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Frequent Detection of the Replicative Form of TT Virus DNA in Peripheral Blood Mononuclear Cells and Bone Marrow Cells in Cancer Patients

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The TT virus (TTV), a member of a family of human viruses related to the *circoviridae* viruses, was associated initially with acute and chronic liver diseases. TTV consists of a single-stranded, circular DNA genome of 3.8 kilobases (kb) and at least three open reading frames (ORFs). The objective of the present study was to determine whether or not TTV replicated in peripheral blood mononuclear cells (PBMCs) and bone marrow cells (BMCs). DNA was extracted from the PBMCs or BMCs of 153 cancer patients and from the PBMCs of 50 healthy blood donors (the controls). By using a single round of polymerase chain reaction (PCR), TTV was detected in 98.6% (141 of 143) of the PBMCs and in 90% (9 of 10) of the BMCs from cancer patients. TTV DNA was detected in significantly fewer control subjects at 86% (43 of 50; $P < 0.05$). Strand-specific PCR (SSPCR) targeting the ORF2 of the common genotypes of TTV was developed specifically to detect TTV positive or negative strand DNA and to examine TTV replication. TTV positive strand DNA, which may be an intermediate of viral replication, was detected in 55.3% (78 of 141) of the TTV-infected PBMCs of the cancer patients and in 7% (3 of 43) of the controls ($P < 0.001$). The replicative form of TTV was also detectable in 55.6% (5 of 9) of the TTV-infected BMCs. The existence of double-strand (positive and negative strands) TTV DNA in PBMCs and BMCs of the cancer patients was also supported by the finding that TTV DNA extracted from these cells was resistant to S1 nuclease. Using in situ hybridization, TTV DNA was also demonstrated to be present in the nucleus of PBMCs. It is concluded that replicative intermediate forms of TTV DNA are present in both PBMCs and BMCs, indicating that blood cells may be a site of TTV replication. ***J. Med. Virol. 66:428–434, 2002.***

KEY WORDS: peripheral blood mononuclear cells; bone marrow cells; PCR

INTRODUCTION

Using representational difference analysis, a new unenveloped DNA virus was isolated from the serum of a patient with posttransfusion hepatitis of unknown etiology in 1997. It was designated TT virus (TTV) after the patient's initials [Nishizawa et al., 1997]. It has been suggested that TTV is a member of a family of human viruses related to the *circoviridae* viruses [Miyata et al., 1999; Mushahwar et al., 1999]. Post-transfusion hepatitis has been attributed to TTV [Nishizawa et al., 1997; Okamoto et al., 1998] and TTV has been detected in patients with acute and chronic liver diseases [Charlton et al., 1998; Hohne et al., 1998; Naoumov et al., 1998; Simmonds et al., 1998; Takayama et al., 1999; Viazov et al., 1998a Desai et al., 1999; Okamura et al., 1999; Okamoto et al., 1999a]. However, TTV infection is also very common among healthy individuals and is known to have a worldwide distribution [Prescott et al., 1998; Takahashi et al., 1998a; Tanaka et al., 1998a; Viazov et al., 1998b; Biagini et al., 1999, 2000; Davidson et al., 1999; Nakano et al., 1999; Niel et al., 1999; Okamoto et al., 1999a; Takayama et al., 1999b; Gallian et al., 2000].

The entire nucleotide sequence of the TTV genome of several genotypes has been determined [Nishizawa et al., 1997; Erker et al., 1999; Okamoto et al., 1999c; Biagini et al., 2000a] and considerable genomic heterogeneity across different isolates has been found [Biagini

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et al., 1999; Tanaka et al., 1998a, 1998b; Viazov et al., 1998b; Okamoto et al., 1999b]. Based on the open reading frame 1 (ORF1) region, TTV has been classified into at least 16 genotypes [Okamoto et al., 1999b]. Although liver tropism has been suggested, TTV also has been found in other organs including kidneys, prostate, mammary glands, brain, bone marrow cells (BMCs), and peripheral blood mononuclear cells [PBMCs; Okamoto et al., 1998, 1999a; Okamura et al., 1999; Pineau et al., 2000]. Although it is not known precisely in which cell(s) TTV replicates, TTV DNA has been detected frequently in the PBMCs [Okamura et al., 1999; Okamoto et al., 1999a] and also has been suggested to infect and replicate in hematopoietic cells in the bone marrow [Kanda et al., 1999; Kikuchi et al., 2000].

Earlier observation by our group had revealed that there was a higher TTV genome load in the PBMCs of cancer patients than in healthy controls [blood donors; Zhong et al., in press]. This could have been related to immune abnormality in cancer patients when compared with the controls, thereby allowing increased TTV replication in the former. The objectives of the present study were to determine whether or not TTV replicated in PBMCs and hematopoietic BMCs of cancer patients and healthy blood donors (controls). In situ hybridization and a novel technique, strand-specific polymerase chain reaction (SSPCR), were developed and used to estimate TTV replication in PBMCs and BMCs.

MATERIALS AND METHODS

Patients and Healthy Blood Donors

One hundred fifty-three patients suffering from breast cancer (n = 48), non-Hodgkin's lymphoma (n = 27), colon cancer (n = 15), hepatocellular carcinoma (n = 8), nasopharyngeal carcinoma (n = 8), multiple myeloma (n = 10), and others (n = 37) were included in this study. As controls, 50 healthy blood donors had samples collected at the Hong Kong Blood Bank. Informed consent was obtained from both the patients and blood donors.

Five milliliters of blood from each of the patients and the blood donors was collected into an EDTA tube. Subsequently, the blood samples were centrifuged at 680*g* at 25°C for 15 minutes to separate the plasma. PBMCs were recovered from the sediment using Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described elsewhere [Okamoto et al., 1999a]. Bone marrow aspirate (0.5 ml) was obtained from 10 subjects with multiple myeloma. Each of the bone marrow aspirates was collected into an EDTA tube and suspended with 5 ml of phosphate-buffered saline (Dulbecco's PBS) three times. These BMCs were recovered from the suspension with Ficoll-Paque and washed with PBS (-) three times as described previously. The final wash did not contain detectable TTV DNA by PCR, indicating that the PBMCs and BMCs isolated were not contaminated by TTV from the plasma.

DNA Extraction From Plasma, PBMCs, and BMCs

DNA was extracted from 100 μl plasma using the phenol and chloroform method as described previously, with the addition of yeast tRNA to act as a carrier [Zhong et al., 1999]. Two microliters of the extracted material, corresponding to a plasma volume of 10 μl, was then subjected to seminested PCR for TTV. The PBMC and BMC pellet was homogenized and incubated in the presence of 400 μg/ml proteinase K and 0.5% sodium dodecyl sulfate (SDS) at 55°C for 14 hours. DNA was extracted with phenol/chloroform/isoamalalcohol and precipitated with ethanol. The DNAs were collected by centrifugation and dissolved in 100 μl of Tris-HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA (TE buffer). Several precautions were taken to avoid contamination, including performing DNA extraction in a separate room, with filtered tips, and using water as a negative control, throughout the extraction and amplification.

PCR Amplification of TTV Sequences

Two microliters of the DNAs extracted from PBMCs and BMCs, containing 50 ng DNA, was subjected to PCR amplification. In contrast to the seminested PCR described by Okamoto et al. [1998], a single PCR was employed using degenerate oligonucleotide primers NG61d 5′-GGM AAY ATG YTR TGG ATA GAC TGG-3′ (1915-1938) and NG63d, 5′-CTG GCA TYT TWC CRT TTC CAA ART-3′ (2162-2185), which were derived from the N22 region, to amplify the most divergent variants described to date [Okamoto et al., 1998]. The reaction mixture contained 1 U *Taq* polymerase (Perkin-Elmer AmpliTaqgold, Roche Molecular Systems, Branchburg, NJ), dNTP (0.2 mM of each nucleotide [Pharmacia]), Tris-HCl (20 mM, pH 8.4), $MgCl_2$ (3.0 mM), and KCl (60 mM) in a volume of 20 μl. Amplification was done in a thermocycler (Model 9700, Perkin-Elmer, Foster City, CA) under the following conditions: after denaturation for 9 minutes at 95°C, 45 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. For each PCR assay, amplified products were identified subsequently on a 2% agarose gel, stained with 0.1% ethidium bromide, and photographed.

SSPCR Detection of TTV Replication

The possibility of viral replication was examined by SSPCR detection in PBMCs and BMCs. DNAs from PBMCs and BMCs were extracted as described above. A 15-cycle linear amplification in the ORF2 was carried out in 50 μl of 10 × PCR buffer II (Perkin-Elmer), 200 μM of each dNTP (Pharmacia), 3.0 mM $MgCl_2$, 1 U AmpliTaqgold DNA polymerase, and 0.02 μM of a single primer: RT+ [5′-CTGTCGTGGCTCTCTCCGCGAGR-CACATAGCAGMGTG-3′] in which the underlined region permits specific hybridization to the positive strand of TTV DNA; or RT-[5′-CTGTCGTGGCTCT-CTCCGCGAGCTCGGGACTGGCCG-3′] in which the underlined region permits specific hybridization to the

negative strand of TTV DNA. These two primers were tailed with a nucleotide sequence [5′-CTGTCGTGG-CTCTCTCCGCGAG-3′], which is not specific for the TTV genome. This step of amplification was carried out with *Taq* polymerase in order to avoid the reverse transcription of RNA viral transcripts.

The product from the linear single primer amplification reaction was submitted to 40 cycles of PCR using a primer specific for the TTV genome (SP [5′-CARTT-CGGGCTCGGGACTGGCCG-3′] in the case of the positive-strand amplification or AP [5′-GGGACACCC-RCACATAGCAGMGTG-3′] in the case of the negative-strand amplification) and a generic primer (G-Gen-P [5′-GCTGTCGTGGCTCTCTCCGCGAG-3′]), which hybridizes to the TTV sequence-independent tail of primers RT + and RT −. This amplification step was carried out at an annealing temperature of 72°C, which does not permit the hybridization of RT + and RT − primers.

To ensure that the final PCR product was obtained by SP or AP and G-Gen-P primers, a 5′ terminal guanosine was added to primer G-Gen-P. The presence of this additional nucleotide in the sequence of PCR products could be used to ensure that they had not been synthesized by the combination of TTV-specific primary PCR primers (RT + or RT −) with TTV-specific secondary PCR primers (SP or AP).

The strandedness of the TTV DNAs was evaluated by treatment with S1 nuclease (Promega, Madison, WI), prior to first-round SSPCR. The DNA extracted from PBMCs and BMCs was treated with 10 U of S1 nuclease at 37°C for 15 minutes.

DNA Sequencing

The amplified products after the second round of PCR were purified from agarose gel using a gel-extraction method (QIAquick, Qiagen, Chatsworth, CA). Subsequently, they were cloned into a pGEM-T vector (Promega), where two to four clones were sequenced. Sequencing reactions were performed in both directions with the universal M13 forward and reverse sequencing primers using the ABI Big Dye sequencing reaction kit (Perkin-Elmer) and run on an ABI 377 DNA automatic sequencer (Perkin-Elmer).

In Situ Hybridization

A nearly-full TTV genome of 3,354 bp amplified using primer NG134/NG136 [Okamoto et al., 1998] from PBMCs of a cancer patient was cloned directly into a pEGM-T vector to obtain the p32(G2) plasmid following the instructions supplied by the manufacturer. Verification of the insert nt sequence was performed by automatic sequencing. The plasmid was digested into four fragments using *Nco I* and *Not I*. Then, the TTV DNA fragments were isolated and purified from the agarose gel using QIAquick. Subsequently, they were labeled with digoxigenin-11-dUTP using a random-primed DNA labeling kit (Roche, Mannheim, Germany) according to the manufacturer's instruction.

PBMCs from four patients were micro-spined onto slides. After digestion with proteinase K (3 μg/ml) at 37°C for 10 minutes, the slides were postfixed in a freshly prepared solution of 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.0, dipped in distilled water, and treated with 0.1 M HCl for 10 minutes at room temperature. Slides were rinsed in 2 × SSC and quickly dehydrated in ethanol. Hybridization was carried out in a solution containing 50% deionized formamide, 10% dextran sulfate, 4 × SSC, 0.5 M DTT, 1 × Denhart's solution, 250 ng/μl sheared salmon sperm DNA, and 5 ng/μl digoxigenin-labeled probe DNA. After incubating 16 hours at 42°C, slides were washed in 2 × SSC for 15 minutes twice and 1 × SSC for another 15 minutes twice at room temperature. The digoxigenin-labeled hybrids were detected with a digoxigenin-antibody alkaline-phosphatase conjugate and an enzyme-substrate chromogen according to the instructions supplied by the manufacturer (digluminesent nucleic acid detection kit, Boehringer Mannheim). Finally, the slides were counterstained with methyl green (Zymed, San Francisco, CA) and mounted with a coverslip.

The specificity of the signal was assessed by (1) digestion of the slides with RNase A (0.2 mg/ml; Sigma, St. Louis, MO) and DNase I (20 U/ml; Sigma) for 2 hours at 37°C before in situ hybridization; (2) competitive hybridization in the presence of an excess of unlabeled TTV probe; and (3) omission of the probe in the hybridization mixture. Visualization of the in situ hybridization signals was performed and photographed using a Olympus BH2 microscope (Olympus, Tokyo, Japan) with 100 × objectives.

Statistical Analysis

Statistical analysis was carried out using the SPSS-PC program (SPSS, Chicago, IL). To compare TTV prevalence and replication in PBMCs or BMCs of the cancer patients and the controls, the chi-square test was used. P values less than 0.05 were considered to be statistically significant.

RESULTS

TTV DNA was Detected Readily in PBMCs and BMCs of Cancer Patients and PBMCs of Controls

TTV DNA was detected frequently in the PBMCs (141 of 143, 98.6%) and BMCs (9 of 10, 90%) from the cancer patients. In the controls, TTV DNA was detected in PBMCs of 43 of the 50 subjects (86%). The prevalence of TTV in PBMCs in the two groups was significantly different ($P < 0.05$; Table I). Specificity was confirmed by hybridization using an oligonucleotide probe and sequencing of cloned PCR products.

Detection of TTV Positive Strand in PBMCs and BMCs

Ten paired DNA samples of plasma and PBMCs of the cancer patients were selected at random for testing

TABLE I. TTV Prevalence of PBMCs and BMCs in Cancer Patients and of PBMCs in Controls

	No. of specimens	TTV prevalence (%)
Cancer patients		
PBMCs	143	141 (98.6)*
BMCs	10	9 (90)
Controls	50	43 (86)

*Chi-square test between PBMCs of cancer patients and controls ($P = 0.008$).

strand-specific detection of TTV DNA. Negative strand TTV DNA could be detected in five of the corresponding plasma DNA, but no positive strand TTV DNA was detected. However, in PBMC samples, both positive and negative strands of TTV DNA were detected in 4 of the 10 PBMCs (Fig. 1). Sequencing data confirmed the presence of the additional guanosine at one end of the amplicon sequences, proving the specificity of the single-strand detection. S1 nuclease treatment did not affect the results. Therefore, existence of double-strand TTV DNA in PBMCs of the cancer patients was supported by the fact that TTV DNA extracted from PBMCs was resistant to S1 nuclease (Fig. 2).

PBMC samples of 141 cancer patients, which were found positive for TTV DNA using a single round of PCR, underwent SSPCR detection (Table II). Of these, 55.6% (78 of 141) were detected to have the positive strand of the viral DNA. In contrast, only 7% (3 of 43) of the TTV-infected PBMCs from the healthy blood donors were found to have detectable positive strand of viral DNA ($P = 0.0001$).

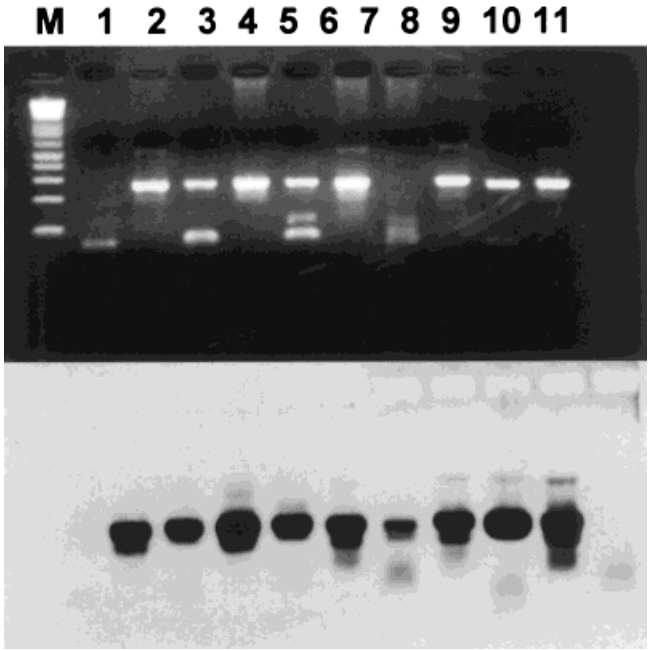

Fig. 1. The positive and negative strands of TTV DNA were detected by SSPCR developed in this study. **Top:** Agarose gel separation of the amplified PCR fragments. Lanes M and 1: 100-bp ladder and negative control (water), respectively; lanes 2–3, 4–5, 6–7, 8–9, 10–11: Negative and positive-strand detection of patients 94, 33, 34, 99, and 31, respectively. **Bottom:** Southern blotting of the gel and hybridization using a labeled probe in the corresponding region.

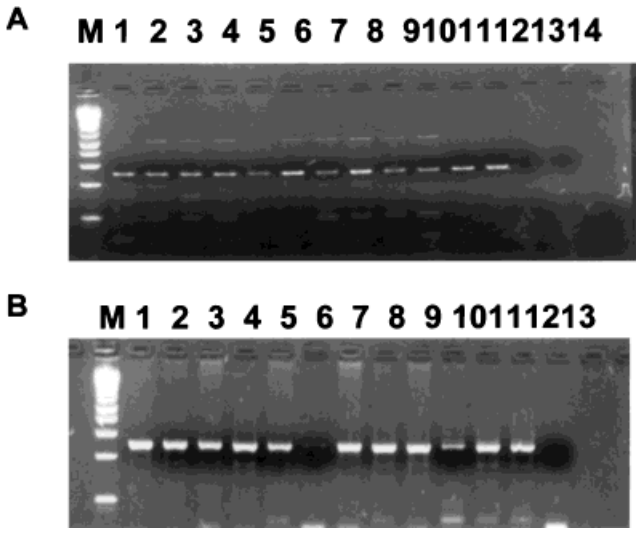

Fig. 2. Susceptibility of TTV DNAs extracted from PBMCs of patients. Lane M: 100-bp ladder. C 1 (lane 13) and C 2 (lane 14) are negative controls for first-round PCR and second-round PCR (only for positive detection). **A:** Positive-strand detection and **B:** negative-strand detection of TTV. Lanes 1–12: PCR amplification of the DNAs from patients 12, 32, 33, 37, 38, 94, 99, 104, 116 156, 158, and 163, respectively.

Similarly, the positive strand of TTV was also detectable in 55.6% (5 of 9) of the TTV-infected BMCs, whereas the negative strand of viral DNA was detected in all samples, eliminating the possibility that other factors may affect PCR efficiency.

In situ Hybridization

TTV genome was detected by in situ hybridization in the PBMC specimens from two of the four patients tested. The hybridization signals were detected mainly in the nucleus (Fig. 3), although the cytoplasm of the cells was also observed in some of the positive cells. Twenty-fifty percent of the PBMCs in the patients studied were infected with TTV.

DISCUSSION

A single round of PCR was employed to detect TTV sequence in PBMCs and BMCs of cancer patients and

TABLE II. Replicative Intermediate Form of TTV DNA in PBMCs and BMCs of Cancer Patients and PBMCs of the Controls

	No. of specimens	TTV prevalence (%)
Cancer patients		
PBMCs	141	787 (55.6)*
BMCs	9	5 (55.6)
Controls	43	3 (7)

*Chi-square test between PBMCs of cancer patients and controls ($P = 0.0001$).

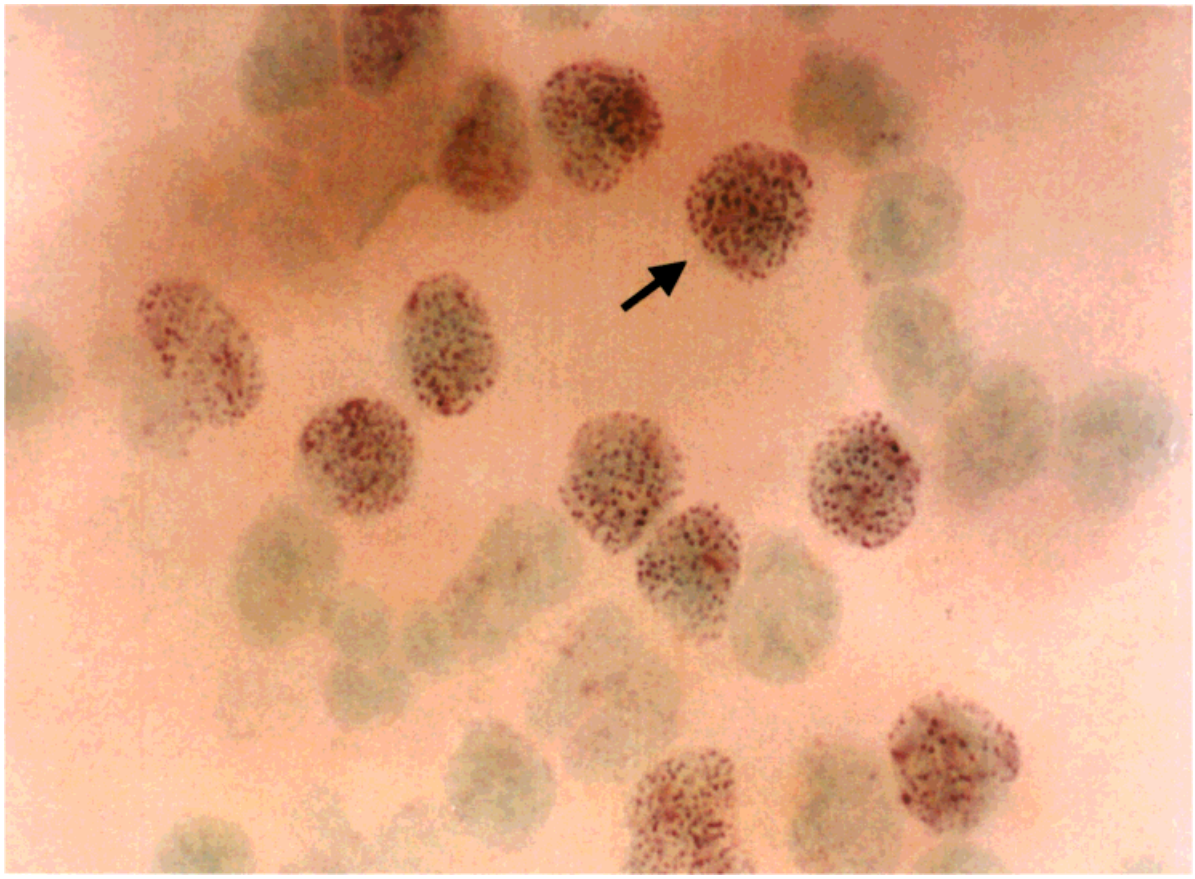

Fig. 3. Detection of TTV DNA in PBMCs. In situ hybridization of TTV DNA in PBMCs from a patient and a control with a full genome TTV DNA probe. Intracellular distribution of TTV DNA was demonstrated in the nucleus and cytoplasm (arrow). The cells were counterstained with methyl green. Original magnification, × 1,000.

PBMCs of blood donors. Very high TTV prevalence rates in the PBMCs of both the cancer patients and the controls were found, suggesting that PBMCs might be preferred to serum or plasma for the study of TTV infection. This could explain why TTV can be detected in many different organs [Pineau et al., 2000]. This observation is also in agreement with our previous study suggesting that TTV is very common in our population [Zhong et al., 2001] as well as in other populations [Tanaka et al., 1998a; Takahashi et al., 1998a,b; Viazov et al., 1998b; Biagini et al., 1999; Davidson et al., 1999; Nakano et al., 1999; Niel et al., 1999; Okamoto et al., 1999b; Takayama et al., 1999b].

TTV resembles most closely the chicken anemia virus (CAV) of the *circoviridae* family. CAV is highly specific for the bone marrow and thymus and can be cultured only in actively proliferating lymphoid and hematopoietic cell lines [Noteborn et al., 1991]. Apart from the liver tropism that has been suggested [Nishizawa et al., 1997; Okamoto et al., 1998], PBMCs and bone marrow hematopoietic cells have been the two suspected sites of TTV replication [Kanda et al., 1999; Okamura et al., 1999; Okamoto et al., 1999a; Kikuchi et al., 2000]. In this study, in agreement with the previous investigators [Okamura et al., 1999; Okamoto et al., 1999], we found TTV to be detected readily in PBMCs and in BMCs. A recent study using a gel migration test identified double-strand TTV DNA in BMCs; however, it was not detected in PBMCs [Okamoto et al., 2000]. Thus, whether or not TTV may replicate in PBMCs remains an unknown. The development of SSPCR, a PCR-based strategy, has allowed us to detect sensitively and specifically the positive and negative strands of the viral genome. Because the TTV genome consists of a circular single strand of DNA, which is of negative polarity, it is very likely that strands of positive polarity constitute the genomic intermediates of replication. TTV may replicate by a rolling circle mechanism as evidenced in other animal and plant *circoviruses* such as CAV [Noteborn et al., 1991]. TTV positive-strand DNA was detected in 55.6% (78 of 141) of the infected PBMCs and in 55.6% (5 of 9) of the infected BMCs of the cancer patients. Existence of double-strand TTV DNA in PBMCs of the cancer patients was supported further by the fact that TTV DNA extracted from PBMCs was resistant to S1 nuclease. Moreover, consistent with such a finding, using

the less sensitive in situ hybridization method, it was demonstrated that TTV is present in the nucleus and cytoplasm of some of the PBMCs. It is possible that infection of immune cells could facilitate escape of the virus from the immune response. Concealed as a "Trojan horse," TTV in PBMCs might serve as a reservoir of TTV for chronicity of the infection and transmission in some clinical and epidemiological settings. A previous study had failed to detect TTV replication in PBMCs of six TTV-infected individuals [Okamoto et al., 2000]. This could have been due to the methods used, which was likely to be less sensitive than our SSPCR assay. The present findings indicate that the virus is able to replicate in both PBMCs and BMCs, and that TTV may be lymphotropic as in the case of CAV. Further investigation will involve coupling of the SSPCR with in situ approaches to determine if TTV replication occurs in a particular subgroup of blood cell lines and to establish the precise cell tropism of TTV.

One of the most striking findings of this study is that the TTV positive strand, a replication intermediate, was detected more frequently in PBMCs of cancer patients when compared with the controls. This may indicate that in cancer patients, TTV may replicate actively in a subgroup of lymphocytes and the function of the infected cells may be affected as a consequence. This was consistent with the observation that TTV was present at a higher frequency in the PBMCs of the cancer patients (98.6%) than in the control blood donors (86%) in this study ($P < 0.05$). In addition, TTV infection was present in a significant proportion of the normal population (86%) in this study, suggesting that TTV infection in cancer patients might be related to recurrent infection of a preexisting infection. Our data indicate that there may be more active replication of TTV in PBMCs of the cancer patients than in the controls. The detection of positive- strand TTV may be due to the different frequencies of TTV replication in PBMCs and BMCs in different clinical settings, suggesting that TTV replication may be associated with an impaired host immune system in these patients. In this regard, quantitative measurement of the TTV genome load, which has detected a higher TTV genome load in PBMCs of the cancer patients than in the controls, supports such a notion [Zhong et al., 2001].

Consistent with the above data, a recent report has shown that phytohemagglutinin (PHA)-stimulated PBMCs release substantial titers of virus into the culture fluid, indicating TTV replication in activated mononuclear cells [Maggi et al., 2001]. However, although the replicative form of TTV was detectable in over one half of the PBMCs and BMCs in the cancer patients, a significant proportion still had undetectable replicative forms by the current SSPCR method despite having been shown to be infected with TTV. This may be due to TTV being present in a nonreplicating state or to its replication level being lower than the threshold of detection by the current method. An alternative explanation is that more than one TTV strain is present in the PBMCs of the cancer patients and that the SSPCR that has been developed can only amplify specific TTV genome types or subtypes.

This is in line with the observation that using in situ hybridization and a genotype- specific probe, TTV DNA was detected in only a fraction of the patients studied. We have not made any attempt to link genotypes based on sequences of ORF1 of the TTV genome with target sequences in the ORF2 region of the TTV genome in the SSPCR. Therefore, we have no information on the ability of the primers to amplify selectively genotypes defined from the ORF1 region.

Based on the findings described above, it is concluded that TTV is a very common infectious agent in PBMCs of cancer patients and normal individuals, and that TTV may replicate in both PBMCs and BMCs. However, TTV replication was detected more frequently in PBMCs of the cancer patients when compared with the controls.

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