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A Novel DNA Virus (TTV) Associated with Elevated Transaminase Levels in Posttransfusion Hepatitis of Unknown Etiology

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By means of representational difference analysis, a viral clone (N22) of 500 nucleotides was isolated from serum of a patient (TT) with posttransfusion hepatitis of unknown etiology. The N22 clone showed a poor homology to any reported sequences. Oligonucleotide primers were deduced from the N22 sequence for detecting it by polymerase chain reaction. N22 sequence in serum banded at a sucrose density of 1.26 g/cm^3, indicating its association with a viral particle which was designated TT virus (TTV). Since nucleic acids of TTV were sensitive to DNase I, it would be a DNA virus. TTV DNA was detected in sera from three of the five patients with posttransfusion non-A to G hepatitis, including the index case (TT). TTV DNA titers closely correlated with aminotransferase levels in the three patients. These results indicate that TTV would be a novel DNA virus with a possible capacity to induce posttransfusion non-A to G hepatitis.

There still is a residual risk of posttransfusion hepatitis in the recipients of blood units screened for hepatitis B virus (HBV) and hepatitis C virus (HCV) (1). A significant population of the patients with chronic hepatitis and cirrhosis is without serum markers of HBV or HCV infection, and therefore, classified as "cryptogenic" (2). Etiology of fulminant hepatitis in most patients is not ascribed to hepatitis A, B, C, D or E virus (3, 4). These backgrounds point to viral agents other than A to E that would induce hepatitis.

A new virus possibly responsible for non-A to E hepatitis has been reported independently by two groups of investigators (5, 6). This virus, designated GB virus C or hepatitis G virus (referred to as HGV hereafter for convenience), is a positive, single-stranded RNA virus of 9,400 nucleotides. HGV resembles members of the *Flaviviridae* family, is distantly related to HCV (5, 6), and covered by a lipoprotein coat (7). It infects 1–2% of the general population, and transmits by transfusions (8). As polymerase chain reaction (PCR) for detecting HGV RNA with specific primers has been applied to extended series of patients with non-A to E hepatitis, however, it has become increasingly evident that HGV would not account for a significant part of the patients with non-A to E hepatitis (9, 10). Hence, there would be a viral agent other than HGV for inducing non-A to E hepatitis.

Using representational difference analysis (11), a viral clone named N22 was isolated from serum of a patient with posttransfusion non-A to E hepatitis who was negative for HGV RNA. The N22 clone was found to originate in the genome of a DNA virus, which was designated TT virus (TTV) after the patient. By means of PCR with specific primers, DNA of TTV was searched for in serial sera from patients with posttransfusion hepatitis of non-A to G etiology. The results indicate TTV to be a candidate virus responsible for non-A to G hepatitis.

MATERIALS AND METHODS

Patients with posttransfusion non-A to G hepatitis. Five patients were studied who developed elevated alanine aminotransferase (ALT) levels > 60 U/L (twice the upper limit of normal) after 1 week and lasting for at least 3 weeks, and had a maximal ALT >150 U/L (five times the upper limit). Case 1 (male, 63 years) received 4 units of blood (one unit: 200 ml) at operation for his dislocated jaw. He had ALT levels elevated during 8–10 weeks after the operation with a peak at 443 U/L. Case 2 (male, 58 years) received 35 units of

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HGV, hepatitis G virus; ALT, alanine aminotransferase; PCR, polymerase chain reaction; TTV, TT virus.

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blood at his heart operation. He had elevated ALT levels 9–11 weeks thereafter (peak: 180 U/L). Case 3 (female, 68 years) received 14 units of blood at her operation for bladder carcinoma. She had her ALT levels elevated at 21–25 weeks (peak: 225 U/L). Case 4 (female, 70 years) received 26 units of blood at her operation for gall bladder carcinoma. Her ALT levels were elevated 9–11 weeks (peak: 182 U/L). Case 5 (male, 56 years) received 21 units of blood at the operation for esophageal carcinoma, and had elevated ALT levels 6–13 weeks (peak: 192 U/L).

Cloning and sequencing a viral isolate. A viral clone (N22) was isolated by representational difference analysis (11). Used as a tester was a 1 : 1 mixture (total: 100 μl) of sera from Case 2 (the patient TT) obtained 8 and 10 weeks after transfusion when his ALT levels were elevated. A driver was his serum (100 μl) taken 2 weeks after transfusion when he had a normal ALT level. Nucleic acids were extracted from tester and driver with ISOGEN-LS (Nippon-Gene, Tokyo, Japan), and converted to cDNA in SuperScript Choice System (Gibco-BRL, Gaithersburg, MD) with random hexamer primer. Obtained cDNA samples were digested with Sau3A I (TaKaRa Shuzo Co., Ltd., Shiga, Japan). Tester and driver thus processed were subjected to representational difference analysis by the method of Lisitsyn et al. (11).

Amplicons obtained after three courses of subtraction were ligated to pT7BlueT-Vector (Novagen Inc., Madison, WI), and with recombinant plasmid obtained by the introduction into *E. coli* TG1, sequences were determined for both strands with ThermoSequenase Fluorescent-labelled primer cycle Sequencing kit (Amersham International plc. Buckinghamshire, England).

Detection of TTV DNA by PCR. Nucleic acids were extracted from serum (50 μl) with proteinase K and sodium dodecyl sulfate (SDS) by the method described previously (12). Extracted DNA was dissolved in 20 μl of Tris-HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA, and heated at 95 °C for 15 min, and then chilled quickly on ice. A half portion thereof was subjected to PCR with Perkin-Elmer AmpliTaq DNA Polymerase (Roche Molecular Systems, Inc., Branchburg, NJ) with nested primers. The first-round PCR was performed with RD037 (sense: 5′-GCA GCA GCA TAT GGA TAT GT-3′) and RD038 (antisense: 5′-TGA CTG TGC TAA AGC CTC TA-3′) for 35 cycles (94 °C 30 sec; 55 °C, 30 sec; and 72 °C, 45 sec [an additional 7 min in the last cycle]) for the amplification of a product of 270 base pairs (bp). The second-round PCR was carried out with RD051 (5′-CAT ACA CAT GAA TGC CAG GC-3′) and RD052 (antisense: 5′-GTA CTT CTT GCT GGT GAA AT-3′) for 25 cycles (94 °C, 30 sec; 55 °C, 30 sec; and 72 °C, 30 sec [an additional 7 min in the last cycle]) for the amplification of a 197-bp product. Amplification products were run by electrophoresis on 2.5% NuSieve 3 : 1 agarose (FMC BioProducts, Rockland ME), stained with ethidium bromide and observed under ultraviolet light.

For sequence analysis, semi-nested PCR was performed with a sense primer NG001 (5′-CAC CAG GAG CAT ATA CAG AC-3′), in place of RD037 and RD051, for the amplification of a 415-bp product in the first round and that of a 396-bp product in the second round. The conditions were the same except that the extension was performed for 60 sec.

PCR after reverse transcription. Nucleic acids extracted from serum of Case 2 (100 μl) were denatured at 70 °C for 5 min, chilled quickly on ice, and converted to cDNA by reverse transcriptase (SuperScript II: Gibco-BRL) with either RD037 or RD038, as described previously (13). The products were denatured at 95 °C for 15 min, and a half portion was subjected to the PCR with nested primers described above.

Ultracentrifugation in a sucrose density gradient. Plasma from a blood donor positive for DNA of N22 clone (250 μl) was added with 5 μl of plasma containing HBV DNA, serving as a size marker, and centrifuged in a micro refrigerated centrifuge (Tomy Seiko, Tokyo, Japan) at 6,200 $\times$ *g* for 5 min. The supernatant (200 μl) was layered onto a stepwise density gradient consisting of 0.7 ml 60% (wt/vol) and 0.2 ml each 50, 40, 30, 20 and 10% sucrose in Tris-HCl buffer (50 mM, pH 8.0) containing 1 mM EDTA and 150 mM NaCl (hereafter referred to as TEN buffer) in a tube with a capacity of 4.4 ml. The tube was overlaid with 2.3 ml of TEN buffer and centrifuged at 179,200 $\times$ *g* for 45 h at 10 °C in a Beckman SW60Ti rotor (Beckman Co., Palo Alto, CA). The tube was pierced at the bottom, and 300-μl fractions were collected. A 100-μl portion of each fraction was treated with proteinase K/SDS, and DNA was extracted and dissolved in 20 μl of Tris-HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA. HBV DNA was determined by PCR with primers deduced from the S gene (14).

Digestion with DNase I. A fraction of ultracentrifugation in a sucrose density gradient enriched in respect to N22 sequence was diluted five-fold with TEN buffer, and a 50-μl portion was made to 6 mM with $MgCl_2$ and digested with 2 μl (70 U/μl) Deoxyribonuclease I (DNase I: TaKaRa Shuzo) at 37 °C for 30 min. The digest was heated at 100 °C for 10 min to inactivate the enzyme. Nucleic acids were extracted with proteinase K/SDS and phenol/chloroform, and subjected to PCR for TTV DNA. Nucleic acids extracted from serum containing TTV DNA was dissolved in TEN buffer containing 10% sucrose and similarly processed, for evaluating their susceptibility to DNase I.

RESULTS

Isolation of the N22 Clone from a Patient with Posttransfusion Non-A to G Hepatitis

Representational difference analysis was performed on paired sera from a patient (TT) with posttransfusion non-A to G hepatitis (Case 2). His sera obtained at peak ALT levels were used as tester and that taken before, while he had a normal ALT level, was used as a driver. After three courses of subtraction, a broad but clear band of 500 bp in size was visualized on electrophoresis. Gel corresponding to the band was cut out, and the extract was connected to pT7Blue T-Vector and introduced to *E. coli.* DNA sequences were determined on the 36 clones obtained. By homology of nucleotide sequence, they were classified into 13 groups varying in size from 281 to 564 bp. Homology research (*vide infra*) was conducted on the representative clone from each group, and clones identical or similar to those registered in data bank were omitted. Four clones were selected in this way, and 20-mer primers were synthesized based on their sequences. By means of PCR with these primers, DNA in tester and driver was amplified, and a single clone was identified the sequence of which was detected in tester but not in driver. There were additional 8 clones among the 36 which showed sequences similar to it. Nucleotide sequences of these 9 clones were determined, and the consensus sequence was adopted as illustrated in Fig. 1.

The N22 clone had a length of 500 bp inclusive of the Sau3A I recognition sequence (GATC) at both ends. An ORF capable of encoding at least 166 amino acids was recognized in a reading frame starting at the third nucleotide; there were no ORF in the other two reading frames starting at the first or second nucleotide, or in any of the three reading frames in the complementary sequence, which could code for >100 amino acids.

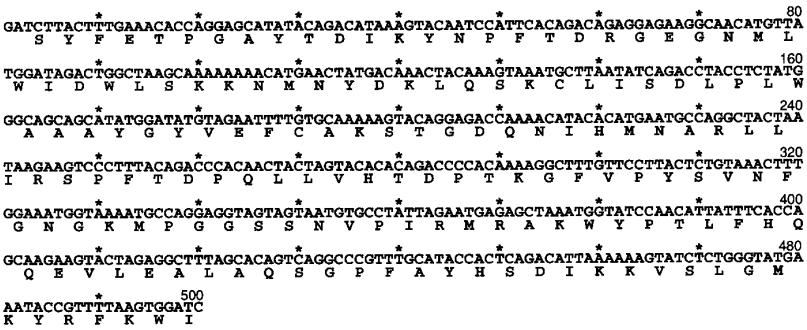

FIG. 1. Nucleotide and deduced amino acid sequences of the N22 clone. The nucleotide sequence is 500-bp long, flanked by the recognition sequence (GATC) of Sau3A I that was used for representational difference analysis.

As of October 2, 1997, 1,731,752 sequences with a total of 1.141,726,059 letters were deposited in DDBJ (National Institute of Genetics, Mishima, Japan). There was not a single sequence with a high homology to the N22 clone among them. There was not a single protein sequence, either, among 154,072 sequences with a total of 51,679,968 amino acids deposited that indicated a high homology to the amino acid sequence of the ORF in the N22 clone.

A DNA Nature of N22

Since the N22 clone was obtained in nucleic acids extracted from the patient's serum, which were then reverse-transcribed, it had to be determined whether it was of a DNA or RNA nature. To determine this, nucleic acids from Case 2 (the source of N22), either reverse-transcribed to cDNA or as they were, were subjected to PCR with primers deduced from N22. Irrespective of cDNA synthesis with sense primer (RD037) or antisense primer (RD038), equally strong signals were found in the PCR products of materials with or without prior reverse-transcription. These results indicated a DNA nature of N22.

A Nonhost Origin of N22

PCR amplification was performed with nested primers deduced from the N22 sequence on four genomic DNA samples including three derived from leukocytes and one from placenta. No amplification was achieved with any of the four genomic DNA samples. These results attested to a non-host origin of N22.

A Viral Origin of N22 Demonstrated by Fractionation in a Sucrose Density Gradient

When plasma from a blood donor positive for N22 sequence was fractionated by ultracentrifugation in a sucrose density gradient, DNA with the N22 sequence appeared in fractions with a peak at 1.26 g/cm^3. HBV DNA serving as a marker distributed in fractions with a peak at 1.24 g/cm^3, as previously reported (15), thereby attesting to the reliability of fractionation. N22 DNA in fractions was resistant to DNase I digestion, unlike that in DNA extracted from plasma of the same donor. These results indicated that DNA with the N22 sequence would be covered by coat in a putative virus. The virus was named TT virus (TTV) after the patient (Case 2) from whom the N22 clone was propagated.

TTV DNA in Sera from Patients with Non-A to G Posttransfusion Hepatitis

When sequential sera from five patients with posttransfusion non-A to G hepatitis were tested for TTV DNA, it was detected in three of them including the original patient TT (Case 2) (Table 1). There was a close association between ALT levels and TTV DNA titers in Cases 2 and 4. Sera from them obtained 4–6 weeks after transfusion were negative for TTV DNA. It turned positive 2–4 weeks before the highest ALT level, and then TTV DNA and ALT peaked simultaneously. Viremia was transient in both cases, and ALT levels normalized as TTV DNA disappeared from serum.

In Case 5, TTV DNA appeared simultaneously with the peak ALT level, and persisted through 21 weeks after transfusion accompanied by moderately elevated ALT levels. TTV DNA gradually increased, peaked 17 weeks after transfusion and then decreased but stayed positive. Overall, TTV DNA was transient but continued to be positive during 4–6 weeks in two cases (Cases 2 and 4) and persisted for longer than 21 weeks in the remaining case (Case 5).

The remaining two cases (Cases 1 and 3) were negative for TTV DNA. Sera from Case 1 were tested at 10 time points covering pre-transfusion and up to 6 months after it. Sera from Case 3 was tested at 8 time points covering pre-transfusion and until 7 months thereafter.

Comparison of TTV Sequences among the Patients

DNA was obtained from sera from Cases 2 and 4 when TTV DNA peaked, 10 and 8 weeks after transfu-

TABLE 1

Detection of TT Virus (TTV) DNA in Sera from the Three Patients with Posttransfusion Non-A to G Hepatitis

Case no.	Age	Sex	Transfusion (units)	Diagnosis	Time after transfusion (weeks)	ALT (U/L)	TTV DNA titer (per ml)
2	58	Male	35	Coronary insufficiency	Before	28	NT*
					2	36	—
					6	37	—
					8	55	10^2
					9	72	NT
					10	180	10^3
					11	94	NT
					12	58	10^1
					17	31	—
					21	43	—
					25	25	—
4	70	Female	26	Cancer of gallbladder	Before	22	NT
					4	14	—
					6	15	10^1
					8	10	10^2
					9	155	NT
					10	182	NT
					11	140	10^3
					12	36	10^1
					15	16	—
					16	7	—
					17	4	—
					21	7	—
5	56	Male	21	Esophageal cancer	Before	17	—
					2	36	—
					4	34	—
					6	192	10^1
					8	172	10^1
					12	84	10^3
					13	85	10^3
					17	49	10^4
					21	28	10^2

* Not tested.

sion, respectively. For Case 5, DNA was extracted from serum at 6 weeks when it was detected for the first time. The three DNA samples were amplified with semi-nested PCR, and sequences of 396 bp were determined. The consensus sequence was determined by comparison of three clones from each patient, and compared among the three cases in Fig. 2. For Case 2, the sequence thus obtained and that determined by representational difference analysis were the same within 396 bp in comparison. Although the three consensus sequences revealed a high homology, they were not identical. The sequence of Case 4 resembled that of Case 2, differing in only 3 (0.8%) nucleotides. By contrast, the sequence of Case 5 differed from those of Cases 2 and 4 in 46–47 (12.9–13.2%) nucleotides. When a sequence of 118 amino acids was compared, they differed in 9– 10 (8%), also. By contrast, there were only 3 (2.5%) substitutions of amino acids between the sequences of Case 2 and Case 4. Based on such an extent of sequence diversity, there would be distinct genotypes of TTV.

DISCUSSION

Using representational difference analysis (11), a viral clone (N22) with 500 nucleotides was identified that developed in the circulation of a patient after he received transfusion at an operation. Primers were deduced from the sequence for detecting it by PCR. The sequence in serum from the patient banded at 1.26 g/cm^3 in sucrose density fractionation, thereby indicating its association with viral particles. Although nucleic acids with the N22 sequence in viral particles were resistant to digestion with DNase I, those extracted from serum were susceptible to it. The DNA sequence did not have a high homology to any of those deposited in data bank, and was not amplified on genomic DNA. By PCR with specific primers, the sequence was found

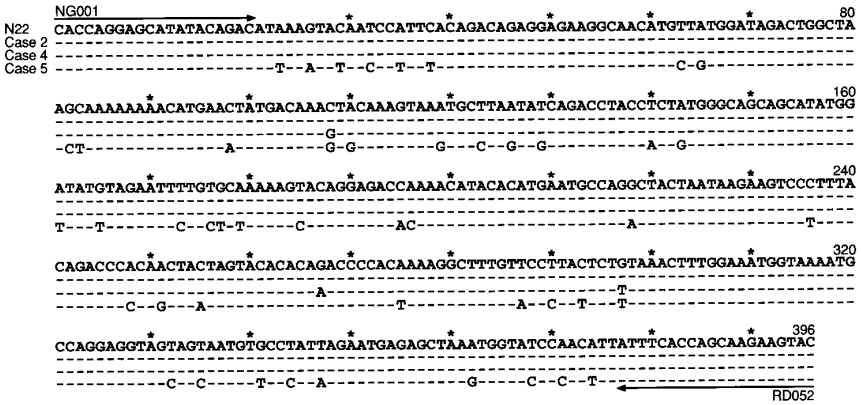

FIG. 2. Comparison of nucleotide sequences of TT virus (TTV) DNA derived from three cases of non-A to G posttransfusion hepatitis. The sequence of N22 clone obtained by representational difference analysis of Case 2 is indicated above. Other sequences are the representative of three clones each recovered from sera of the three cases.

in three patients with non-A to G posttransfusion hepatitis with titers in a close association with ALT levels. Hence, the sequence would originate in a novel DNA virus, which would be associated with non-A to G hepatitis. The DNA virus with the N22 sequence was designated TT virus (TTV) after the patient (Case 2) from whom it was derived. By a pure coincidence, TTV stands for a "transfusion-transmitted virus", also.

TTV infected the recipients of transfusions, either transiently or persistently, in association with elevated ALT levels. It would have to be evaluated if TTV replicates in the liver, and induces hepatitis in an extended cases of posttransfusion non-A to G hepatitis as well as in the patients with cryptogenic hepatitis/cirrhosis. Also, the epidemiology of TTV infection needs to be surveyed in the general population and patients with various liver diseases. These studies are required for incriminating TTV in non-A to G hepatitis, which are feasible now by PCR with primers specific for it. Experimental transmission of TTV to primates for evaluating hepatitis-inducing capacity would be mandatory, also. Such efforts would be worthwhile at present, as a role of HGV in inducing non-A to E hepatitis has not evolved as it was initially hoped for (9, 10).

Evidence for at least two viruses, responsible for non-A, non-B hepatitis, has been reported repeatedly in experimental transmission studies in chimpanzees (16-18). Bradley et al. (18) found two kinds of hepatitis agents, one of which is susceptible to treatment with chloroform while the other not. The chloroform-sensitive agent was deduced to be covered with viral coat (18), and was identified to be HCV later (19). The other, chloroform-resistant agent was suspected to be without an envelope. Although it induces elevated transaminase levels in chimpanzees (18), the hepatitis-inducing capacity in human beings is not verified, because there are no reliable methods for the detection.

Many groups of investigators have reported viral particles with a diameter of 25–30 nm by immune electron microscopy in sera from chimpanzees and marmosets experimentally infected with putative non-A, non-B hepatitis agents (16, 17, 20-23). The relevance of such viral particles with human hepatitis has not been confirmed. As antibodies to TTV are presumed to be raised by the host, probably responsible for a transient infection in two cases reported here, they would be used to detect any such particles in patients and stored chimpanzee sera.

In addition to HBV, some DNA viruses have been implicated in human hepatitis. One of them possesses DNA of 5 kilobases long (DDBJ/GenBank/EMBL databases, accession no. X53411), and has been implicated in sporadic non-A, non-B hepatitis among German patient (24); the sequence of N22 clone does not show any homology to it, however.

For a further characterization of TTV, the full genomic sequence should be determined. Even within a short sequence of TTV, however, considerable diversity was found among DNA clones from three cases of posttransfusion non-A to G hepatitis. Hence, TTV would have distinct genotypes like those of HCV (25) and HGV (26).

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