History of Discoveries and Pathogenicity of TT Viruses

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Contents

Abstract Since 1997, groups of novel nonenveloped DNA viruses with a circular, single-stranded (negative sense) DNA genome of 3.6–3.9 kb, 3.2 kb, or 2.8–2.9 kb in size have been discovered and designated Torque teno virus (TTV), Torque teno midi virus (TTMDV), and Torque teno mini virus (TTMV), respectively, in the floating genus *Anellovirus*. These three anelloviruses frequently and ubiquitously infect humans, and the infections are characterized by lifelong viremia and great genetic variability. Although TTV infection has been epidemiologically suggested to be associated with many diseases including liver diseases, respiratory disorders, hematological disorders, and cancer, there is no direct causal evidence for links between TTV infection and specific clinical diseases. The pathogenetic role of

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TTMV and TTMDV infections remains unknown. The changing ratio of the three anelloviruses to each other over time, relative viral load, or combination of different genotype(s) of each anellovirus may be associated with the pathogenicity or the disease-inducing potential of these three human anelloviruses. To clarify their disease association, polymerase chain reaction (PCR) systems for accurately detecting, differentiating, and quantitating all of the genotypes and/or genogroups of TTV, TTMDV, and TTMV should be established and standardized, as should methods to detect past infections and immunological responses to anellovirus infections.

History of Discoveries

Discovery of Original TT Virus

In 1997, while searching for an as-yet-unidentified hepatitis viruses, Nishizawa et al. found a novel DNA virus in a Japanese patient (initials T.T.) with posttransfusion hepatitis of unknown etiology (Nishizawa et al. 1997). The patient was 58 years old, and had received 35 units of blood during heart surgery. He had elevated alanine aminotransferase (ALT) levels 9–11 weeks after the surgery (peak, 180 IU/l at 10 weeks after transfusion). Representational difference analysis (RDA) (Lisitsyn et al. 1993) was performed for the specific amplification of nucleic acid sequences present in the serum of the patient during the period of his acute hepatitis, but which were absent before transfusion. After three courses of subtraction, a broad but clear band 0.5 kb in size was visualized on electrophoresis, and subjected to molecular cloning. Among the 36 clones obtained, varying in size from 281 to 564 bp, 9 clones of 500 bp were similar to each other, whose sequence was detectable only during the period of acute hepatitis in the index patient. A representative clone (N22) with the consensus sequence showed poor homology to any of the 1,731,752 sequences deposited in DNA databases as of 2 October 1997 (Nishizawa et al. 1997).

The N22 clone was found to originate from the genome of a nonenveloped, single-stranded DNA virus based on data using a PCR method with N22-derived primers RD037 and RD038 in the first round and RD051 and RD052 in the second round. The virus was provisionally named “TT” virus (TTV) after the initials of the index patient (Nishizawa et al. 1997). In brief, since the N22 sequence was not amplified from any of four human genomic DNA samples, a nonhost origin of N22 was attested. Furthermore, since the N22 sequence fractionated in sucrose gradient at 1.26 g/cm^3 and was resistant to DNase I, it was concluded to be encapsidated and thereby of viral origin. Furthermore, serum-derived TTV DNA was sensitive to mung bean nuclease but resistant to RNase A and restriction enzymes. Hence, TTV was believed to be a DNA virus that had a single-stranded genome (Nishizawa et al. 1997; Okamoto et al. 1998b). Since the density of Tween 80-treated TTV remained unchanged in sucrose gradient, TTV was understood to be a nonenveloped virus (Okamoto et al. 1998b).

The N22 sequence was extended to 3,739 nt in the prototype TTV isolate (TA278) obtained from a 34-year-old male blood donor with an elevated ALT level of 106 IU/l and containing TTV DNA in high titer (10^5 copies/ml) detectable by PCR with nested N22 primers, but its extreme 5′- and 3′-end sequences remained undetermined at that time (Okamoto et al. 1998b). In 1999, the presence of a GC-rich sequence of approximately 120 nt was reported (Miyata et al. 1999; Mushahwar et al. 1999), leading to the recognition of the circular nature of the TTV genome with negative polarity. The entire genomic length of the TA278 isolate was finally determined to be 3,853 nt, with the unique stem-and-loop structures in the GC-rich region, which would play a pivotal role in viral replication (Okamoto et al. 1999b, c).

Virological Characterization of TTV

Subsequent studies revealed the following characteristics of TTV. The buoyant density in cesium chloride (CsCl) was found to be 1.31–1.33 g/cm^3 for TTV in serum and 1.33–1.35 g/cm^3 for TTV in feces (Okamoto et al. 1998a). TTV particles in the circulation were bound to immunoglobulin G (IgG), forming immune complexes (Itoh et al. 2000). Therefore, TTV-associated particles with a diameter of 30–32 nm recovered from the sera of infected humans were observed as aggregates of various sizes on electron microscopy. In contrast, TTV particles in feces exist as free virions. TTV particles of genotype 1a with a diameter of 30–32 nm and banding at 1.33–1.35 g/cm^3 have been visualized in fecal supernatant by immune electron microscopy using γ-globulins from human plasma containing TTV genotype 1a-specific antibodies (Itoh et al. 2000; Tsuda et al. 1999).

Kamahora et al. (2000) analyzed the messenger RNAs (mRNAs) transcribed from a plasmid containing the whole genome construct of TTV in COS1 cells. They recovered three spliced mRNAs of 3.0 kb, 1.2 kb, and 1.0 kb with common 5′- and 3′-termini, and showed that the splicing sites link distant open reading frames (ORFs) to create two new ORFs capable of encoding 286 amino acids (aa) and 289aa. Such spliced mRNAs of TTV have also been observed in actively replicating cells including bone marrow cells in infected humans (Okamoto et al. 2000d). The proposed genomic organization of the prototype TTV isolate (TA278; Accession No. AB017610) is illustrated in Fig. 1.

Discovery of Many TTV-Like Variants in Humans

After the discovery of the original TTV isolate, with the use of primers based on a conserved untranslated region, many TTV variants with marked genetic variability were identified (Hallett et al. 2000; Hijikata et al. 1999b; Khudyakov et al. 2000; Muljono et al. 2001; Okamoto et al. 1999a, c, d, 2000d, 2001; Peng et al. 2002;

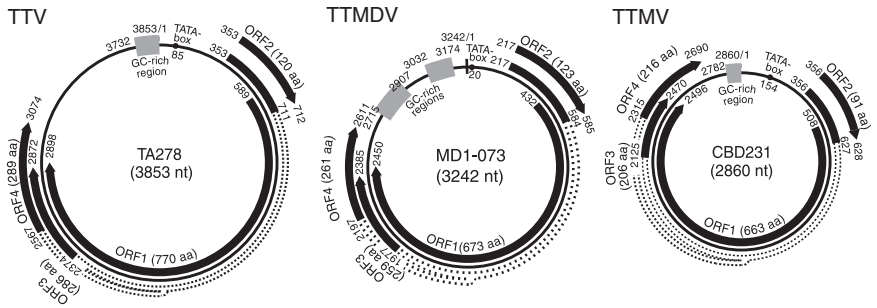

Fig. 1 Genomic organization of the prototype TTV, TTMDV, and TTMV isolates. The *circumference* of each circle represents the relative size of the genome. The *closed arrows* represent ORFs (ORF1 to ORF4). The *open boxes* located between an *upstream closed box* and *downstream closed arrow* in ORF3 and ORF4, which encode joint proteins, represent areas corresponding to introns in the mRNA (Kamahora et al. 2000; Okamoto et al. 2000c). The *shaded box* indicates the GC-rich stretch and the *small closed circle* represents the position of the TATA box. (Reproduced from Ninomiya et al. 2007a, with permission)

Takahashi et al. 2000a; Ukita et al. 2000) and segregated into at least 39 genotypes with a difference of greater than 30% or five major genetic groups with a difference of greater than 50% difference (Okamoto et al. 2004; Peng et al. 2002; Fig. 2). Independently, the SEN virus (SENV) was discovered by RDA (patent application WO 00/28039, 2000). However, it soon became apparent that it represented different genotypes of TTV (Tanaka et al. 2001).

In addition to ubiquitous distribution of many TTV-like variants in various tissues and body fluids of humans, multiple genotypes of TTV may be found within an infected individual, often with different genotype combinations predominating in different tissues (Okamoto et al. 2001). This suggests that certain virus genotypes might be better adapted to particular cell or tissue types.

Discovery of Torque Teno Mini Virus

In 2000, a small virus that was distantly related to TTV was accidentally discovered by PCR of human plasma samples using TTV-specific primers that partially matched homologous sequences but generated a noticeably shorter amplicon than expected for TTV, and was provisionally named as TTV-like mini virus (TLMV) (Takahashi et al. 2000b). The genome of TLMV consists of a circular, single-stranded DNA of approximately 2,800–2,900 nt with negative polarity (Fig. 1). The size of a TLMV virion has been estimated to be less than 30 nm in diameter (Takahashi et al. 2000b). TLMV resembles TTV in genomic structure, and also contains an arginine-rich N-terminus as well as Rep-motifs in the ORF1 region and a chicken anemia virus (CAV)-like motif in the ORF2 region (Biagini et al. 2001b, 2007; Okamoto

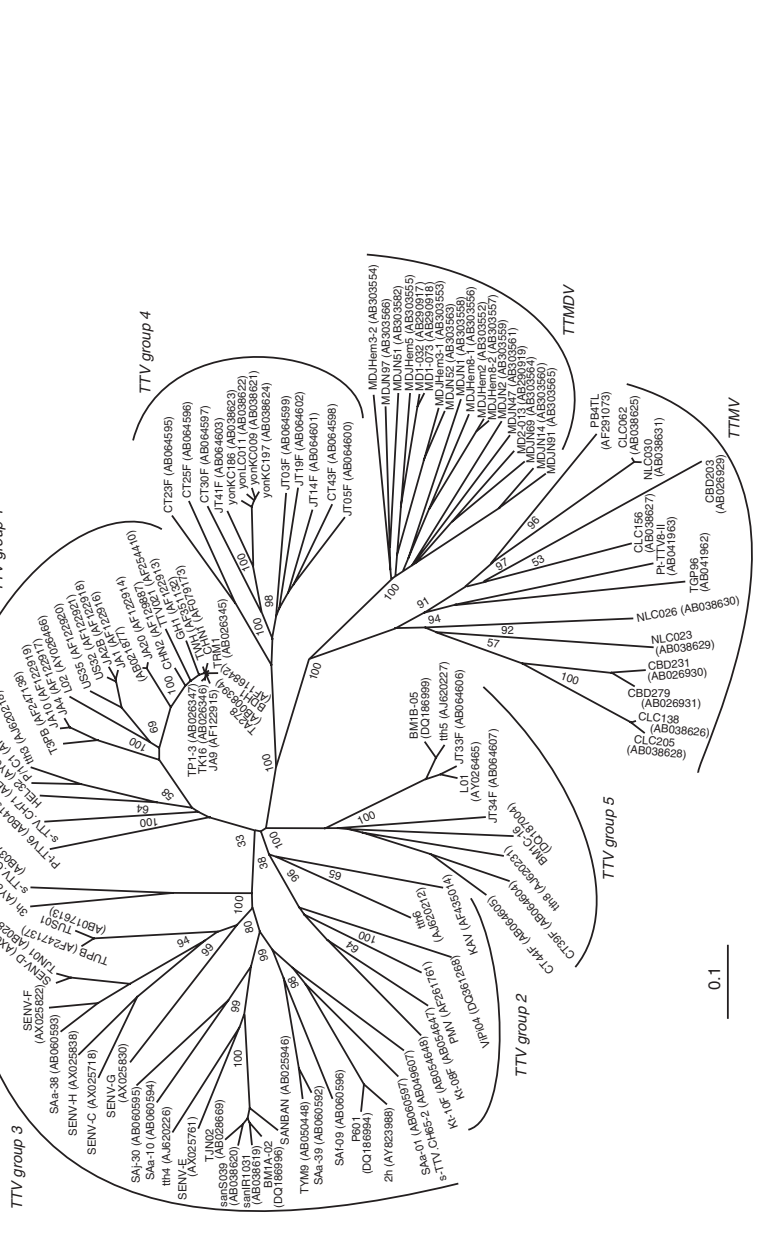

Fig. 2 Phylogenetic tree based on the entire nucleotide sequence of ORF1 of the 81 TTV, 18 TTMDV, and 13 TTMV isolates by the neighbor-joining method (Saitou and Nei 1987). The percentage of bootstrap values generated from 1,000 samplings of the data is shown near the nodes. The *scale bar* represents the number of nucleotide substitutions per position. (Reproduced from Ninomiya et al. 2008, with permission)

et al. 2000a; Takahashi et al. 2000b). TLMV is also highly divergent: the first three TLMV sequences reported by Takahashi et al. (2000b) differed from each other by 42% at the nucleotide level and by 67% at the amino acid level.

TLMV was found to be distributed worldwide among healthy individuals (Biagini et al. 2001b; Niel et al. 2001). The prevalence of TLMV DNA among blood donors is reported to be 48%–72% (Biagini et al. 2006b; Moen et al. 2002; Niel et al. 2001). TLMV has been isolated from various body fluids and tissues, such as plasma/serum, peripheral blood mononuclear cells (PBMC), feces, saliva, bone marrow, spleen, and cervical swabs (Biagini et al. 2001b; Fornai et al. 2001; Thom et al. 2003; Vasconcelos et al. 2002).

Recently, the International Committee on Taxonomy of Viruses (ICTV) officially designated TTV and TLMV as Torque teno virus (TTV) and Torque teno mini virus (TTMV), respectively, deriving from the Latin terms *torque* meaning "necklace" and *tenuis* meaning "thin", and classified them into a novel floating genus, *Anellovirus*. These terms were chosen to reflect the organizational arrangement of the TTV genome, without changing the abbreviation TTV (Biagini et al. 2005).

Discovery of Torque Teno Midi Virus

By means of the DNase-sequence independent single primer amplification (SISPA) method, two new TTV-like viruses named small anellovirus 1 (SAV1) and small anellovirus 2 (SAV2) were isolated from the sera of patients with acute viral infection syndrome (Jones et al. 2005). SAV1 possessed a genomic DNA of 2,249 nt with three putative ORFs, while SAV2 had a genomic DNA of 2,635 nt with five ORFs. These two viruses (collectively, SAVs) were provisionally classified as anelloviruses on the basis of the circular nature of the genomic DNA and the presence of regions homologous to TTV and TTMV in the largest ORF (ORF1) and noncoding region. The SAV ORF2 region was shown to possess a similar CAV-like motif as TTVs and TTMVs (Andreoli et al. 2006). Similar to TTVs and TTMVs, SAV isolates showed wide genomic variation of up to 41%. SAV has also been isolated from various body fluids and tissues, including saliva and PBMC (Biagini et al. 2006a) as well as nasopharyngeal aspirates (Chung et al. 2007). Similar to TTVs and TTMVs, SAVs were found to be common among healthy individuals and were present in 20% of French blood donors (Biagini et al. 2006a) and in 34.5% of Korean children (Chung et al. 2007). In addition, using a combined rolling-circle amplification (RCA) and SISPA approach, isolates related to SAV, but with even shorter genomes (2,002 nt and 2,454 nt) have been identified (Biagini et al. 2007); they differed from SAVs by approximately 40%.

Recently, in the process of amplifying the SAV sequence in human sera, amplicons longer than expected were obtained, and the full-length clones were 3,242–3,253 nt, with all of the characteristics of TTV-like viruses (Fig. 1). Most importantly, the previously described SAVs were found to be deletion mutants or artifacts generated during amplification of these longer isolates. These newly identified isolates were

named Torque teno midi virus (Ninomiya et al. 2007a). Upon analyzing 15 additional TTMDV sequences over the entire genome (Fig. 2), it was found that they form a large clade of isolates differing in length (3,175–3,230 nt) and in sequence (up to 33% divergence at the nucleotide level and 61% divergence at the amino acid level of ORF1; Ninomiya et al. 2007b). In addition to other TTV-like characteristics, three Rep-motifs were identified in the ORF1 region, as well as putative stem-loop structures in the GC-rich region. Therefore, TTMDV is provisionally classified as the third group in the genus *Anellovirus* (Ninomiya et al. 2007a, b).

Multiple Infections of Three Human Anelloviruses (TTV, TTMDV, and TTMV) in Humans

The development of methods for specific detection of human anelloviruses has been made more difficult by the discovery of the third human anellovirus, TTMDV. In fact, TTMDV DNA can be erroneously amplified by previously reported TTMV PCR assays (Biagini et al. 2006b; Vasconcelos et al. 2002). Biagini et al. (2006a) reported that TTMDV/SAV showed a 20% prevalence among French blood donors, which is comparable to the 9% frequency of TTMDV/SAV DNA among Italian blood donors (Andreoli et al. 2006). The selection of PCR primers and the length of the genomic region for PCR amplification crucially influence the detection of TTV, which has an extremely divergent genome (Biagini et al. 2001a; Itoh et al. 1999; Okamoto et al. 1999d). Recent surveys using primers specific for individual genotypes or genogroups of TTV, or those that differentiate TTV from TTMV sequences indicated that approximately 90% of study populations (generally healthy adults) were viremic for TTV or TTMV, with co-infection of TTV and TTMV in 44% (Biagini et al. 2006b). Therefore, it is likely that the actual prevalence of TTV, TTMV, or TTMDV DNA is higher than was previously reported.

PCR Assays for Differential Detection of Three Human Anelloviruses

Despite marked divergence with a difference of greater than 50% among TTV genomes (Khudyakov et al. 2000; Okamoto et al. 2004; Peng et al. 2002; Thom et al. 2003), a difference of up to 33% among TTMDV genomes (Ninomiya et al. 2007b), and a difference of up to 40% among TTMV genomes (Biagini et al. 2001, 2007; Okamoto et al. 2000a; Takahashi et al. 2000b), there exists a highly conserved area of 130 nt located just downstream of the TATA-box in the TTV, TTMDV, and TTMV genomes. Taking advantage of this particular genomic area that is conserved among known anelloviruses, virus species-specific PCR assays were developed in which the genomic DNA of all three anelloviruses is amplified by

first-round PCR with universal primers; TTV DNA, TTMDV DNA, or TTMV DNA is separately amplified by each of the three second-round PCR assays with species-specific primers (Ninomiya et al. 2008). All 257 molecular clones of the PCR products amplified by universal primers obtained from three subjects (subjects D1 to D3) who were co-infected with TTV, TTMDV, and TTMV were classifiable into TTV, TTMDV, or TTMV by the three differential PCR assays. The reliability of these assays for classification was confirmed by phylogenetic analysis (Fig. 3). When the newly developed PCR assays were applied to serum samples from adults in the general population in Japan, high prevalence rates of TTV DNA (100%), TTMV DNA (82%), and TTMDV DNA (75% or 75/100) were found.

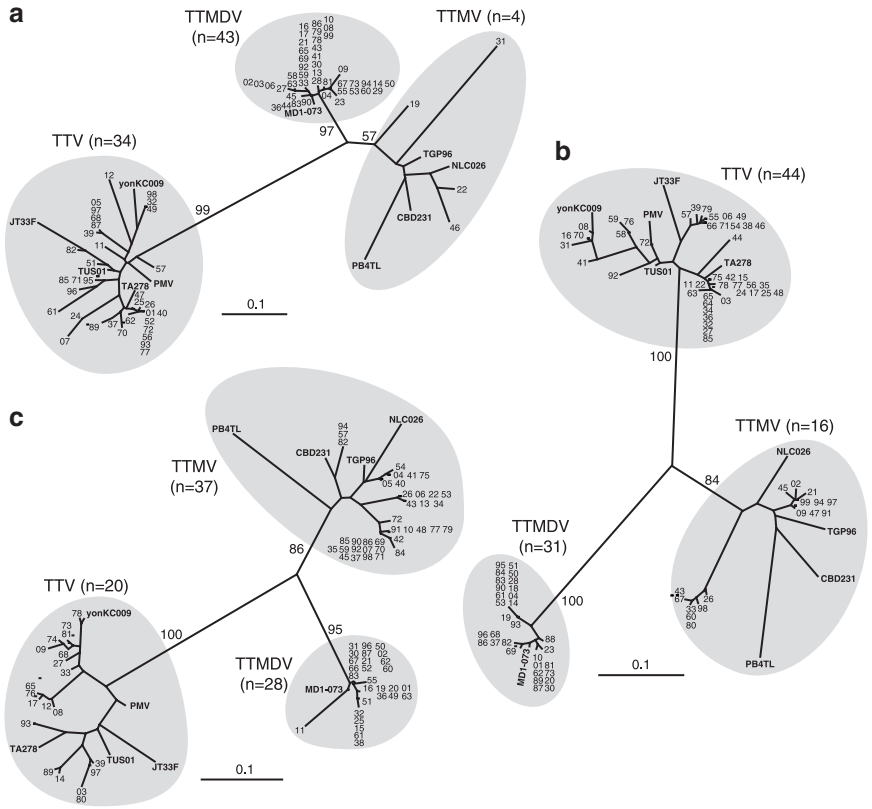

Fig. 3 a–c Phylogenetic trees constructed by the neighbor-joining method based on the partial nucleotide sequences (85–97 nt) of TTV, TTMDV, and TTMV isolates obtained from subject D1 (**a**), subject D2 (**b**), and subject D3 (**c**). The representative TTV, TTMDV, and TTMV isolates whose full-length sequence is known are indicated in *boldface*. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data. *Bar*, 0.1 nucleotide substitutions per site. (Reproduced from Ninomiya et al. 2008, with permission)

Early Acquisition of Dual or Triple Infection of three Human Anelloviruses During Infancy

Ten cord blood samples, 44 serum samples from infants (<1 year of age), and 261 serum samples from individuals age 1 year or older were tested for TTV, TTMDV, and TTMV DNA. Although none of the 10 umbilical cord blood samples had the genomic DNA of any of TTV, TTMDV, and TTMV, rapidly rising prevalence rates of TTV, TTMDV, and TTMV infections were noted over the subsequent months (Table 1), reaching a rate of 100% within the first 2 years of life (Ninomiya et al. 2008). This finding corroborated earlier studies reporting that TTV infection prevails in childhood (Kazi et al. 2000; Lin et al. 2002; Toyoda et al. 1999; Vasconcelos et al. 2002). Therefore, maternal transmission may play only a minor role in early acquisition of TTV and TTMV as well as TTMDV in infants, and early acquisition via horizontal infection during infancy may be common to all three anelloviruses (Ninomiya et al. 2008). TTV and TTMV genomes have been detected in feces (Biagini et al. 2001b; Okamoto et al. 1998a), saliva (Biagini et al. 2001b; Deng et al. 2000; Gallian et al. 2000; Ishikawa et al. 1999; Vasconcelos et al. 2002), and breast milk (Gerner et al. 2000; Toyoda et al. 1999), suggesting transmission of anelloviruses including TTMDV via horizontal routes.

Due to the frequent dual or triple infection of TTV, TTMDV, and TTMV noted in infants and adults, it remains unknown whether each of the three anelloviruses can replicate and maintain the persistent carrier state independently or in concert with one or two of the other anelloviruses. However, the presence of single infection of TTV or TTMV in some infected hosts suggests that TTV, TTMDV, and TTMV can independently infect susceptible hosts and replicate in their tissues or organs (Ninomiya et al. 2008).

Table 1 Age-specific prevalence of TTV, TTMDV, and TTMV DNAs. (Reproduced from Ninomiya et al. 2008, with permission)

Age (years) and sample type	No. of samples tested	No. of samples (%) with: TTV DNA	TTMDV DNA	TTMV DNA
<1				
Cord blood	10	0	0	0
Serum[a]	44	35 (79.5)	22 (50.0)	33 (75.0)
Subtotal	54	35 (64.8)	22 (40.7)	33 (61.1)
≥1 (serum)				
1	28	28 (100)	28 (100)	28 (100)
2–4	29	29 (100)	28 (96.6)	28 (96.6)
5–9	42	41 (97.6)	36 (85.7)	41 (97.6)
10–19	62	61 (98.4)	48 (77.4)	55 (88.7)
20–49	32	32 (100)	22 (68.8)	24 (75.0)
50–81	68	68 (100)	53 (77.9)	58 (85.3)
Subtotal	261	259 (99.2)	215 (82.4)	234 (89.7)
Total	315	294 (93.3)	237 (75.2)	267 (84.8)

[a] The serum samples were from infants <1 year of age (9–364 days old)

Pathogenesis and Clinical Manifestations

Pathogenesis

Recently, two cell culture systems supporting virus replication and virion formation of transfected cloned TTV have been reported, but in neither case was efficient virus propagation achieved (Kakkola et al. 2007; Leppik et al. 2007). Infection with TTV is characterized by persistent lifelong viremia in humans, with circulating levels of up to 10^6 copies/ml in the general population (Hu et al. 2005; Pistello et al. 2001). As an indication of its great replicative capacity in vivo, a study on the kinetics of clearance of TTV viremia suggested that a daily production rate of $>10^{10}$ virions is required to maintain the observed levels of viremia (Maggi et al. 2001b). TTV replicates in the liver, as documented by in situ hybridization and/or quantitative PCR (Ohbayashi et al. 2001; Rodríguez-Iñigo et al. 2000), as well as the detection of double-stranded replicative intermediates in the liver (Okamoto et al. 2000e) and a high level of TTV excreted in bile (Luo et al. 2000; Nakagawa et al. 2000; Ukita et al. 1999). Excretion of TTV in bile may be the main source of TTV in the gastrointestinal tract and its fecal shedding. Replication of TTV is not restricted to the liver. High viral loads, double-stranded replicative forms of TTV DNA, and mRNA transcripts have also been detected in lung tissues (Okamoto et al. 2001), pancreas (Okamoto et al. 2001), bone marrow (Fanci et al. 2004; Kikuchi et al. 2000; Okamoto et al. 2000b), spleen (Okamoto et al. 2001; Jelcic et al. 2004), and other lymphoid tissues (Kakkola et al. 2004). TTV DNA is frequently detectable in PBMCs (Barril et al. 2000; Lopez-Alcorocho et al. 2000; Okamoto et al. 1999a, 2000c; Okamura et al. 1999). In these lymphoid cells, TTV shows a very broad tropism with viral DNA detected not only in T and B lymphocytes, monocytes, and natural killer (NK) cells (Maggi et al. 2001; Takahashi et al. 2002; Zhong et al. 2002) but also in granulocytes and other polymorphonuclear cells (Maggi et al. 2001a; Takahashi et al. 2002).

Host's Immune Response

As described above, TTV infection is likely acquired early in infancy, which may lead to substantial immune tolerance, as is well known for other viruses such as hepatitis B virus. The persistent nature of infection and co-infection of multiple TTV variants in the circulation, representing repeated rounds of concurrent infection, may suggest the presence of mechanisms of immune evasion that have evolved in TTV to establish persistent infection in immunocompetent individuals. However, antibodies against native TTV virions (Tsuda et al. 1999) or recombinant ORF1 proteins (Handa et al. 2000; Ott et al. 2000) are detected in viremic and nonviremic individuals, and TTV particles in the circulation are often bound to IgG, forming immune complexes (Itoh et al. 2000). Nevertheless, at present there is no evidence

to indicate an association with diseases evoked by the deposition of immune complex, such as glomerulonephritis.

TTV viral loads have been shown to increase in human immunodeficiency virus (HIV)-infected patients who are progressing toward AIDS, and a high TTV viral load was associated with a low CD4 cell count, indicating a potential role of the immune system in controlling TTV replication (Christensen et al. 2000; Shibayama et al. 2001; Thom and Petrik 2007; Touinssi et al. 2001; Zhong et al. 2002). Although it remains unclear what role the immune system plays in the natural course of TTV infection, TTV may act as an opportunistic pathogen in immunocompromised hosts, analogous to human cytomegalovirus in AIDS patients.

Disease Associations

Although TTV, TTMDV, and TTMV are potentially related to many diseases, conflicting opinions exist on their disease-causing potential due to their nearly universal presence in human populations (i.e., lack of controls). It is possible that certain genotypes/genogroups of TTV, TTMDV, and TTMV may be specifically pathogenic. Interestingly, the expression of genotype 1–ORF1 in transgenic mice, leading to production of a spliced protein, caused pathological changes in the kidneys. Expression of the TTV protein seemed to interfere with differentiation of renal epithelial cells (Yokoyama et al. 2002). It has recently been suggested that subgenomic fragments of TTV identified in human sera could have some role in diseases as is the case with plant geminiviruses (Leppik et al. 2007). Unfortunately, however, there are only a few reports to support the disease-inducing potential of TTV.

Possible Association of TTV with Liver Diseases

When the original TTV isolate was discovered, TTV (genotype 1) detectable by N22 PCR was found in three of five patients with posttransfusion acute hepatitis of unknown etiology, and the presence of TTV genotype 1 was closely associated with the serum ALT level (Nishizawa et al. 1997). When a serum sample obtained from an 11-month-old infant with acute hepatitis of unknown etiology who had been transiently infected with genotype 1 TTV (10^5 copies/ml) was inoculated intravenously into a naive chimpanzee, TTV DNA was transiently detected in the chimpanzee at 5–15 weeks postinoculation (PI), with the titer peaking at 12–13 weeks PI (Tawara et al. 2000). This viremia was accompanied by an abrupt elevation of the serum α-glutathione-*S*-transferase level and mild elevation of ALT level. Histological changes in biopsied liver samples (ballooning degeneration of hepatocytes) were observed in association with the reduction in TTV DNA titer and the appearance of IgM-class and IgG-class anti-TTV (genotype 1) antibodies, suggesting that TTV genotype 1 has hepatitis-inducing capacity. Shibata et al. (2000) reported

that TTV genotype 1 may play a role in the pathogenesis of non-A, -B, or -C fulminant hepatic failure (FHF), since the TTV-positive rate was significantly higher among the group with non-A, -B, or -C FHF (6/7 or 86%) than among the group with non-A, -B, or -C acute hepatitis (4/17 or 24%; p=0.005). Tajiri et al. (2001) presented three infants diagnosed with idiopathic neonatal hepatitis and intrahepatic fatty degeneration and whose livers were infected with TTV detectable by N22 PCR. Several other studies also suggested that TTV of genotype 1 may be more pathogenic than other genotypes of TTV in children with liver disease of unknown etiology (Okamura et al. 2000; Sugiyama et al. 2000). On the other hand, infection with TTV genotype 12 or 16, which was described as SENV-D and SENV-H, respectively, was found to be much more prevalent among patients with transfusion-associated non-A to -E hepatitis than among transfused patients without hepatitis in the United States (92% vs 24%, p<0.001) (Umemura et al. 2001). Foschini et al. (2001) reported an Italian case of TTV (genotype 13)-related acute recurrent hepatitis, with clinicopathological findings reinforcing the suggestion that TTV can be responsible for a mild form of liver disease. Other investigators (Charlton et al. 1998; Ikeda et al. 1999; Kanda et al. 1999; Okamura et al. 2000; Tanaka et al. 1998, 2000; Tuveri et al. 2000) also showed an association between the prevalence of TTV and/or TTV load and various hepatic disorders. It was also suggested that persistent TTV infections could contribute to cryptogenic hepatic failure in hemophiliacs (Takayama et al. 1999).

However, contradictory results showing that TTV is not associated with ALT levels or with any form of hepatitis (posttransfusion, chronic idiopathic, acute or fulminant) have also been presented (Hijikata et al. 1999a; Hsieh et al. 1999; Naoumov et al. 1998; Niel et al. 1999; Prati et al. 1999; Viazov et al. 1998). Additionally for SENV, it was reported that infection is not related to hepatitis or other liver disease (Akiba et al. 2005; Kao et al. 2002; Schroter et al. 2003; Umemura et al. 2001).

Tokita et al. (2002b) reported that a high TTV viral load was independently associated with the complication of hepatocellular carcinoma (HCC) and that it may have prognostic significance in patients with hepatitis C virus (HCV)-related chronic liver disease. There are two possible explanations for the findings in this report. One explanation is that high TTV viremia has an adverse effect on the progression of chronic liver disease in concert with concurrent HCV infection and may be associated with the development of HCC. Zein et al. (1999) reported that TTV infection was more prevalent among patients with advanced HCV-associated liver disease (decompensated cirrhosis and HCC) than among those with stable disease (chronic hepatitis and compensated cirrhosis). Moriyama et al. (2001) reported that the score of irregular regeneration of hepatocytes among TTV-infected cirrhotic patients with chronic hepatitis C was higher than that among patients who were not infected with TTV. These findings suggest that TTV plays a role in the development of cirrhosis and subsequent complications. However, another explanation is possible. A correlation between high TTV titer and a low CD4 T cell count among patients infected with HIV type 1, and the possible prognostic significance of TTV viral load in immunocompromised patients, has been reported (Christensen et al. 2000; Shibayama

et al. 2001). Therefore, it is likely that an impaired immune system or suppression of the immune system is involved in elevated TTV viremia in HCC patients.

At present, despite evidence for hepatic replication of TTV, TTV does not fulfill the criteria for being a hepatitis virus. For TTV to be characterized as a hepatitis virus, direct causal evidence of cytopathology or specific inflammatory changes associated with replication as well as statistical difference in comparison with controls in terms of TTV prevalence, loads, sequence variation, genotype distribution, or co-infection among liver disease patients have to be demonstrated in future studies.

Possible Association of TTV with Respiratory Diseases

It has been suggested that TTV infection has a potential role in children with respiratory diseases. Importantly, TTV replication has been shown to occur in lung tissues (Okamoto et al. 2001; Bando et al. 2001). Infection with TTV coincided with mild rhinitis in a neonate (Biagini et al. 2003), and children hospitalized with acute respiratory disease or with bronchiectasis showed higher TTV viral loads than controls (Maggi et al. 2003b, c; Pifferi et al. 2006). In addition, children with high TTV loads in nasal specimens were shown to have worse spirometric values, and TTV was suggested to contribute to the pathogenesis of asthma (Pifferi et al. 2005). Although the precipitating factors of idiopathic pulmonary fibrosis have not been elucidated, Bando et al. (2001) first reported the influence of TTV infection on the disease activity and prognosis of idiopathic pulmonary fibrosis. Furthermore, the association between TTV infection and the complication of lung cancer in patients with idiopathic pulmonary fibrosis has been reported (Bando et al. 2008).

These observations raise interesting questions about the pathophysiological significance of TTV in the respiratory tract of infected humans. However, it remains undetermined if TTV is the cause or the result of the disease. Interestingly, it was suggested that TTV replication could twist the immunobalance toward the T helper 2 cell (Th2) response that is known to have a role in the pathogenesis of asthma (Pifferi et al. 2005).

Possible Association of TTV with Hematological Disorders

Hepatitis-associated aplastic anemia mainly occurs after acute non-A, non-B, non-C hepatitis (Brown et al. 1997). A high level of TTV replication in bone marrow has been suggested as being responsible for hepatitis-associated aplastic anemia of unexplained etiology (Kikuchi et al. 2000). A possible association between TTV infection and aplastic anemia has also been suggested by others (Miyamoto et al. 2000). However, contradictory results were also presented indicating that TTV is not associated with post-hepatitis aplastic anemia (Poovorawan et al. 2001; Safadi et al. 2001).

As for the association of TTV with hematopoietic malignancies, TTV DNA was detected in lymphocytes circulating in the lymph nodes of patients with B-cell

lymphomas and those with Hodgkin's disease (Garbuglia et al. 2003). It was postulated that TTV could somehow modulate the infected T cells and thus play some role in the pathogenesis of lymphomas.

Possible Association of TTV with Cancer

In addition to hepatocellular carcinoma, lung cancer, and hematopoietic malignancies, the possible involvement of TTV infection in other malignancies or malignant changes has been suggested. TTV DNA has been detected in a wide variety of neoplastic tissues (de Villiers et al. 2002). Co-infection of TTV genotype 1 and human papillomavirus was related to poor outcome of laryngeal carcinoma (Szladek et al. 2005). However, similar to other small DNA viruses such as parvoviruses and circoviruses, there is no plausible causal association of TTV infection with tumorigenesis or malignant transformation of cells.

Conclusions

Although TTV was discovered relatively recently in 1997 (Nishizawa et al. 1997), it seems to be a well-adapted virus of humans that has been a persistent source of infection since the distant past. TTV infections are extremely prevalent even in healthy individuals. The high prevalence of TTV is not usual per se among viruses; for example, papillomaviruses and herpesviruses are frequently found in healthy individuals. However, TTV differs from all other known viruses in its ability to sustain lifelong viremia, i.e., to actively replicate and continuously produce virus in the blood for decades, even in healthy individuals. Due to its global distribution and persistent viremia in human populations, there is no definitive causal association of TTV infection with the diseases that have been investigated. It may be possible that TTVs per se do not cause any disease and do not have any adverse effect whatsoever on human health (Griffiths 1999; Simmonds et al. 1999). On the other hand, as with opportunistic pathogens, disease may appear only under exceptional circumstances. In some virus infections, the viral load is a critical determinant of development of disease. It has been suggested that TTV could be a commensal in normal conditions, incapable of exceeding the threshold of a disease-causing load (Griffiths 1999), and if TTV is a genuine symbiont, the virus should benefit the host, which is an intriguing aspect hitherto unexplored with TTV.

At present, however, we cannot rule out the possibility that some isolates/genotypes could be more pathogenic than others (Maggi et al. 2003a, 2007; Okamura et al. 2000; Sugiyama et al. 2000; Tokita et al. 2002a), as is well known for human papillomaviruses, either alone or co-infecting with other TTV strains or other pathogens, that they have an effect on the outcome or progression of some disease(s), and that the level of TTV in tissue and/or in the bloodstream could affect any of the disease conditions.

Our newly developed PCR method with high sensitivity and reliability has revealed frequent dual or triple infection of these three anelloviruses, even in infants (Ninomiya et al. 2008). The pathogenetic role of TTMV and TTMDV infections remains unknown. The changing ratio of the three anelloviruses to each other over time, their relative viral load, or the combination of different genotypes of each anellovirus may be associated with the pathogenicity or the disease-inducing potential of these three human anelloviruses. In this context, further efforts are warranted to develop methods to separately or simultaneously quantify the genomic DNA of the three anelloviruses and to clarify their disease association. PCR systems for detecting, differentiating, and quantitating all of the genotypes and/or genogroups of TTV, TTMDV, and TTMV should be established and standardized, as should methods to detect past infections and immunological responses to anellovirus infections.

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