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Dynamics of Persistent TT Virus Infection, as Determined in Patients Treated with Alpha Interferon for Concomitant Hepatitis C Virus Infection

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TT virus (TTV) is a recently identified widespread DNA virus of humans that produces persistent viremia in the absence of overt clinical manifestations. In an attempt to shed light on the dynamics of chronic infection, we measured the levels of TTV in the plasma of 25 persistently infected patients during the first 3 months of alpha interferon (IFN-α) treatment for concomitant hepatitis C virus (HCV) infection. The first significant decline of TTV loads was observed at day 3 versus day 1 for HCV. Subsequently, the loads of TTV became progressively lower in most patients, but some initial responders relapsed before the end of the follow-up, suggesting that at least in some subjects the effects of IFN on TTV can be very short-lived. No correlation between the responses of TTV and HCV to therapy was found. Fitting the viremia data obtained during the first week of treatment into previously developed mathematical models showed that TTV sustains very active chronic infections, with over 90% of the virions in plasma cleared and replenished daily and a minimum of approximately 3.8×10^{10} virions generated per day. Low levels of TTV were occasionally detected in the peripheral blood mononuclear cells of patients who had cleared plasma viremia, thus corroborating previous results showing that these cells may support TTV replication and/or persistence.

TT virus (TTV) is a recently described single-stranded DNA virus of humans that shares considerable similarities with the chicken anemia virus and other circoviruses of animals. Unlike related viruses, TTV is characterized by a great genetic heterogeneity and by a seemingly absolute lack of pathological effects for infected hosts. In fact, the original suggestion that TTV might be responsible for at least some of the acute and chronic forms of hepatitis that still remain cryptogenetic has not been substantiated, and no other illness has yet been associated with certainty to the virus.

Although sensitive PCR assays have documented that TTV produces long-lasting, possibly permanent infections in most infected individuals and that plasma viremia is highly prevalent in the general population throughout the world (1, 6, 8, 24, 31), the natural history of TTV infection is still poorly understood. Suggestions that the liver is a major site of replication (26) have been questioned (9). On the other hand, the observation that TTV is consistently found associated with extensively washed peripheral blood mononuclear cells (PBMC) and grows in cultured phytohemagglutinin-stimulated PBMC (13, 25, 27) is evidence for a more or less pronounced tropism for lymphoid tissues.

Analysis of viral dynamics following initiation of antiviral treatments has been helpful in resolving the natural history of several persistent viruses, including human immunodeficiency virus type 1 (HIV-1), hepatitis B virus, and hepatitis C virus (HCV), and has provided useful clues for optimizing therapeutic protocols (17, 22, 30). HCV is an important cause of chronic liver damage and may lead to decompensated cirrhosis and hepatocellular carcinoma, usually after an indolent course of two or more decades. Prolonged administration of alpha interferon (IFN-α), alone or in combination with the synthetic purine analogue ribavirin, remains crucial in efforts to reduce the detrimental effects of HCV infection on the liver, even though sustained virological responses are observed in only a minority of treated patients (5, 11). In an attempt to shed light on TTV-host interactions, we have investigated the dynamics of TTV, and for comparison HCV, in patients chronically infected with both viruses during the early stages of IFN treatment. The results have shown that, as in the case of other chronic plasma viremia-inducing viruses previously investigated, persistent TTV viremia implies an extremely active ongoing virus replication.

MATERIALS AND METHODS

Patients and samples. The study group consisted of 25 randomly selected patients (19 males and 6 females; mean age, 44 ± 10 years; range, 26 to 63 years) with chronic dual HCV and TTV infection. All patients were free from hepatitis B virus and human HIV-1 and -2 infections and, prior to initiation of the study, had received no antiviral or immunosuppressive treatments. The patients were subcutaneously administered 3 (7 subjects) or 6 (18 subjects) international megaunits of recombinant IFN-α2 (rIFN-α2 [Intron A]; Schering-Plough, Madison, N.J.) three (15 subjects) or seven (10 subjects) times per week throughout the 3 months of follow-up, and 4 also received 1,000 mg of ribavirin per day after the last month. Because the study was not designed to evaluate the effects of different forms of therapy and no differences were noted in the limited subgroups studied, the patients were pooled into a single group. After informed consent, patients were bled with EDTA tubes at day 0, 1, 2, 3, 7, 14, 30, and 90 of therapy. Total PBMC were obtained by standard Ficoll-Hypaque density-gradient centrifugation (Lympho separation medium; ICN Biomedicals, Aurora, Ohio),

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TABLE 1. TTV and HCV detection and loads in the plasma of 25 doubly infected patients at selected times of IFN treatment

Day of treatment	TTV			HCV		
	No. positive/tested (%)[a]	Load[b]		No. positive/tested (%)[a]	Load[b]	
		Mean ± SE	Range[c]		Mean ± SE	Range[c]
0	25/25 (100)	6.7 ± 0.2	4.9–9.3	25/25 (100)	6.0 ± 0.2	4.7–7.4
1	25/25 (100)	6.7 ± 0.2	4.9–10.3	22/23 (96)	5.2 ± 0.2**	3.4–6.3
2	18/18 (100)	6.8 ± 0.2	5.6–9.4	12/15 (80)	5.0 ± 0.3**	3.7–6.6
3	12/12 (100)	5.9 ± 0.3*	3.1–7.1	15/17 (88)	5.2 ± 0.2**	4.9–6.7
7	13/14 (93)	5.5 ± 0.3**	3.3–7.1	14/16 (87)	5.1 ± 0.3***	4.8–6.6
14	14/19 (74)	5.4 ± 0.4**	5.2–8.6	17/20 (85)	4.5 ± 0.2***	3.3–6.7
30	6/14 (43)	3.8 ± 0.3***	3.6–7.0	10/18 (55)	4.3 ± 0.3***	5.3–6.2
90	7/17 (41)	4.1 ± 0.4***	3.5–7.6	6/18 (33)	4.2 ± 0.4***	5.3–7.1

[a] Not all samples were available for all patients at all times.
[b] Log_{10} copies of viral genome per milliliter of plasma. Viral loads below the detection assay sensitivity were arbitrarily scored as 3 log_{10} for calculation. *, **, ***, significantly different from day 0 at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively (Student's t test).
[c] Positive patients only.

washed with abundant phosphate-buffered saline, and treated to achieve maximum elimination of adsorbed extracellular virus as previously described (13). Plasma and PBMC samples were immediately stored at −80°C and kept frozen until use. Viral nucleic acids were extracted by the method included in the Amplicor Monitor version 2.0 (Roche Diagnostic Systems, Basel, Switzerland) for HCV and QIAamp DNA Blood Mini kit (QIAgen, Chatsworth, Calif.) for TTV. Four doubly infected patients, who had agreed to be left untreated and to be bled monthly, were used to measure the extent of spontaneous viremia fluctuations in the absence of antiviral treatments.

Viral load assays. TTV DNA quantification was carried out with a TaqMan real-time PCR targeted to a segment of the untranslated region (UTR) of the viral genome highly conserved among the ones deposited in gene banks at the time of writing: forward primer 5′-GTGCCGIAGGTGAGTTTA-3′, position 177 to 194; reverse primer 5′-AGCCCGGCCAGTCC-3′, position 226 to 239; probe 5′-TCAAGGGGCAATTCGGGCT-3′, position 205 to 223. The procedures used for copy number quantification and evaluation of intra- and interassay accuracy and reproducibility have been previously described (13, 31). The lower limits of sensitivity were 1.0×10^3 and 1.0×10^2 TTV DNA copies per ml of plasma or per μg of total PBMC DNA, respectively. All samples from each patient were assayed simultaneously in triplicate, and at least two independent DNA extractions for each sample were examined. Samples positive in only one replicate and/or with a coefficient of variation of 50% or greater were reextracted and tested again in triplicate. Levels of HCV RNA in plasma were measured with the Monitor 2.0 Roche assay with a lower limit of sensitivity of 1.0×10^3 copies per ml.

Data analysis. Viral load data in the first week of treatment were analyzed by using the model developed by Neumann et al. (17) to investigate the dynamics of HCV infection in IFN-treated patients. In this analysis, we obtained least-square estimates of the parameters t_0, c and ε by fitting the equation $V(t) = V_0 \{1 - \varepsilon + \varepsilon \, exp\,[-c(t - t_0)]\}$ to the observed data. Here, $V(t)$ is the viral load predicted t days after initiation of therapy, ε is the efficacy of therapy at reducing virus release from infected cells, c is the virion clearance rate, and exp represents the exponential function. The model assumes that viral decay begins at a certain time, t_0, after the start of therapy, at a viral load V_0. In order to keep the number of parameters estimated from data at a minimum, we used the observed pretreatment viral load values for V_0 in the equation presented above. Standard errors of parameter estimates were obtained by the numerical resampling method known as jackknifing (33). Virion half-life ($t_{1/2}$) was obtained by the equation $\ln(2)/c$. Daily production of plasma virions (P) was calculated from cV_0, multiplied by the extracellular body fluid volume, which was arbitrarily set at 3.0×10^3 ml.

TTV genotyping. TTV genotype was determined by sequencing a 222-bp segment of open reading frame 1 (ORF1) as previously described (12) and, for selected isolates, also a 204-bp segment of UTR according to Leary et al. (10). Cycle sequencing was carried out with an automatic DNA sequencer (ABI model 373; PE Biosystems, Foster City, Calif.).

RESULTS

IFN-induced changes in plasma TTV viremia. Twenty-five previously untreated patients doubly infected with TTV and HCV were monitored for levels of the two viruses in plasma at selected time points during the first 3 months of IFN therapy. Overall data are shown in Table 1. At baseline, TTV values ranged between 4.9 and 9.3 log_{10} DNA copies per ml of plasma with a mean of 6.7 log_{10}, confirming that plasma TTV loads may vary extensively in individual patients, but tend to be relatively high (31). At days 1 and 2 of therapy, two patients showed a marginal reduction of plasma TTV levels, but the others had either unchanged or slightly increased values relative to baseline. Thus, the first significant decline of mean plasma TTV load occurred at day 3. Subsequently, increasing numbers of patients showed levels of viral DNA in plasma below detection sensitivity (3 log_{10} copies/ml), and up to day 30, the mean loads became progressively lower. At day 90, the mean TTV load was slightly increased relative to that at day 30, although the increase did not reach statistical significance. Table 1 also shows that in the same patients, baseline levels and responses to treatment of HCV were in line with published data (3, 12, 19). A substantial decline in mean HCV viremia was clearly evident at day 1 of treatment, when one patient was already below the assay sensitivity limit (3 log_{10} copies/ml). Subsequently, HCV viremia declined at a lower rate up to day 90, when the maximum proportion of patients who had cleared the virus to an undetectable level (67%) was also observed. In accord with previous findings (4, 18), in four untreated patients, monthly measurement of TTV and HCV levels in plasma showed essentially stable values, with fluctuations of no more than 0.6 and 0.5 log_{10} copies/ml, respectively (data not shown).

Figure 1 shows the sequential modifications of plasma TTV levels of the patients for whom a minimum of six sequential samplings were available. One subject showed a maximum decline of viremia of approximately 1 log_{10} copy/ml throughout the observation period. The other 13 patients in this group responded favorably to treatment, as demonstrated by viremia levels under the detection limit at one or more sampling points. It is noteworthy that only in three patients was the beneficial effect of IFN evident from the first week of treatment. It is also important to note that, in spite of continued treatment, three responders had a relapse of TTV viremia during the observation period. It should also be noted that, at least in this limited group, negativity or positivity of TTV

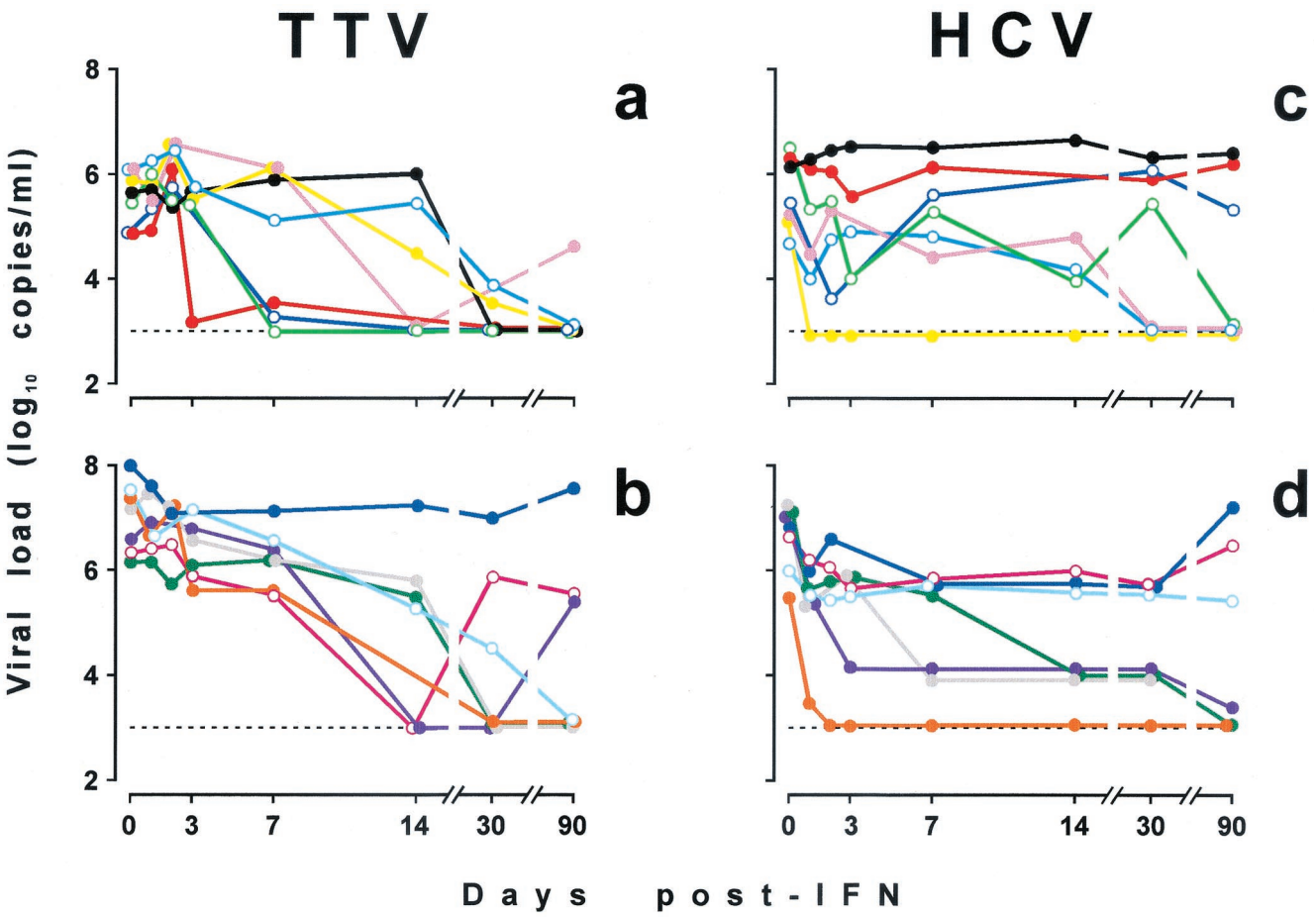

FIG. 1. Effects of 3 months of IFN therapy on plasma loads of TTV (a and b) and HCV (c and d) in 14 doubly infected patients for whom a minimum of six samplings were available. a and c, patients with baseline TTV load lower than the median; b and d, patients with baseline TTV load higher than the median. Individual patients are color coded (solid symbols, patients infected with subtype 1 TTV; open symbols, patients infected with other TTV genotypes). The horizontal broken line represents the lower limit of detection sensitivity.

viremia at 90 days appeared essentially unrelated to virus genotype and baseline viremia levels (Fig. 1).

Consistent with previous findings (3, 11, 17), the changes of HCV plasma loads produced by IFN treatment were also varied. After a prompt viremia reduction of variable extent and duration observed in virtually all patients, HCV loads continued to decline in some individuals, remained essentially stable in others, and rebounded to values equal to or greater than pretreatment ones in still others (Fig. 1). The data were used to calculate whether a correlation existed between baseline or intratreatment levels of TTV and HCV in plasma. At no time, were loads and responses to IFN treatment of the two viruses found to correlate.

IFN-induced changes in PBMC-associated TTV. TTV has previously been shown to circulate in blood closely associated with PBMC as well as free virions (13, 25, 28). In eight patients of Fig. 1, we also determined the loads of TTV DNA found in thoroughly washed sequential PBMC samples. Consistent with previous findings (13, 27), all baseline samples tested TTV positive at copy numbers that varied widely in individual patients (Table 2). Following initiation of IFN treatment, PBMC-associated TTV levels underwent a decline that was similar in extent and kinetics to what was found for the corresponding plasma samples at all times tested, except, possibly, it started a little earlier, as suggested by the slightly reduced mean load detected at day 2. In fact, viral loads in plasma and PBMC were moderately to highly correlated in individual patients ($r = 0.31$

TABLE 2. TTV detection and loads in the PBMC and plasma at selected times of IFN treatment

Day of treatment	No. of patients tested[a]	TTV DNA			
		PBMC		Plasma	
		No. of patients positive	Log_{10} copies/μg of total DNA (mean ± SE)[b]	No. of patients positive	Log_{10} copies/ml (mean ± SE)[b]
0	8	8	5.8 ± 0.5	8	6.2 ± 0.4
1	8	8	5.9 ± 0.6	8	6.1 ± 0.3
2	8	8	5.3 ± 0.6	8	6.5 ± 0.2
3	5	5	4.9 ± 0.6*	5	5.2 ± 0.5*
7	8	8	5.2 ± 0.6	8	5.3 ± 0.4*
30	7	7	4.1 ± 0.5*	5	4.2 ± 0.6**
90	7	5	3.6 ± 0.6**	3	4.1 ± 0.6**

[a] Not all samples were available for all patients at all times.
[b] TTV loads below detection sensitivity were arbitrarily scored as 2 and 3 log_{10} copies/μg of total DNA or per ml of plasma, respectively. * and **, significantly different from day 0 at $P < 0.05$ and $P < 0.01$, respectively (Student's t test).

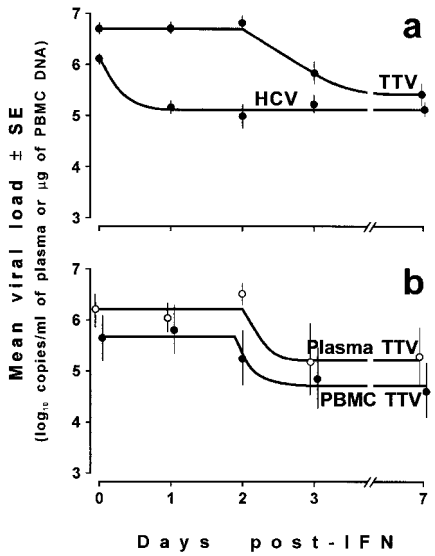

FIG. 2. Mean TTV loads and best fit of the model with plasma of all 25 patients in Table 1 (a) and in the PBMC and plasma of the 8 patients in Table 2 (b). For comparison, panel a also shows the corresponding curve for HCV. Bars represent 95% confidence limits of the mean.

at baseline and $r = 0.87$ at day 2). It should be noted, however, that at late sampling points, some PBMC samples tested TTV positive (in one case at 5.11 $\log_{10}$ per μg of extracted DNA) in spite of the fact that the corresponding plasma samples were under the level of detection sensitivity (Table 2). This is in line with findings showing that PBMC are not merely contaminated by virus in plasma, but likely represent a site of active TTV replication (13).

Dynamics of TTV. In chronically infected individuals, TTV viremia fluctuates very little over periods of weeks or months (described above), indicative of the existence of a quasi-steady-state virus-host equilibrium resulting from balanced virus production and elimination. We hence assumed that, prior to treatment, the viral loads in the study patients were at steady state and exploited the declines of TTV plasma viremia brought about by IFN to look into the dynamics of chronic TTV infection. Plasma viremia data from day 0 to day 7 of all 25 study patients were analyzed with a previously developed mathematical model (17). By using nonlinear regression analysis, we estimated the overall values for delay of response to IFN (t_0), efficacy of the drug (ε), and virion clearance rate (c) by fitting the model equation to mean viral loads. Figure 2a and Table 3 show that TTV elimination from plasma started after day 2 and was maximum between days 2 and 3, while mean IFN effectiveness was 0.94. On the other hand, the clearance rate of TTV from plasma was 2.5 days^{-1}, and the $t_{1/2}$ of TTV in plasma, calculated with such a value, was 0.27 days, with large standard error values reflecting the wide variability in response to treatment exhibited by individual patients. Assuming that the rate of natural TTV clearance from the circulation did not vary as a result of IFN treatment, this implies that, on average, 94% of the TTV virions found in plasma of chronically infected subjects turn over daily and that the minimum number of cell-free TTV particles cleared and replenished daily to keep plasma viremia levels steady is 3.8×10^{10}. However, in view of the fact that most plasma TTV is complexed with antibodies (20), it is possible that its clearance rate is significantly affected by IFN treatment. Thus, calculated values should be considered as approximate. As depicted in Fig. 2b, the best-fit elimination curve of PBMC-associated TTV was identical in shape to that of TTV in plasma, except that the phase of the maximum elimination rate started somewhat earlier ($t_0 = 1.85 \pm 0.014$ versus 2.07 ± 0.012; $P < 0.001$).

In accord with previous findings (17), the same patients showed a clearly biphasic elimination curve of plasma HCV, with a very steep slope from day 0 to 1, followed by a phase of slow or no decline (Fig. 2a). Calculated parameters for HCV dynamics (Table 3) compared well with previously published data (17, 34): in particular, $t_{1/2}$ was 0.14 days, corresponding to over 98% virions renewed per day and at least 2.7×10^{10} virions produced and cleared daily.

DISCUSSION

The present observation that in the early stages of IFN treatment for hepatitis C most patients exhibited a marked decline of concomitant TTV viremia confirms earlier reports that TTV infection responds favorably to IFN treatment (2, 21). However, in spite of continued treatment, some initial responders showed a TTV relapse before the end of the 3-month study period, suggesting that at least in some subjects, this beneficial effect can be very short-lived. A previous report that hepatitis C patients examined after 6 or more months of IFN therapy had levels of TTV essentially unchanged relative to pretreatment values (12) points to the same conclusion. The observed lack of correlation between the responses of TTV and HCV to therapy suggests that the factors that control the responsiveness to IFN of the two viral infections are at least partly independent. The parameters generally used as predictors of HCV responsiveness to IFN therapy include virus ge-

TABLE 3. TTV and HCV dynamics in doubly infected patients treated with IFN

Virus	Effectiveness of drug ε (mean ± SE)	Viral parameter (mean ± SE)			
		Delay of drug response (t_0 [days])	Clearance rate (c [days^{-1}])	Virion half-life ($t_{1/2}$ [days])[a]	Minimal input and clearance of plasma virions/day (10^{10})[b]
TTV	0.94 ± 0.002	2.07 ± 0.004	2.5 ± 3.1	0.27 ± 0.31	3.8 ± 0.6
HCV	0.85 ± 0.002	<1	5.0 ± 4.1	0.14 ± 0.03	2.7 ± 0.4

[a] Calculated by the equation $t_{1/2} = \ln(2)/c$.
[b] Calculated by the equation $P = cV_0$, assuming that plasma and extracellular fluids were 3.0×10^3 ml in total and in equilibrium. In this equation P represents daily production of virions in plasma.

notype, viral load, and quasispecies complexity (29). Here, patients carrying genotype 1 or non-genotype 1 TTV or having different pretreatment TTV loads showed no differences in their ability to clear TTV in response to IFN, but further studies involving larger series of patients will be needed to draw conclusions on this matter. Being targeted to a highly conserved domain of the viral UTR, the real-time PCR we used to measure TTV loads detects a wide range of diverse TTV genomes, some of which have recently been proposed to represent distinct viral species (16, 32). Thus, it is most likely that among the large variety of viral genomes currently labeled as TTV, there are definite subtypes or species with different IFN sensitivities.

Undoubtedly, the most interesting information that emerged from this study is the estimate of the kinetics of chronic TTV viremia we obtained by analyzing the observed IFN-induced changes of TTV load with a mathematical model that has provided valuable insights on other chronic viral infections (17, 30). The analysis showed that TTV sustains very active chronic infections, with over 90% of the virions in plasma cleared and replenished on a daily basis and a minimum of approximately 3.8×10^{10} virions generated per day. Thus, in this respect, TTV resembles the other viruses that produce chronic plasma viremia in humans, namely HCV, HIV-1, and hepatitis B virus, which have previously been shown to sustain highly dynamic chronic infections (5, 22, 30). For example, the estimated numbers of HCV virions produced daily by chronically infected patients have ranged between 10^9 and 10^{12} in different studies (17, 34) and averaged 2.7×10^{10} in our patients.

Different from the other known viruses of humans that produce chronic plasma viremia, TTV lacks an external cell-derived envelope that might permit the egress of virions from intact cells with little or no cytopathology. Thus, unless virus yield per infected cell is unusually high, the copious production of virions that occurs during chronic TTV infection should be expected to produce substantial cytolysis. Because no pathological effects have unequivocally been ascribed to TTV, this in turn might imply that it replicates in cell types that tolerate extensive destruction with no clinical consequences, possibly due to the existence of large reservoirs and/or regenerative potential. Although the cells supporting TTV replication in infected hosts are still poorly defined, current evidence points to liver (26) and lymphoid tissues (9, 13), two districts which would correspond to such a requisite. Resembling what was previously observed with HIV-1 following antiretroviral chemotherapy (7), the levels of PMBC-associated and plasma TTV declined at about the same rate as a result of IFN therapy. However, low levels of TTV were occasionally detected in the PBMC of patients who had cleared the virus from plasma, thus corroborating results showing that lymphoid tissues may support TTV replication (13). Interestingly, while in analogy with previous reports (3, 17), the decline of HCV viremia was already clearly evident within 1 day from initiation of IFN treatment, that of TTV became detectable only at day 3. Because it seems unlikely that this lag reflects differences in the efficacy of IFN at inhibiting virus replication in different cell types or body sites, it is plausible that TTV release into the circulation continues for some time after initiation of IFN administration, due to continuing lysis of cells that have already progressed through virus replication to a phase no longer susceptible to IFN. Alternatively, the bulk of TTV replication might occur in districts in which exchanges with blood are relatively slow, hence representing a sort of virus reservoir. According to a recent study (14), that the decline of plasma HIV-1 loads induced by highly active antiretroviral therapy is slower to start than that the decrease in plasma HCV loads induced by IFN should be attributed to the fact that most HIV-1 is produced in lymphoid tissues, whereas most HCV replicates in the liver and is therefore virtually released directly into blood. The latter explanation would also imply that the estimate presented above for total daily TTV production is indeed minimal and should be considerably increased.

When incubated at 37°C in vitro, TTV detectability exhibited an estimated half-life of over 5 days versus approximately 7 h in vivo (data not shown). This confirms that TTV is a very stable virus, as is predictable from inferred structural properties (15, 23) and indicated by a previous study (13), and also suggests that powerful mechanisms are operative in vivo that continuously and very effectively clear the virus from blood. Possible mechanisms of TTV clearance from the circulation include virus adsorption to susceptible cells and uptake by phagocytic cells. Although immune responses to TTV are just beginning to be unveiled, current data (20) and our own unpublished results show that a variable but generally large portion of the cell-free virus in plasma of persistently infected individuals is in the form of immune complexes. It is hence feasible that antiviral antibodies play an important role in clearing the virus from the circulation, by opsonizing the virions for phagocytic cells or other means. Further studies should clarify whether the abundant immune-complexed TTV circulating in chronically infected individuals is still capable of initiating cell infection, thus possibly shedding light on why antibodies are incapable of eradicating a putatively cytocidal virus that circulates so extensively in plasma.

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