final concentrations (0.5 U Taq/50 $\mu$L, 2.5 mM MgCl$_2$, 0.2 mM dNTP 1× Q-reagent, 1× buffer, 0.5 $\mu$M primers). Reactions were performed in a Perkin Elmer thermal cycler 9600 (Perkin Elmer, Norwalk, CT) using the following program steps: 1 min of denaturation at 94°C followed by 55 cycles of 93°C (20 s), 56°C (30 s), and 72°C (30 s) and finally 7 min of extension at 72°C. PCR products were separated through 3% agarose and were visualized by ethidium-bro-
mide staining. PCR reactions were done by use of primers 5′-GCTACGTCATAACCCAGGTG-3′ (T801) and 5′-CTBCGGGTGTGTAACCTAC-3′ (T935), which resulted in 199-bp fragments, as described by Takahashi et al. [7]. These primers target the relatively stable area upstream open-reading frame (ORF) 2 of the TTV genome. A sample of the PCR products were sequenced, and GenBank Basic Local Alignment Search Tool search (AF122913 and AB017610 as hits) was used for verification that fragments were derived from TTV. Patient DNA samples were diluted 10-fold until PCR resulted in bands in the range of 1.0–30 ng. Quantification was done by use of a GeneGenius gel documentation station (Syngene, Cambridge, UK). A gel-purified PCR product-stock derived from TTV by use of the Takahashi primers was quantified, and a dilution series of this stock was used as a template for a set of PCR reactions. These reactions composed the basis for establishing a standard curve that correlated TTV genome copy number in sample dilutions with amounts of PCR product in ethidium bromide–stained bands, within the approximately linear range of 1–30 ng. Viremias were estimated by multiplying dilution factors and thus determined copy numbers of each end-diluted sample. The detection limit determined from the same standard curve was 40 DNA templates per 50 μL–PCR reaction or 10^3 TTV genomes per milliliter of serum.

Statistical analysis. Data were analyzed by use of SPSS version 8 (SPSS, Chicago). Comparison of proportions was analyzed by use of the χ² test. Comparison of unpaired observations was analyzed by use of the Mann-Whitney U test. Survival was analyzed by use of the Kaplan-Meier method, and groups were compared by use of the log-rank test. Median values among groups were compared by 1-way ANOVA analysis. Univariate risk ratios for available prognostic factors were estimated. A multivariate Cox proportional-hazards regression model was constructed from the set of significant univariate predictors, excluding those that did not remain significant (P < .05).

Results

One hundred forty of the 185 tested HIV-infected patients were TTV-positive (76%). This was significantly higher than the prevalence among Danish blood donors, of whom 18 (7%) of 252 were TTV-positive (P < .0001). Among HIV-infected who were TTV-positive, TTV viremia ranged from the limit of detection to 9 × 10^3 copies/mL of serum. Among blood donors, viremia ranged from the limit of detection to 7 × 10^4 copies/mL of serum. TTV titers in HIV-infected patients were significantly higher (median, 3.9 × 10^3 copies/mL; range, 1 × 10^3 to 9 × 10^4 copies/mL) than those observed in blood donors (median, 3.1 × 10^3 copies/mL; range, 1 × 10^3 to 7 × 10^4 copies/mL; P < .001).

Effect of TTV infection on HIV progression. In HIV-infected patients, there was no significant difference in survival when considering TTV-negative versus TTV-positive status (log-rank, P = .19). To investigate whether TTV viremia influenced survival, we divided HIV-infected patients into 3 groups: (1) patients who were TTV negative, (2) patients with low TTV viremia (<5 times greater than the highest titer observed among blood donors, which is equivalent to 3.5 × 10^5 TTV/mL of serum), and (3) patients with titers equal to or above this value. Route of HIV transmission and distribution of prognostic factors for these groups are shown in table 1.

As shown in the Kaplan-Meier plot (figure 1A), there was no difference in survival between the TTV-negative patients (group 1) and patients with low TTV viremia (group 2; P = .79). Survival was significantly decreased in patients with high TTV viremia (group 3), compared with that seen in patients with no (group 1) or low (group 2) TTV viremia (P < .001). These results justified a regrouping of patients into 2 groups: (1) patients with no or low TTV viremia (low viremia group) and (2) patients with high TTV viremia (high viremia group). The high TTV viremia group had decreased survival, compared with the low TTV viremia group (figure 1B; P < .001).

CD4+ T cell counts and TTV viremia. TTV viremia in HIV-infected patients was found to be higher than that seen in normal blood donors. This indicates that TTV viremia might be controlled by cellular immune mechanisms. Accordingly, we found a significantly higher CD4+ T cell count among patients with no or low TTV viremia, compared with that seen in patients with high TTV viremia (P = .006).

### Table 1. Route of human immunodeficiency virus (HIV) transmission and prognostic variables in 185 HIV-infected patients, according to TTV virus (TTV) viremia.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No TTV (n = 45)</th>
<th>Low TTV viral load (n = 68)</th>
<th>High TTV viral load (n = 72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route of transmission</td>
<td>Homosexual men</td>
<td>34</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Injection-drug users</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Transfusion</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Hemophiliacs</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Heterosexual</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Prognostic variables</td>
<td>CD4+ cell counts/mL</td>
<td>202 (4–504); n = 44</td>
<td>265 (1–1116); n = 65</td>
</tr>
<tr>
<td></td>
<td>Log HIV viral load/mL</td>
<td>5.07 (2.30–6.86); n = 34</td>
<td>4.86 (2.85–6.36); n = 52</td>
</tr>
<tr>
<td></td>
<td>β2 microglobulin level</td>
<td>246 (0–610); n = 44</td>
<td>238 (103–601); n = 65</td>
</tr>
<tr>
<td></td>
<td>Age, years</td>
<td>38.2 (22–55)</td>
<td>39.1 (16–66)</td>
</tr>
</tbody>
</table>

a Data are no.
b Data are mean (range).
TTV viremia as a prognostic factor in HIV progression. In univariate analyses, CD4+ T cell count, age, HIV viral load, β2 microglobulin, and IFN-γ measured on phytohemagglutinin-stimulated peripheral blood mononuclear cells, as well as TTV viremia, were significant predictors of death. To evaluate the degree to which TTV viremia affects survival, independent from other factors significant in univariate analysis, we included these parameters in a multivariate Cox regression model. Complete data were available from 128 patients.

In this model, the following factors remained significant and independent predictors of death: CD4+ T cell count (P < .001), age (P < .001), log HIV viral load (P < .001), β2 microglobulin (P < .02), and high TTV viral load (P < .01; relative hazard, 1.93). The effect of TTV viremia remained significant when scored as a continuous variable, instead of the TTV groupings used in the Kaplan-Meier plots of figure 1B, or when log-transformed CD4+ counts values were included. Thus, for a given CD4+ T cell count, HIV viral load, age, and β2 microglobulin, a TTV serum level ≥3.5 × 10^5 became associated with a 2-fold increased risk of death.

Discussion

We find that TTV infection is very common among HIV-infected patients (76% TTV-positive). This is similar to what was found in a recent study on TTV prevalence among HIV-infected patients [12]. We found that 7% of Danish blood donors are TTV-positive. This appears low compared with other studies that use the T801/T935 set of PCR primers [7, 12, 13]. One possible explanation is that ≥1 TTV strains prevalent in Denmark are not amplified by the primers used in this study. We chose the Takahashi primers to target the region upstream ORF2, because sequence alignment of reported TTV sequences obtained by use of Clustal X software (available from http://www-igbmc.u-strasbg.fr/bioinfo/) showed that this region is the most stable and conserved region of TTV. We have not made any attempt to sequence N22 regions linked to our target sequences upstream ORF2. Therefore, we have no information on the ability of the study primers to selectively amplify genotypes defined from the N22 region.

A more likely explanation for the relatively low TTV prevalence found in blood donors is that our PCR protocol was optimized for maximum specificity and accuracy, to enable reliable determination of TTV viremia. This was achieved at the expense of optimal sensitivity (our detection limit was 40 DNA templates per PCR reaction or 10^3 TTV genomes per milliliter of serum). According to our experience with the Takahashi primers, sensitive conditions give rise to unspecific bands that compromise reliable quantification, which may also cause false positives. However, as our new, major finding relates to relative TTV viremias among HIV-infected patients infected, we find the problem of low sensitivity to be of less importance.

The difference in TTV viremia between HIV-infected patients and blood donors indicates that TTV viral load is controlled by cellular immune mechanisms, which is supported by the inverse correlation between CD4+ T cell counts and TTV viremia.

Finding a high TTV infection rate and large variation in levels of TTV viremia in HIV-infected patients led us to investigate whether TTV viral load might influence the course of HIV infection. We chose to subdivide the population of HIV-infected patients into 2 groups: (1) the high TTV viremia group that consisted of HIV-infected patients whose TTV viral load is ≥5 times greater than that observed among blood donors (i.e., 3.5 × 10^5 TTV copies/mL of serum) and (2) the low TTV viremia group that included the negative group together with those who had titers below this concentration. Thus, the TTV-
positive HIV-infected patients were divided into 2 groups of similar size. As depicted in figure 1B, patients in the high TTV viremia group had significantly decreased survival, compared with patients in the low TTV viremia group. In a Cox regression model, CD4+ T cell count, log HIV viral load, age, β2 microglobulin, and high TTV viremia were found to be significant independent predictors of survival.

We put forward the hypothesis based upon these findings that TTV titers increase in parallel with the impairment of cellular immune status. TTV at a certain viral load becomes an opportunistic infection, with its own independent influence on HIV disease progression and survival. Alternatively, high TTV titer might reflect another previously unrecognized factor, rather than being the factor directly responsible for the worse prognosis.

The CHIC is composed of HIV-infected patients enrolled from 1991 to 1992 at one of the major HIV clinics in Copenhagen. Serum samples from 165 (47%) patients were not available for the present study. Although the population studied was not selected in any other way, the patients enrolled in the TTV study had higher HIV viral load and lower CD4+ T cell counts and fared worse than the patients not enrolled. It is unclear how this unintentional selection bias might influence the present findings. However, it seems reasonable to assume that the pattern found in the study population would be the same in the population not included and that an analysis based on data from all patients would strengthen, rather than weaken, our conclusion.

We chose to limit the period of observation until May 1996 when the first patient received HAART. HAART effectively reduces HIV replication, restores immune function, and improves survival. Since TTV levels reflect the degree of immune impairment, one would expect TTV levels to decrease once HAART is initiated with the individual patient. This hypothesis should be examined in a longitudinal study of TTV replication in HIV-infected patients undergoing HAART.

If it is true that TTV is an opportunistic pathogen with a significant independent effect on the course of HIV infection, then what is the clinical equivalent to TTV infection with high viremia? In the CHIC database, no formal prospective registration of liver-associated diseases or transaminase levels was performed. We are in the process of a blinded assessment of patient records to look for a possible association between high TTV viremia and any recorded clinical symptoms and established HIV-related diseases.

Our hypothesis presented here is based on preliminary findings and should be evaluated in other large cohorts of HIV-infected patients. The interplay among TTV, immune impairment, and effect of HAART should be investigated in longitudinal studies of HIV-infected patients.

In conclusion, TTV infection is prevalent among HIV-infected patients, and TTV titers are increased among patients with impaired immune status. TTV is suspected to be an opportunistic pathogen with a significant effect on HIV disease progression, independent of other classic HIV-progression markers. Alternatively, high TTV titer might reflect another previously unrecognized factor, rather than being the factor directly responsible for the poorer prognosis.

Acknowledgments

We are grateful to Dr. Jannik Helweg-Larsen for statistical assistance; Terese L. Katzenstein for measurement of plasma HIV-RNA; Ebtisam Khalil Ibrahim for dedicated technical assistance; and the blood banks in Aarhus, Aalborg, Odense, and Copenhagen for providing serum samples from blood donors.

References