CLEARANCE OF HEPATITIS TT VIRUS INFECTION AMONG THALASSEMIA CHILDREN AND IVDU

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Abstract. The novel transfusion transmissible hepatitis virus TTV first isolated by a group from Japan has predominantly been detected in members of groups at high risk for contracting blood borne viruses. Aside from elevated liver enzymes, the symptoms associated with its infection have been reported to range from asymptomatic to hepatic failure. The purpose of the present study was to determine if and to what extent the host's immune response is capable of clearing TTV infection. Hence, we extracted DNA from sera obtained from altogether 201 intravenous drug users (IVDU) and 80 thalassemia children - both groups at high risk of parenteral exposure - and performed PCR using semi-nested primers. Those positive for TTV DNA were once again subjected to PCR after approximately one year in order to determine how many still harbored the virus. Our results showed TTV DNA to be absent in merely 20.6% of the formerly positive IVDU, whereas it was still present in all the thalassemia children who could be tested for the second time. Based on the small sample size and the high-risk environment, these results ought to be interpreted with caution and definitely merit further investigation.

INTRODUCTION

Hepatitis TT virus (TTV) represents a novel hepatitis virus first isolated by representational difference analysis as a clone of 500 nucleotides from the serum of a Japanese patient with post-transfusion non-A, B, C hepatitis, who exhibited elevated ALT levels indicating liver inflammation (Nishizawa *et al*, 1997). Subsequently, the same team of researchers have molecularly cloned and characterized the agent as a non-enveloped, single-stranded DNA virus. By now, approximately 3.7 kb of its genome harboring two potential open reading frames have been sequenced (Okamoto *et al*, 1998).

In order to improve the diagnostic efficacy and to elucidate the genetic characteristics of TTV, a Japanese group of researchers sequenced a ~2.4 kb segment of the TTV genome derived from eight Japanese isolates. The region sequenced was found to contain a long open reading frame (ORF-L) coding for a protein of 768-770 amino acids highly rich in arginine at its N-terminus and harboring three or four asparagine-linked glycosylation sites clustered in its central portion. Comparison of this long ORF encoded protein with those of known single-stranded DNA viruses suggested a possible phylogenetic similarity of TTV with chicken anemia virus, which belongs to the family of Circoviridae rather than that of Parvoviridae (Takahashi *et al*, 1998).

To date, five different genotypes of TTV have been isolated from sera of infected individuals in Japan. In Thailand, phylogenetic analysis revealed three different genotypes of TTV comprising six distinct subtypes (Tanaka *et al*, 1998). In either environment, the virus has been found highly prevalent in patients at risk for contracting blood-borne viruses, such as hemoplilia and hemodialysis patients, or intravenous drug users (IVDU). Likewise, TTV was detected among patients with non-A-to-G fulminant hepatitis and chronic liver disease at a frequency amounting to almost 50% (Okamoto *et al*, 1998). Similarly, our group has identified TTV infection among members of high-risk groups in Thailand at a prevalence ranging from 9.2 to 32.7 % (Poovorawan *et al*, 1998a).

Whether TTV acts as an etiologic agent of viral hepatitis has not yet been proven and likewise, the data available to date are far from sufficient regarding its implication in the development of chronic liver disease. For example, the group of Yamamoto investigated the prevalence of TTV DNA in liver tissue of 20 hepatocellular carcinoma (HCC) patients. Their results showed 3 of 8 HBV- and HCV-negative (NBNC) HCC patients and 5 of 12 HBV- or HCV-associated HCC patients to be TTV posi-

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tive. Moreover, TTV was proven neither to be specific for NBNC HCC, nor was it found integrated into host hepatocyte DNA in those patients positive for TTV DNA (Yamamoto *et al*, 1998). Also, the implications of coinfection with TTV in the natural history of chronic HBV or HCV infection are far from clear. In the present study, we investigated if TTV infection could be cleared by the host's immune system in the two population groups who due to the nature of their disorder are readily available for follow-up, namely, IVDU and thalassemia children.

MATERIALS AND METHODS

Population study

From December 1997 until mid January 1998, samples of venous blood were collected from altogether 201 IVDUs all of whom had been using drugs intravenously for varying periods of time and who for the purpose of routine check-up attended the outpatient service at the Drug Addict Center no. 7, Bangkok Metropolitan Health Center no. 7 and Drug Addict Center no. 16, Health Center no. 16, Health Department, Bangkok Metropolitan City, in order to receive methadone therapy. Before drawing blood, each individual was interviewed by means of a standard questionnaire. In addition, every one of the IVDUs tested was asked to sign a form thereby giving his/her informed consent to participating in the study.

In addition, eighty thalassemia children having received multiple blood transfusions who attended the hematology clinic during the period of July to September 1996 were included in the study. The blood was taken during the group matching for the therapeutic transfusion. There were 38 male and 42 female children, their age ranging from 1 to 16 years. The frequency of blood transfusions varied from 2 to 243.

From both groups, those patients found positive for TTV DNA were invited for follow-up and again, blood was drawn and serum was subsequently separated for a repeated PCR-based test for the presence of TTV DNA performed in October, 1998, in order to investigate the potential of immunological clearance of the virus.

At this juncture, it turned out that from the initially 62 TTV-positive IVDU only 34 appeared for the follow-up study, whereas from the initially 16 TTV-positive thalassemia children merely 12 were available for the second PCR-based test.

Detection of TTV

TTV-DNA extraction: DNA was isolated employing the alkaline extraction method (Kaneko *et al*, 1989). Briefly, a 10 µl aliquot of serum was pipetted into a 0.5 ml microcentrifuge tube and incubated with NaOH at a final concentration of 0.1 M at 37° C for 60 minutes. The solution was subsequently spun for 15 seconds in a microcentrifuge and neutralized with HCl at a final concentration of 0.1 M.

TTV-DNA detection: TTV-DNA was detected by polymerase chain reaction using semi-nested primers. The amplification reaction was performed in a 50 µl reaction volume containing 1 U of Taq polymerase (Perkin Elmer Cetus), and each of four deoxynucleotide triphosphates at a concentration of 200 µM, primer pairs NG 059 and NG 063 for the first round, and NG061 and NG 063 for the second round, respectively, at a concentration of 1 µM each, 10 mM Tris, 1.5 mM $MgCl_2$ and 5 µl of each DNA sample. According to Okamoto *et al* (1998) the nucleotide sequences of the TTV primers derived from the N-22 region, which represents the most conserved sequence of the 5 genotypes described to date, were: NG 059 (5′ CAG ACA GAG GAG AAG GCA ACA TG 3′), NG 061 (5′ GGC AAC ATG TTA TGG ATA GAC TGG 3′) and NG 063 (5′ CTG GCA TTT TAC CAT TTC CAA AGT T 3′). The first round amplification reaction using primer pair NG 059 and NG 063 was performed for 30 cycles (denaturation at 94°C for 36 seconds, annealing at 55°C for 42 seconds, and extension at 72°C for 90 seconds, final extension at 72°C for 10 minutes). The second round of amplification was performed using 2 µl of the PCR product along with primer pair NG 061 and NG 063 for 30 cycles under identical conditions in a final reaction volume of 20 µl. Upon conclusion of the PCR the reaction mixture was spun for 1 minute at 10,000 rpm, and 10 µl each of the amplified DNA were fractionated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light. The product band will show at 271 base pairs. The gels were photographed on a UV light box. Sera obtained from IVDU and known to be positive for TTV-DNA and sterile water were used as positive and negative controls, respectively.

Liver function test

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (AP) levels were determined from each specimen by automated chemical analyzer (Hitachi 911) at the central laboratory, Chulalongkorn Hospital. The normal levels obtained with healthy adults are within the

range of 0-38 U/l for AST/ALT and 98-279 U/l for AP, respectively.

Statistical analysis

Comparison between groups of patients with ALT elevation was made by Fisher's exact test for categorical variables. A p value below 0.05 was considered significant.

RESULTS

The results of the first PCR-based test revealed 62 of the 201 IVDU (30.8%) and 16 of the 80 thalassemia children (20.0%), respectively, to carry TTV DNA. Within the first group, the liver enzymes were elevated in 11 patients out of the 34 available for follow-up (32.3% of 34). In the group of thalassemia children this test was not performed.

The second PCR-based test performed on samples obtained from identical sources, but approximately one year later, showed that 27 out of 34 formerly TTV- positive IVDU, who were still available for follow-up (79.4% of 34) still carried the virus, whereas 7 (20.6% of 34) had apparently cleared it. In the group of thalassemia children, the 12 patients still available for follow-up all proved positive for TTV DNA.

The results as to the prevalence of TTV positivity determined in both groups at various points in time have been depicted in Fig 1.

Among the IVDU, the liver enzymes tested for the second time were still elevated in 10 patients (29.4% of 34), 3 of who were negative for TTV DNA. No statistically significant difference was detected between TTV-positive and TTV-negative individuals, respectively.

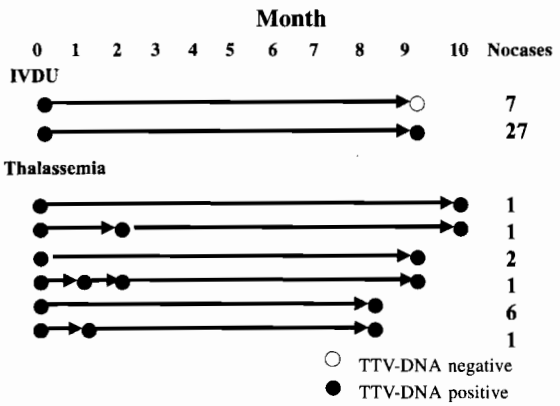

Fig 1–Clearance of hepatitis TT virus DNA from sera of IVDU and thalassemia patients.

DISCUSSION

The results garnered from the present study exclusively performed on the members of two groups at particular risk of contracting blood-borne infectious agents, namely, IVDU and thalassemia children, at least hint at the possibility of immune clearance of the novel transfusion transmissible hepatitis TT virus in that 7 (20.6%) of the 34 TTV-positive IVDU still available for follow-up nine months after the initial examination apparently had cleared the viral DNA from their sera. Still, these data ought to be viewed with caution especially as the information available on this agent, due to its novelty, is too scarce by far to draw solid conclusions from.

By the same token, the elevation of the liver enzymes detected in the first as well as the second test performed in all but 10 of the IVDU, 3 of who were TTV-DNA negative, might be attributable to one or more of several factors other than TTV infection as for example, any other viral infection and/or the long-term impact of prolonged drug abuse, the exact composition of which is and most probably will remain impossible to be traced.

On the other hand, if we go along with Nishizawa *et al* (Nishizawa *et al*, 1997) and presume antibodies to TTV to be raised by the host, irrespective of TTV being a non-enveloped virus, and if we further presume that these antibodies account for the transient infections described by the above group of researchers in two cases, as well as in the seven cases of IVDU observed by us, then it would still be premature to speak of immune clearance in the true sense of the word. Hence, the apparent clearance of TTV in 7 of the 34 TTV-positive IVDU might be deceptive in that these people might have been examined at the time when they had turned negative, yet might have been re-infected by a different strain of TTV by now. In other words, with the small number of persons tested, who furthermore belong to high-risk groups, the results obtained cannot be interpreted conclusively.

Comparison between the clearance rate of TTV with that of HGV, another blood-borne hepatotropic virus predominantly detected among members of high-risk groups, unequivocally shows that HGV can be cleared much more efficiently than TTV, in that HGV RNA was undetectable in the sera of approximately 50% of the formerly HGV-RNA posi-

tive thalassemia children after a period of 14-16 months (Poovorawan *et al*, 1998b). This might be due to either less variability in HGV as opposed to TTV genotypes, or to the HGV envelope protein representing an antigenic epitope triggering both the cellular and humoral immune response, whereas any TTV encoded core protein could merely elicit a humoral reaction.

Last not least, as the DNA samples examined were exclusively derived from serum and therefore, those found TTV-DNA positive indicate circulating and actively replicating viral particles, whereas the presence of viral DNA within PBMC, for example, which would indicate integrated and hence latent virus, has not been established in the present study.

The results of the present study thus clearly emphasize the necessity for further research into this novel virus in order to determine its tropism as well as the host's capacity for immune clearance to a degree less equivocal.

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