

Detection of TTV in Peripheral Blood Mononuclear Cells of Intravenous Drug Users

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POOVORAWAN, Y., THEAMBOONLERS, A., VIMOLKET, T., JANTARADSAMEE, P., KAEW-IN, N. and HIRSCH, P. *Detection of TTV in Peripheral Blood Mononuclear Cells of Intravenous Drug Users.* Tohoku J. Exp. Med., 1999, 188 (1), 47-54 — In order to further elucidate the tropism of the novel hepatitis TT virus (TTV) we investigated 22 intravenous drug users (IVDU) for the presence of viral DNA in their peripheral blood mononuclear cells (PBMC) by means of seminested polymerase chain reaction using a set of primers specific for the conserved region of its genome. We detected TTV DNA in 63% of those individuals who had previously been found positive for the agent in their serum, whereas the three remaining ones not displaying TTV DNA in their serum and hence, serving as controls also proved negative in their PBMC. The results presented here further support earlier findings by other authors and their conclusion as to the virus employing a parenteral route of transmission. Further investigation will be required in order to clarify the mechanism of viral infection. ———— TTV; PBMC; IVDU © 1999 Tohoku University Medical Press

The discovery of novel hepatotropic viruses has been reported in the hope of providing explanations for the occurrence of non-A-G acute and chronic hepatitis. Along those lines, hepatitis TT virus (TTV) constitutes the most recent acquisition in the already veritable collection of hepatitis viruses. The agent was first isolated by representational difference analysis as a clone of 500 nucleotides from the serum of a Japanese patient with post-transfusion non-A-G hepatitis, whose liver function test showed elevated enzyme levels indicating liver inflammation (Nishizawa et al. 1997). Thereupon, it was molecularly cloned and characterized by the same group as a non-enveloped, single-stranded DNA virus. To date, approximately 3.7 kb of its genome containing two potential open reading frames have been sequenced (Okamoto et al. 1998a).

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With the aim of improving diagnostic efficacy, as well as elucidating the genetic characteristics of TTV, another Japanese group sequenced a ≈ 2.4 kb segment of the TTV genome obtained from eight Japanese isolates. This region was found to contain a long open reading frame (ORF) coding for a protein of 768–770 amino acids particularly rich in arginine residues at its N-terminus and furthermore, 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, a member of the Circoviridae family (Takahashi et al. 1998).

Initially, five different genotypes of TTV have been isolated from sera of infected persons in Japan where the virus appears to be highly prevalent among patients at risk for contracting blood-borne viruses, as for example, hemophilia and hemodialysis patients, or intravenous drug users (IVDU). Moreover, TTV was recovered from patients with non-A-G fulminant hepatitis and chronic liver disease at a frequency of almost 50% (Okamoto et al. 1998a). In that context, our group has attained similar results in that we identified TTV infection among members of high-risk groups in Thailand with a prevalence between 9.2 and 32.7% (Poovorawan et al. 1998b). Also, TTV does not appear geographically limited as it has been detected by a group of researchers in Germany performing polymerase chain reaction (PCR) on several distinct patient groups in 19% of patients awaiting orthotopic liver transplantation due to decompensated cirrhosis, in 16% of non-A-G hepatitis patients, in one out of seven patients with autoimmune hepatitis and in one IVDU (Hohne et al. 1998). Very recently, a phylogenetic analysis performed on the open reading frame I sequence of 93 TTV isolates obtained from various geographical areas revealed six different genotypes of the virus including three, which to that day had not been reported (Tanaka et al. 1998b).

The question as to whether TTV actually is the etiologic agent of viral hepatitis has as yet not been answered satisfactorily and the data available with respect to its implication in the development of chronic liver disease are also rather insufficient. Moreover, the findings published by several researchers regarding the cell or tissue type in which TTV was detected can almost be considered conflicting. For example, the team of Okamoto (Okamoto et al. 1998a) discovered TTV in liver specimens from five hepatitis patients with TTV in serum at titers equal to or between 10- to 100-times higher, whereas the liver sample of one patient without the virus in serum proved equally negative. Tanaka's group (Tanaka et al. 1998a), on the other hand, reported TTV DNA to occur more frequently in patients with liver cirrhosis and hepatocellular carcinoma than in those with chronic hepatitis. Also, they did not find any differences in age, sex, or markers of infection with hepatitis B, C and GBV-C/HGV viruses, from which they concluded the mode of transmission of TTV to be

different from that of the other hepatitis viruses. In support of this, Okamoto's team (Okamoto et al. 1998b) reported in yet another publication to have detected TTV DNA in feces from three patients with type B or C hepatocellular carcinoma, including two with high viral titers in serum. Hence, the excretion of TTV into feces hints at it being transmitted by a fecal-oral route in addition to parenteral transmission proven by its presence in serum.

The purpose of our present study has been to shed some light on the rather obscure tropism of this novel virus by subjecting the peripheral blood mononuclear cells (PBMC) of 22 intravenous drug users, 19 of who were TTV DNA positive and 3 TTV DNA negative in their respective serum, to the polymerase chain reaction using seminested primers deduced from the nucleotide sequence of the original N22 clone (Nishizawa et al. 1997) situated within the long open reading frame.

MATERIALS AND METHODS

Population studied

Nineteen IVDU known to be positive for TTV DNA in their sera were included in the study, along with 3 additional IVDU the sera of who were TTV negative and who therefore served as controls. After having informed the subjects of the purpose of the study, peripheral blood was taken administering ethylenediaminetetraacetic acid (EDTA) as anticoagulant for PBMC separation, as well as clotted blood for serum analysis. Written informed consent was obtained from all subjects.

Laboratory methods

PBMC separation. Sera were obtained by centrifugation of the clotted blood at 1500 rpm for 10 minutes (Beckman refrigerated centrifuge). PBMC were separated by spinning the EDTA-treated blood on a Ficoll-Hipaque (Pharmacia, Uppsala, Sweden) gradient at 2500 rpm at 4°C for 15 minutes (Beckman refrigerated centrifuge) followed by four consecutive washing steps with phosphate buffered saline (PBS) at 2500 rpm at 4°C for 15 minutes each. The washing buffer remaining after the last washing step was also subjected to PCR in order to examine the plasma for TTV contamination. The PBMC thus obtained were suspended in 1 ml PBS and after staining with methylene blue their respective concentration was determined in an improved Neubauer ruling chamber. All specimens were kept at -70°C until further tested.

TTV DNA

TTV-DNA extraction. DNA was extracted from sera, PBMC, as well as from PBS kept after the last washing step by incubating the respective sample in Tris/SDS-buffer containing proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. Pellets were resuspended in 20 μ l sterile water each

and directly subjected to the polymerase chain reaction.

TTV-DNA detection. TTV DNA was detected applying the method described elsewhere (Poovorawan et al. 1998b).

Detection of β -globin DNA

The β -globin gene was selected to serve as internal control. We performed 35 cycles of PCR in a total reaction volume of 50 μ l containing 1.25 U of Taq polymerase (Perkin Elmer Cetus) and each of four deoxynucleotide triphosphates at a concentration of 200 μ M, primers GH 20 (5'-AAGAGCCAAGG ACAGGTAC-3') and PC04 (5'-CAACTTCATCCACGTTCAACC-3') at a concentration of 0.05 μ M, 10 mM Tris buffer, 4 mM MgCl₂, and 10 μ l of each DNA sample. The PCR product will show after fractionation on a 2% agarose gel containing ethidium bromide as a 260 base-pair band under UV light.

GBV-C/HGV RNA

GBV-C/HGV RNA was detected applying the method described elsewhere (Poovorawan et al. 1998a).

Anti-HIV

The sera were examined for anti-HIV antibody using three consecutive enzyme linked immunosorbent assays (Abbott Laboratoris, North Chicago, IL, USA; Enzygnost, Behring Co., Germany; Genelaviamist, Pasteur Merieux, France).

RESULTS

As shown in Table 1, the requirements for the polymerase chain reaction were met with all samples as obvious from the ubiquitous amplification of the β -globin gene. Also, the negative results obtained upon amplification performed on the washing buffer (PBS) proved our PBMC preparations to be free from contamination by plasma-borne virus particles. Furthermore, the three IVDU negative in their respective serum for TTV DNA, were also negative in their PBMC which had an average concentration of 2600–6000 cells/mm³. Of the 19 IVDU whose serum contained TTV DNA, 12 (63%) harbored the virus in their PBMC. Also, in the present study 6 out of those 19 individuals have been shown positive for GBV-C/HGV RNA in their sera, and in four anti-HIV antibody has been detected by three consecutive ELISA-tests.

DISCUSSION

As previously reported (Poovorawan et al. 1998b), infection with the novel hepatitis TTV appears to be rather common among intravenous drug users, who clearly constitute members of a high-risk group for contracting blood-borne viruses. As with TTV DNA in serum, we could not detect any bias regarding age

TABLE 1. *Viral parameters and β globin gene in PBMC and serum of IVDU*

No.	Age (Year)	Sex	PBMC concentration (cells/mm ³)	Serum TTV- DNA	PBS TTV- DNA	PBMC		Serum	
						β globin Gene	TTV- DNA	GBV- C RNA	anti- HIV
1	40	M	5000	—	—	+	—	—	+
2	20	F	5600	—	—	+	—	—	—
3	45	M	5800	—	—	+	—	—	—
4	40	M	5800	+	—	+	+	—	—
5	30	M	6000	+	—	+	—	—	—
6	28	M	2600	+	—	+	+	—	—
7	23	M	5000	+	—	+	—	—	—
8	59	M	3000	+	—	+	+	—	—
9	38	M	4000	+	—	+	—	—	—
10	43	M	4600	+	—	+	+	—	—
11	19	F	5000	+	—	+	+	—	—
12	31	M	5600	+	—	+	+	—	—
13	26	M	5000	+	—	+	—	—	—
14	47	M	3400	+	—	+	+	+	—
15	19	F	4000	+	—	+	—	—	—
16	40	M	4200	+	—	+	+	+	—
17	29	M	5000	+	—	+	+	+	—
18	45	M	5600	+	—	+	+	—	—
19	30	M	5800	+	—	+	—	+	—
20	25	M	6000	+	—	+	+	—	+
21	22	M	5200	+	—	+	—	+	+
22	38	M	5800	+	—	+	+	+	+

or sex distribution. Yet, regarding the latter bias this ought to be interpreted with caution since the female gender has been rather under-represented in our study population.

The assumption of TTV representing a blood-borne virus has been further corroborated by the findings of Simmonds' team (Simmonds et al. 1998) who reported TTV viremia to be frequent in the blood-donor population, as well as blood products to be contaminated with the agent. Based on these findings, the suggestion of TTV employing a fecal-oral route of transmission as it was detected in the feces of hepatocellular carcinoma patients (Okamoto et al. 1998a) not merely ought to be viewed with caution due to the small case number, but also calls for additional data collected from asymptomatic carriers. Moreover, in addition to being detected in their respective sera, in 63 % of those individuals it could also be found in PBMC, a finding which calls the results of various researchers to mind who have reported the presence of hepatitis B virus, hepatitis

C virus, Epstein-Barr virus, cytomegalovirus, human immunodeficiency virus, and human T cell leukemia virus (Rinaldo et al. 1978; Reinherz et al. 1980; Gelmann et al. 1983; Pontisso et al. 1984; Laskus et al. 1997; Sebire et al. 1998) in mononuclear cells. Since all these agents are undeniably transmitted by the parenteral route the novel hepatitis TTV may simply be added to their number. Moreover, in order to ascertain that TTV DNA detected in PBMC had not originated from minute serum contamination nonspecifically attached to the cell surface, the PBMC were subjected to extensive washing before DNA extraction. The negative PCR results obtained with the final washing buffer confirm the success of our precaution.

In a very recent publication from Japan, TTV DNA has been found in PBMC of all the 30 healthy individuals tested who had been known to harbor the virus in their plasma (Okamoto et al. 1999). The reason why we could detect TTV DNA in only 63% of the PBMC extracted from IVDU 100% positive for TTV in their sera, as opposed to the 100% detection rate in PBMC reported from Japan, remains open to speculation. For example, it might be due to a particular permissiveness for TTV infection potentially genetically determined within certain populations in analogy to the CCR5 chemokine receptor deletion especially prevalent among certain geographically delineated groups in connection with HIV infection (Kim et al. 1998; Lucotte and Mercier 1998).

The mechanism of this viral infection remains speculative and further investigation will be required in order to establish if it is related to the presence of cellular receptors on the cells, or to the state of cell differentiation, moreover, if TTV uses the mononuclear cells as a vehicle for transport simultaneously supplying it with the means required for its replication, transcription and translation, or if on top of that, the virus causes long-term immunological abnormalities.

Therefore, further investigation will be required in order to establish if TTV DNA can exist as an integrated form in the host DNA or, in case of the PBMC supplying it with the cellular components essential for viral proliferation and expression, if it is B- or rather T-lymphocytes that serve this purpose.

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