Biochemical and Biophysical Research Communications **290,** 242–248 (2002)
doi:10.1006/bbrc.2001.6183, available online at http://www.idealibrary.com on IDEAL®

TT Virus Is Distributed in Various Leukocyte Subpopulations at Distinct Levels, with the Highest Viral Load in Granulocytes

Masaharu Takahashi,* Shinichi Asabe,* Yuhko Gotanda,† Junichi Kishimoto,‡ Fumio Tsuda,§ and Hiroaki Okamoto*,[1]

**Immunology Division and Division of Molecular Virology, Jichi Medical School, Tochigi-Ken 329-0498, Japan; †Japanese Red Cross Saitama Blood Center, Saitama-Ken 338-0001, Japan; ‡Institute of Immunology, Tokyo 112-0004, Japan; and §Department of Medical Sciences, Toshiba General Hospital, Tokyo 140-8522, Japan*

Received November 30, 2001

When TT virus (TTV) DNA was quantitated in whole blood and plasma aliquots from 27 viremic individuals by real-time detection PCR that can detect essentially all TTV genotypes, the TTV load was 6.9 ± 3.5 (mean ± standard deviation)-fold higher in the whole blood than in the plasma samples [$P < 0.002$ (paired t test)]. To clarify the reason for this difference, peripheral blood cells of various types including red blood cells, granulocytes (CD15+), B cells (CD19+), T cells (CD3+), monocytes (CD14+), and NK cells (CD3−/CD56+) were separated at a purity of 95.4–99.5% from each of three infected individuals with relatively high TTV viremia, and their TTV viral loads were determined. Red blood cells were uniformly negative, but the other cell types were positive for TTV DNA at various titers. In all three patients, the highest TTV load was found in granulocytes (4.2×10^4–3.1×10^5 copies/10^6 cells), followed by monocytes (1.4–2.2×10^4 copies/10^6 cells) and NK cells (5.4–6.5×10^3 copies/10^6 cells); B and T cells were positive, with a low viral load (6.7×10^1–2.7×10^3 copies/10^6 cells). These results indicate that TTV is distributed in various peripheral blood cell types at distinct levels, with the highest viral load in granulocytes, and that a significant proportion of the TTV DNA in peripheral blood is not identified by the standard plasma/serum DNA detection methods.

Key Words: **TT virus; real-time PCR; peripheral blood cells; cell separation.**

TT virus (TTV) was first identified in a patient with posttransfusion hepatitis of unknown etiology (1). It is an unenveloped, single-stranded, circular DNA virus of negative polarity with a diameter of 30–32 nm and a total genomic length of approximately 3.8 kilobases (2–5). Due to its unique genomic structure, transcriptional profile and sequence variability, TTV has tentatively been placed into the *Circoviridae* family or in a novel virus family, the *Circinoviridae* or the *Paracircoviridae* (3, 6); the most closely related known virus is chicken anemia virus (CAV) (7). TTV can replicate in multiple tissues including the liver, bone marrow and lung, as circular, double-stranded TTV DNA in the replicative form and TTV mRNA have been detected in various tissues, although viral replication levels in each tissue differ among infected individuals (8–11).

TTV has an extremely wide range of sequence divergence, and at least 38 TTV genotypes and five major phylogenetic groups (groups 1–5) have been identified (10, 12–15). Eight genotypes of the SEN virus (SENV, SENV-A to SENV-H) have been classified into TTV group 3, which is represented by the TUS01 and SANBAN isolates (5, 16–18). The extent to which the TTV sequence has been preserved varies by genomic region. Hence, the genomic area that is selected as the target region for polymerase chain reaction (PCR) amplification of TTV DNA, considerably influences the rate of detection of TTV DNA by PCR (12). Accordingly, in the present study, PCR was performed using primers based on the well-conserved sequence of an untranslated region that can detect nearly all genotypes/genetic groups (12, 15, 19).

Previous studies have indicated the association of TTV with peripheral blood mononuclear cells (PBMC) and the distinct distribution of TTV genotypes between plasma and PBMC (19–24). However, little is known about the distribution of TTV in the various types of peripheral blood cells. In the present study, we found that the titer of TTV DNA is significantly higher in

[1] To whom correspondence and reprint requests should be addressed at Immunology Division and Division of Molecular Virology, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-Machi, Tochigi-Ken 329-0498, Japan. Fax: +81-285-44-1557. E-mail: hokamoto@jichi.ac.jp.

0006-291X/02 $35.00

whole blood than in the corresponding plasma. To elucidate the precise reason for this discrepancy, peripheral blood cells including red blood cells, B- and T-lymphocytes, monocytes, NK cells, and granulocytes were separated at a high purity of >95% and the TTV DNA in these cells was quantitated by real-time detection PCR. The present study indicated that, contrary to the previous study by Maggi *et al.* (24), TTV was most abundant in granulocytes among the studied peripheral blood cells, having a viral load comparable to that in whole blood.

MATERIALS AND METHODS

Subjects. Blood samples were drawn from 30 healthy Japanese individuals [25 males and 5 females; age, 42.0 ± 9.3 years (mean ± standard deviation, SD), range 26–64 years]. Four ml of venous blood was mixed with 0.4 ml of 0.15 M sodium citrate, and 200 μl of this mixture was divided into four aliquots and kept as "whole blood" samples at −80°C until testing. Plasma samples were separated from 4.2 ml of the remaining citrated blood by centrifugation at 800*g* at 25°C for 20 min, and stored at −80°C until testing. For the purpose of cell separation, 30 ml of venous blood was collected from each of three individuals with high TTV viremia (3 males; age, 47, 49, and 53 years), and mixed with 3 ml of 0.15 M sodium citrate. All of the studied subjects were negative for serological markers of hepatitis B virus (MyCell HBsAg RPHA; Institute of Immunology, Co. Ltd., Tokyo, Japan), hepatitis C virus (Abbott HCV PHA 2nd Generation; Dainabot, Tokyo, Japan) and human immunodeficiency virus type I (SERODIA-HIV; Fujirebio, Tokyo, Japan).

Percoll gradient centrifugation. Using 33 ml of the citrated peripheral blood, PBMC and polymorphonuclear leukocytes (PMNL) were separated from red blood cells by density gradient centrifugation at 800*g* at 25°C for 20 min over Percoll (Amersham–Pharmacia Biotech AB, Uppsala, Sweden), according to the manufacturer's instructions. The recovered PBMC fraction was mixed with 4 volumes of Hanks' Balanced Salt Solution (HBSS; Sigma, St. Louis, MO), and briefly centrifuged (200*g* at 25°C for 10 min). The resulting pellet was rinsed twice with 2% (v/v) fetal calf serum (FCS) in HBSS and suspended in 1 ml of 10% FCS in RPMI 1640 medium (Sigma) to obtain the "PBMC-enriched fraction." Similarly, the PMNL fraction obtained as a result of density gradient centrifugation over Percoll was mixed with 3 vol of 0.75% (w/v) NaCl solution, and, after a brief centrifugation, the pellet was washed once with 50 ml of saline, suspended in 1 ml of phosphate-buffered saline (PBS) supplemented with 2 mM EDTA, and subjected to treatment with 50 ml of lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA–2Na at room temperature for 6 min, followed by a brief centrifugation. The resulting pellet was rinsed with 50 ml of PBS supplemented with 2 mM EDTA, and suspended in 1 ml of 10% FCS in RPMI 1640 medium to obtain the "PMNL-enriched fraction." Five milliliters of red blood cells recovered from the bottom layer was rinsed three times with 50 ml of saline and used as the "red blood cell fraction." The "PBMC-enriched fraction" and "PMNL-enriched fraction" were subjected to the cell sorting described below.

Cell separation by magnetic cell sorting. Monocyte-enriched and monocyte-depleted populations were prepared from the "PBMC-enriched fraction" with the use of magnetic beads. In brief, cells were stained with anti-CD14-fluorescein isothiocyanate (FITC) (BD PharMingen, San Diego, CA), and monocytes (CD14+) were purified with anti-FITC MicroBeads, magnetic cell separator MidiMACS and LS separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For purification, hereafter, magnetic cell separator MiniMACS and MS separation columns (Miltenyi Biotec GmbH) were used instead of MidiMACS and LS columns, respectively. B lymphocytes (CD19+) were prepared with the use of anti-CD19-phycoerythrin (PE) (BD PharMingen) and anti-PE MicroBeads (Miltenyi Biotec GmbH) from 0.6 vol of the monocyte-depleted population, and T lymphocytes (CD3+) were purified with use of anti-CD3-PE (BD PharMingen) and anti-PE MicroBeads from the remaining 0.4 vol of the monocyte-depleted population. Further, from the T lymphocyte-depleted fraction, NK cells (CD3−/CD56+) were prepared with the use of anti-CD56-PE (BD PharMingen) and anti-PE MicroBeads. In addition, from the "PMNL-enriched fraction," granulocytes (CD15+) were purified with the use of anti-CD15-FITC (BD PharMingen) and anti-FITC MicroBeads. To increase the purity of each cell population, each cell sorting procedure using an LS or MS separation column was performed twice. Aliquots of 10^5–10^6 cells were prepared for each cell fraction and kept at −80°C for future analysis. Purity was assessed by fluorescence-activated cell sorting (FACS) analysis on a FACSCalibur machine (Becton–Dickinson, Franklin Lakes, NJ).

Detection and quantitation of TTV DNA. Nucleic acids were extracted from 50 μl of whole blood or plasma, or from 10^5–10^6 cells of red blood cells, monocytes, B-lymphocytes, T-lymphocytes, NK cells or granulocytes using the High Pure Viral Nucleic Acid kit (Roche Diagnostics GmbH, Mannheim, Germany), and dissolved in 50 μl of nuclease-free distilled water. Plasma samples were tested for TTV DNA by nested polymerase chain reaction (PCR) in the presence of Perkin–Elmer AmpliTaq Gold (Roche Molecular Systems, Inc., Branchburg, NJ), according to the same method described previously (19) except for the following primer pairs. The first-round PCR was performed using primers NG472 (sense, 5′-GCG TCC CGW GGG CGG GTG CCG-3′ [W = A or T]) and NG352 (antisense, 5′-GAG CCT TGC CCA TRG CCC GGC CAG-3′ [R = A or G]), and the second-round PCR was carried out using primers NG473 (sense, 5′-CGG GTG CCG DAG GTG AGT TTA CAC-3′ [D = G, A, or T]) and NG351 (antisense, 5′-CCC ATR GCC CGG CCA GTC CCG AGC-3′), which were derived from a well-conserved area in the untranslated region of the TTV genome. The size of the amplification product of the first-round PCR was 91 bp [nucleotide (nt) 162–252], and that of the second-round PCR was 71 bp (nt 174–244): the nucleotide positions are in accordance with the prototype TTV isolate (TA278) of 3853 nt (5). The sensitivity was estimated to be 300–500 copies/ml when nucleic acids corresponding to 10 μl of plasma were used for the PCR assay.

TTV DNA was quantitated by real-time detection PCR using 1–10 μl of the nucleic acid solution as a template, primers NG473 and NG352, a dual fluorophore-labeled probe [NG369-P, 5′-(Fam)-AGT CAA GGG GCA ATT CGG GCT CGG GA-(Tamra)-3′; nt 203–228], and the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Diagnostics GmbH). PCR amplification was started with an initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 10 s and annealing-extension at 62°C for 30 s. All reactions were performed in a LightCycler System (Roche Diagnostics GmbH). The quantification limit of the system was 3 to 5 copies per test capillary (20 μl of reaction mixture). The overall variation was less than 0.5 log. All samples were tested in triplicate from independent extractions and the mean values are shown under Results.

RESULTS

Comparison of TTV DNA Titer in Paired Whole Blood and Plasma Samples

Among the 30 plasma samples tested, 27 (90%) were positive for TTV DNA by nested PCR, the positive rate being comparable with previous reports (15, 19). Then, the whole blood and plasma samples from these 27 healthy individuals were subjected to quantitation of

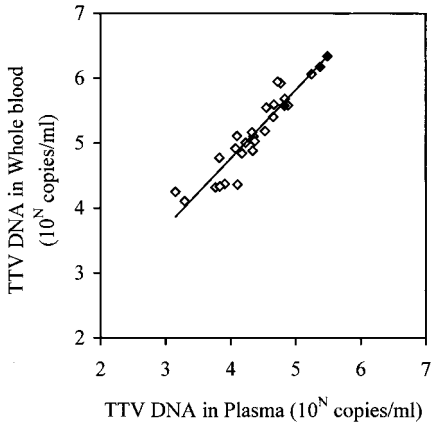

FIG. 1. Comparison of TTV DNA titers in plasma and whole blood samples obtained from 27 symptom-free TTV carriers. There was a significant correlation between the TTV DNA titer in plasma and that in whole blood [$r = 0.9414$; $P < 0.0001$ (Pearson's correlation coefficient test)]. The TTV DNA titer was significantly higher in whole blood than in plasma [$P = 0.0012$ (paired t test)]. The samples indicated by closed boxes are from subjects 1–3 in Tables 1 and 2.

TTV DNA, and the titers were compared (Fig. 1). The TTV viral load in the whole blood samples was correlated with that in the paired plasma samples (correlation coefficient, 0.9414). However, the TTV viral load in the whole blood samples was significantly higher than that in the plasma samples [$3.7 \times 10^5 \pm 5.3 \times 10^5$ vs $5.2 \times 10^4 \pm 7.4 \times 10^4$ copies/ml, $P = 0.0012$ (paired t test)]. In other words, the TTV load in whole blood was 6.9 ± 3.5 (range 1.8–16.8)-fold higher than that in the paired plasma. To clarify the reason for this discrepancy, the various types of peripheral blood cells were purified and their TTV viral loads were determined.

Purity and TTV Load of Fractionated Peripheral Blood Cells

Using venous blood samples obtained from three healthy individuals with high TTV viremia (subjects 1–3), different leukocyte subsets including monocytes, B cells, T cells, NK cells, and granulocytes were prepared by Percoll gradient centrifugation followed by magnetic cell sorting. Cell purity was assessed by FACS analysis using CD3, CD14, CD15, CD19, and CD56 as cell surface markers, and it was found to be 97.7–99.0% for the monocytes (CD14+), 96.2–98.1% for B cells (CD14−/CD19+), 99.5% for T cells (CD14−/CD3+), 95.4–99.2% for NK cells (CD14−/CD3−/CD56+) and 98.0–98.4% for granulocytes (CD15+) (Table 1). The purity of the red blood cells was >99.5% when counted under microscopic observation.

The red blood cells were uniformly negative for TTV DNA, whereas the other cell types were positive for TTV DNA at various titers ranging from 6.7×10^1 to 3.1×10^5 copies/10^6 cells. The highest viral load was found in granulocytes in the three individuals tested (4.2×10^4–3.1×10^5 copies/10^6 cells), followed by monocytes (1.4–2.2×10^4 copies/10^6 cells) and NK cells (5.4–6.5×10^3 copies/10^6 cells); the B and T cells of the three viremic subjects were positive for TTV DNA, with a low viral load (6.7×10^1–2.7×10^3 copies/10^6 cells) (Table 1). Although the term "granulocyte" includes neutrophils, eosinophils, and basophils, neutrophils were found to predominate in the recovered CD15+ granulocyte fraction by microscopic observation. Therefore, the terms "neutrophil" and "granulocyte" are used interchangeably in this report.

TABLE 1

TTV Loads in Whole Blood, Plasma, and Fractionated Peripheral Blood Cells of Various Types

Specimen[a]	Subject 1		Subject 2		Subject 3	
	Purity[b] (%)	TTV viral load (copies/ml or 10^6 cells)	Purity (%)	TTV viral load (copies/ml or 10^6 cells)	Purity (%)	TTV viral load (copies/ml or 10^6 cells)
Whole blood		3.8×10^5		1.5×10^6		2.2×10^6
Plasma		6.7×10^4		2.4×10^5		3.1×10^5
PBMC						
Monocytes (CD14+)	99.0	2.2×10^4	98.9	1.4×10^4	97.7	1.6×10^4
B lymphocytes (CD19+)	98.1	5.7×10^2	96.5	1.5×10^3	96.2	2.7×10^3
T lymphocytes (CD3+)	99.5	6.7×10^1	99.5	3.0×10^2	99.5	8.5×10^2
NK cells (CD3−/CD56+)	99.2	6.5×10^3	95.4	5.4×10^3	96.9	6.5×10^3
PMNL						
Neutrophils (CD15+)[c]	98.4	4.2×10^4	98.0	3.1×10^5	98.2	1.3×10^5
Red blood cells	>99.5	(−)[d]	>99.5	(−)	>99.5	(−)

[a] Abbreviations used: PBMC, peripheral blood mononuclear cells; PMNL, polymorphonuclear leukocytes.

[b] Purity was assessed by FACS analysis (see Materials and Methods) or by microscopic observation for red blood cells.

[c] Among PMNL, both neutrophils and eosinophils are known to have CD15 as a cell surface marker, but no eosinophils were observed on microscopic observation of the purified CD15+ population.

[d] Negative for TTV DNA.

TABLE 2

Estimated Distribution of TTV in Plasma and Fractionated Peripheral Blood Cells of Various Types Recovered from 1 ml of Whole Blood

Specimen[a]	Subject 1		Subject 2		Subject 3	
	Volume/cell count	TTV viral load (copies equivalent to 1 ml blood)	Volume/cell count	TTV viral load (copies equivalent to 1 ml blood)	Volume/cell count	TTV viral load (copies equivalent to 1 ml blood)
Whole blood[b]	1 ml	3.8×10^5	1 ml	1.5×10^6	1 ml	2.2×10^6
Plasma	0.52 ml	3.4×10^4	0.52 ml	1.2×10^5	0.53 ml	1.6×10^5
PBMC[c]						
Monocytes (CD14+)	2.2×10^5	4.8×10^3	2.0×10^5	2.9×10^3	2.2×10^5	3.6×10^3
B lymphocytes (CD19+)	1.7×10^5	9.6×10^1	2.8×10^5	4.2×10^2	2.4×10^5	6.5×10^2
T lymphocytes (CD3+)	1.7×10^6	1.1×10^2	1.7×10^6	5.2×10^2	1.9×10^6	1.6×10^3
NK cells (CD3−/CD56+)	1.2×10^6	8.1×10^3	3.4×10^5	1.8×10^3	7.4×10^5	4.8×10^3
PMNL						
Neutrophils (CD15+)[d]	4.0×10^6	1.7×10^5	3.5×10^6	1.1×10^6	4.9×10^6	6.3×10^5

[a] Abbreviations used: PBMC, peripheral blood mononuclear cells; PMNL, polymorphonuclear leukocytes.

[b] Hematocrit and white blood cell count were 48.5% and 7.3×10^6/ml in subject 1, 48.0% and 6.0×10^6/ml in subject 2, and 47.0% and 8.0×10^6/ml in subject 3.

[c] PMBC and PMNL were counted on microscopic observation of a blood smear, and the cell count of each PBMC subpopulation was estimated based on the cell ratio determined by FACS analysis.

[d] Among PMNL (granulocytes) that had CD15 as a cell surface marker, only neutrophils were observed on microscopic observation.

Estimation of Relative Amount of TTV DNA in Each Peripheral Blood Cell Type in 1 ml of Whole Blood

The total white blood cell count was 7.3×10^6/ml in subject 1, 6.0×10^6/ml in subject 2, and 8.0×10^6/ml in subject 3. The cell count of each peripheral blood cell type was estimated based on those counted on the blood smear by microscopic observation and based on the cell ratio as determined by FACS analysis. As shown in Table 2, the amount of TTV DNA in granulocytes recovered from 1 ml of whole blood was estimated to be $1.7 \times 105–1.1 \times 10^6$ copies, and was 3.9- to 9.2-fold higher than that in the corresponding plasma and comparable with that in whole blood. Although the estimated TTV load in granulocytes was only 30% to 70% of the actual TTV load in whole blood, this supports that TTV in granulocytes may predominantly contribute to the high TTV titer in whole blood.

DISCUSSION

At present, plasma/serum DNA detection methods are standard for the detection of TTV DNA. When we were quantitating TTV DNA in a heavily hemolyzed plasma sample, we noticed that the TTV titer in this sample was significantly higher than that in a nonhemolyzed plasma sample collected from the same patient one day before. With regard to hepatitis C virus (HCV), it is well known that whole blood contains a higher titer of viral RNA than plasma (25), and it has been suggested that whole-blood assays may be more reliable for HCV-RNA quantitation in hepatitis C patients with cryoprecipitate (26). These results prompted us to compare the TTV load in whole blood and paired plasma samples. In the present study, as expected, we found clear evidence that the TTV load in whole blood is significantly higher than that in the corresponding plasma aliquot ($P = 0.0012$). Then, to answer the questions as to why the TTV load is significantly higher in whole blood than in the paired plasma, and what component(s) in peripheral blood contributes to increasing the relative TTV titer in whole blood, we performed quantitation of TTV in fractionated leukocyte subpopulations. As a result, we found that TTV is distributed in various types of peripheral blood cells at distinct viral levels, with the highest load in granulocytes, which is contrary to the first report (24) that described the distribution of TTV in various peripheral blood cell types, with no preferential association of TTV to these cell types. The precise reason for this discrepancy is not known. However, it should be noted that the cell purity differs considerably in the two studies: it was >95% in the present study and >85% in the earlier study (24). It is possible that contamination of cells with high TTV load into each fraction, even a trace amount, led to their results of no apparent preference for any specific cell type.

In the present study, the highest TTV load was found in granulocytes or neutrophils among the various peripheral blood cell types. The clinical significance and virological implication of the preferential association of TTV with neutrophils remain unknown. Neutrophils are involved in the body's main defense against invasion by bacteria and fungi (27). After a variable timeperiod of storage in the bone marrow as stab neutro-

phils or segmented neutrophils, the neutrophils are known to be released into the peripheral blood where they circulate for about 6 hr in dynamic equilibrium with a marginal pool of cells that are loosely adherent to the vascular endothelium (27). TTV was distributed abundantly in peripheral blood cells; the TTV viral load decreased in the order of neutrophils, monocytes, NK cells, B-lymphocytes and T-lymphocytes. The association of TTV with neutrophils and monocytes, the cell types with the first and second highest TTV viral loads, may be explained by the phagocytic role these cells play in peripheral immunosurveillance. In our previous studies, double-stranded TTV DNA of replicative intermediate form and TTV mRNAs were detected in bone marrow cells, but not in PBMC (9, 28). However, TTV mRNAs were detected in the neutrophils from the three studied subjects (Takahashi and Okamoto, unpublished observation), suggesting that TTV is transcribed and TTV-specific proteins may be expressed in these cells. It has been suggested that TTV can replicate under altered physiological conditions; for example, TTV replication occurred in PBMC upon cellular activation with phytohemagglutinin *in vitro* (24). However, among the various peripheral blood cell types, neutrophils may preferentially support the replication of TTV. Although the studies seeking evidence of viral replication in the peripheral lymphocytes of HCV-infected patients have yielded conflicting results, the presence of antigenomic HCV RNA as the replicative intermediate form in neutrophils has recently been reported (29). Epstein–Barr virus (EBV) infects neutrophils, modulates protein expression, and is associated with immunosuppressive effects (30). These data warrant further studies on the possible role of TTV in peripheral blood cells involved in the host's immunosurveillance.

It has been reported that TTV replicates in multiple tissues in infected hosts, and this could contribute to the viremia level and heterogenous population of TTV in the circulation among infected individuals (10). In the present study, we found that neutrophils contribute to the viral load in the circulation to a much higher degree than PBMC, which have also been considered to act as a reservoir of TTV infection (19–24). TTV DNA has been detected in a variety of clinical specimens other than peripheral blood obtained from infected individuals, such as the bile, feces, saliva, throat swabs, breast milk, semen, and cerebrospinal fluid (31–37), some of which may have been contaminated with peripheral blood cells including neutrophils and monocyte. Neutrophils infiltrate infected tissues, and monocytes migrate from the bloodstream into tissues by converting to macrophages. Therefore, careful attention is required to evaluate the tissue/organ tropism of TTV. For this purpose, the presence of double-stranded TTV DNA and TTV mRNAs should be examined in every tissue specimen in future studies. In addition, extended studies are required to elucidate the exact location of TTV by *in situ* hybridization as was performed for the study of TTV in the liver (38) and detection of virus-encoded proteins in various tissues testing positive for TTV DNA must be performed. Several reports have indicated the heterogeneity of the TTV population in the circulation and tissues of infected subjects (10, 19, 39, 40). Although the underlying basis for the finding that multiple and distinct viral genotypes are present in various tissues of the same individual, remains unknown, genotypic analysis may be helpful in evaluating the tissue-dependent distribution of TTV, as was performed in our previous report (10).

In conclusion, our data demonstrate the frequent distribution of TTV in various peripheral blood cell types of infected humans at distinct levels, with the highest viral load in neutrophils, and that a significant proportion of the TTV DNA in peripheral blood is not identified by the standard plasma/serum DNA detection methods, although the clinical implication of detection and quantitation of TTV DNA in whole blood must be clarified in future studies. Further investigations are needed to fully understand the exact properties and pathogenic role of this newly recognized, ubiquitous human virus that is distributed not only in the plasma/serum but also in peripheral blood cells and various tissues of the infected body.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Ministry of Health, Labour, and Welfare of Japan. The authors are grateful to Professor M. Mayumi for his advice and encouragement during this study.

REFERENCES

1. Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1997) A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem. Biophys. Res. Commun.* **241,** 92–97, doi:1006/bbrc.1997.7765.
2. Okamoto, H., Nishizawa, T., Kato, N., Ukita, M., Ikeda, H., Iizuka, H., Miyakawa, Y., and Mayumi, M. (1998) Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. *Hepatol. Res.* **10,** 1–16.
3. Mushahwar, I. K., Erker, J. C., Muerhoff, A. S., Leary, T. P., Simons, J. N., Birkenmeyer, L. G., Chalmers, M. L., Pilot-Matias, T. J., and Desai, S. M. (1999) Molecular and biophysical characterization of TT virus: Evidence for a new virus family infecting humans. *Proc. Natl. Acad. Sci. USA* **96,** 3177–3182.
4. Miyata, H., Tsunoda, H., Kazi, A., Yamada, A., Khan, M. A., Murakami, J., Kamahora, T., Shiraki, K., and Hino, S. (1999) Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. *J. Virol.* **73,** 3582–3586.
5. Okamoto, H., Nishizawa, T., Ukita, M., Takahashi, M., Fukuda,

M., Iizuka, H., Miyakawa, Y., and Mayumi, M. (1999) The entire nucleotide sequence of a TT virus isolate from the United States (TUS01): Comparison with reported isolates and phylogenetic analysis. *Virology* **259,** 437–448, doi:10.1006/viro.1999.9769.

6. Takahashi, K., Iwasa, Y., Hijikata, M., and Mishiro, S. (2000) Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. *Arch. Virol.* **145,** 979–993.
7. Todd, D., McNulty, M. S., Mankertz, A., Lukert, P. D., Randles, J. W., and Dale, J. L. (2000) Family *Circoviridae. In* Virus Taxonomy. Classification and Nomenclature of Viruses, Seventh Report of the International Committee on Taxonomy of Viruses (van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R., and Wickner, R. B., Eds.), pp. 299–303, Academic Press, San Diego.
8. Okamoto, H., Ukita, M., Nishizawa, T., Kishimoto, J., Hoshi, Y., Mizuo, H., Tanaka, T., Miyakawa, Y., and Mayumi, M. (2000) Circular double-stranded forms of TT virus DNA in the liver. *J. Virol.* **74,** 5161–5167.
9. Okamoto, H., Takahashi, M., Nishizawa, T., Tawara, A., Sugai, Y., Sai, T., Tanaka, T., and Tsuda, F. (2000) Replicative forms of TT virus DNA in bone marrow cells. *Biochem. Biophys. Res. Commun.* **270,** 657–662, doi:1006/bbrc.2000.2481.
10. Okamoto, H., Nishizawa, T., Takahashi, M., Asabe, S., Tsuda, F., and Yoshikawa, A. (2001) Heterogeneous distribution of TT virus of distinct genotypes in multiple tissues from infected humans. *Virology* **288,** 358–368, doi:10.1006/viro.2001.1097.
11. Suzuki, F., Chayama, K., Tsubota, A., Akuta, N., Someya, T., Kobayashi, M., Suzuki, Y., Saitoh, S., Arase, Y., Ikeda, K., and Kumada, H. (2001) Pathogenic significance and organic virus levels in patients infected with TT virus. *Intervirology* **44,** 291–297.
12. Okamoto, H., Takahashi, M., Nishizawa, T., Ukita, M., Fukuda, M., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1999) Marked genomic heterogeneity and frequent mixed infection of TT virus demonstrated by PCR with primers from coding and noncoding regions. *Virology* **259,** 428–436, doi:10.1006/viro.1999.9770.
13. Takahashi, K., Hijikata, M., Samokhvalov, E. I., and Mishiro, S. (2000) Full or near full length nucleotide sequences of TT virus variants (types SANBAN and YONBAN) and the TT virus-like mini virus. *Intervirology* **43,** 119–123.
14. Muljono, D. H., Nishizawa, T., Tsuda, F., Takahashi, M., and Okamoto, H. (2001) Molecular epidemiology of TT virus (TTV) and characterization of two novel TTV genotypes in Indonesia. *Arch. Virol.* **146,** 1249–1266.
15. Peng, Y. H., Nishizawa, T., Takahashi, M., Ishikawa, T., Yoshikawa, A., and Okamoto, H. (2001) Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. *Arch. Virol.,* in press.
16. Hijikata, M., Takahashi, K., and Mishiro, S. (1999) Complete circular DNA genomes of a TT virus variant (isolate name SANBAN) and 44 partial ORF2 sequences implicating a great degree of diversity beyond genotypes. *Virology* **260,** 17–22, doi:10.1006/viro.1999.9797.
17. Tanaka, Y., Primi, D., Wang, R. Y. H., Umemura, T., Yeo, A. E. T., Mizokami, M., Alter, H. J., and Shih, J. W. K. (2001) Genomic and molecular evolutionary analysis of a newly identified infectious agent (SEN virus) and its relationship to the TT virus family. *J. Infect. Dis.* **183,** 359–367.
18. Mushahwar, I. K. (2000) Recently discovered blood-borne viruses: Are they hepatitis viruses or merely endosymbionts? *J. Med. Virol.* **62,** 399–404.
19. Okamoto, H., Takahashi, M., Kato, N., Fukuda, M., Tawara, A., Fukuda, S., Tanaka, T., Miyakawa, Y., and Mayumi, M. (2000) Sequestration of TT virus of restricted genotypes in peripheral blood mononuclear cells. *J. Virol.* **74,** 10236–10239.
20. Okamoto, H., Kato, N., Iizuka, H., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1999) Distinct genotypes of a nonenveloped DNA virus associated with posttransfusion non-A to G hepatitis (TT virus) in plasma and peripheral blood mononuclear cells. *J. Med. Virol.* **57,** 252–258.
21. Okamura, A., Yoshioka, M., Kubota, M., Kikuta, H., Ishiko, H., and Kobayashi, K. (1999) Detection of a novel DNA virus (TTV) sequence in peripheral blood mononuclear cells. *J. Med. Virol.* **58,** 174–177.
22. Lopez-Alcorocho, J. M., Mariscal, L. F., de Lucas, S., Rodriguez-Inigo, E., Casqueiro, M., Castillo, I., Bartolome, J., Herrero, M., Manzano, M. L., Pardo, M., and Carreno, V. (2000) Presence of TTV DNA in serum, liver and peripheral blood mononuclear cells from patients with chronic hepatitis. *J. Viral Hepatitis* **7,** 440–447.
23. Martinez, N. M., Garcia, F., Garcia, F., Alvarez, M., Bernal, M. C., Piedrola, G., Hernandez, J., and Maroto, M. C. (2000) TT virus DNA in serum, peripheral blood mononuclear cells and semen of patients infected with HIV. *AIDS* **14,** 4–6.
24. Maggi, F., Fornai, C., Zaccaro, L., Morrica, A., Vatteroni, M. L., Isola, P., Marchi, S., Ricchiuti, A., Pistello, M., and Bendinelli, M. (2001) TT virus (TTV) loads associated with different peripheral blood cell types and evidence for TTV replication in activated mononuclear cells. *J. Med. Virol.* **64,** 190–194.
25. Schmidt, W. N., Wu, P., Han, J. Q., Perino, M. J., LaBrecque, D. R., and Stapleton, J. T. (1997) Distribution of hepatitis C virus (HCV) RNA in whole blood and blood cell fractions: Plasma HCV RNA analysis underestimates circulating virus load. *J. Infect. Dis.* **176,** 20–26.
26. Schmidt, W. N., Stapleton, J. T., LaBrecque, D. R., Mitros, F. A., Kirby, P. A., Phillips, M. J. P., and Brashear, D. L. (2000) Hepatitis C virus (HCV) infection and cryoglobulinemia: Analysis of whole blood and plasma HCV-RNA concentrations and correlation with liver histology. *Hepatology* **31,** 737–744.
27. Yeghen, T., and Devereux, S. (2001) Granulocyte transfusion: A review. *Vox Sang.* **81,** 87–92.
28. Okamoto, H., Nishizawa, T., Tawara, A., Takahashi, M., Kishimoto, J., Sai, T., and Sugai, Y. (2000) TT virus mRNAs detected in the bone marrow cells from an infected individual. *Biochem. Biophys. Res. Commun.* **279,** 700–707, doi:10.1006/bbrc.2000.4012.
29. Crovatto, M., Pozzato, G., Zorat, F., Pussini, E., Nascimben, F., Baracetti, S., Grando, M. G., Mazzaro, C., Reitano, M., Modolo, M. L., Martelli, P., Spada, A., and Santini, G. (2000) Peripheral blood neutrophils from hepatitis C virus-infected patients are replication sites of the virus. *Haematologica* **85,** 356–361.
30. Larochelle, B., Flamand, L., Gourde, P., Beauchamp, D., and Gosselin, J. (1998) Epstein–Barr virus infects and induces apoptosis in human neutrophils. *Blood* **92,** 291–299.
31. Okamoto, H., Akahane, Y., Ukita, M., Fukuda, M., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1998) Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. *J. Med. Virol.* **56,** 128–132.
32. Deng, X., Terunuma, H., Handema, R., Sakamoto, M., Kitamura, T., Ito, M., and Akahane, Y. (2000) Higher prevalence and viral load of TT virus in saliva than in the corresponding serum: Another possible transmission route and replication site of TT virus. *J. Med. Virol.* **62,** 531–537.
33. Inami, T., Konomi, N., Arakawa, Y., and Abe, K. (2000) High prevalence of TT virus DNA in human saliva and semen. *J. Clin. Microbiol.* **38,** 2407–2408.
34. Ishikawa, T., Hamano, Y., and Okamoto, H. (1999) Frequent detection of TT virus in throat swabs of pediatric patients (letter). *Infection* **27,** 298.

35. Ross, R. S., Viazov, S., Runde, V., Schaefer, U. W., and Roggendorf, M. (1999) Detection of TT virus DNA in specimens other than blood. *J. Clin. Virol.* **13,** 181–184.

36. Ukita, M., Okamoto, H., Kato, N., Miyakawa, Y., and Mayumi, M. (1999). Excretion into bile of a novel unenveloped DNA virus (TT virus) associated with acute and chronic non-A-G hepatitis. *J. Infect. Dis.* **179,** 1245–1248.

37. Maggi, F., Fornai, C., Vatteroni, M. L., Siciliano, G., Menichetti, F., Tascini, C., Specter, S., Pistello, M., and Bendinelli, M. (2001) Low prevalence of TT virus in the cerebrospinal fluid of viremic patients with central nervous system disorders. *J. Med. Virol.* **65,** 418–422.

38. Rodriguez-Inigo, E., Casqueiro, M., Bartolome, J., Ortiz-Movilla, N., Lopez-Alcorocho, J. M., Herrero, M., Manzarbeitia, F., Oliva, H., and Carreno, V. (2000) Detection of TT virus DNA in liver biopsies by *in situ* hybridization. *Am. J. Pathol.* **156,** 1227–1234.

39. Ball, J. K., Curren, R., Berridge, S., Grabowska, A. M., Jameson, C. L., Thomson, B. J., Irving, W. L., and Sharp, P. M. (1999) TT virus sequence heterogeneity *in vivo:* Evidence for co-infection with multiple genetic types. *J. Gen. Virol.* **80,** 1759–1768.

40. Forns, X., Hegerich, P., Darnell, A., Emerson, S. U., Purcell, R. H., and Bukh, J. (1999) High prevalence of TT virus (TTV) infection in patients on maintenance hemodialysis: Frequent mixed infections with different genotypes and lack of evidence of associated liver disease. *J. Med. Virol.* **59,** 313–317.