Detection, Semiquantitation, and Genetic Variation in Hepatitis C Virus Sequences Amplified from the Plasma of Blood Donors with Elevated Alanine Aminotransferase

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Abstract

Hepatitis C virus (HCV) is the predominant etiologic agent of posttransfusion non-A, non-B hepatitis, characterized by undulating elevation of alanine aminotransferase (ALT) and chronic liver disease. A commercial enzyme-linked immunosorbent assay detected antibodies to HCV (anti-HCV) in 11 specimens among 101 nontransfusable plasma units obtained from asymptomatic, volunteer blood donors with elevated levels of ALT. Using a combined reverse-transcription polymerase chain reaction (RT-PCR) assay developed by us, HCV RNA was detected in 0.6 ml of plasma from 8 of 11 (73%) of the anti-HCV-positive but in none of the 90 anti-HCV-negative specimens. The relatively low concentration of HCV RNA could be detected in the remaining three anti-HCV-positive specimens when 2.4 ml of plasma was analyzed. The plasma concentration of virions was estimated to range from 10^2 to 5 $\times 10^7$/ml. Direct sequencing performed on the PCR-amplified HCV cDNAs (210 base pairs) from three specimens revealed heterogeneity between 2.5 and 8.6% at the nucleotide level and < 4% at the amino acid level. Our findings demonstrate that RT-PCR can be performed with 2.4 ml of plasma, providing an assay for the direct detection of HCV RNA and confirming the existence of an asymptomatic carrier state for HCV infection in the apparently healthy anti-HCV-positive donors. (*J. Clin. Invest.* 1990, 86:1609-1614.) Key words: anti-hepatitis C virus • hepatitis C virus carriers • hepatitis C virus confirmatory assay • non-A, non-B hepatitis • polymerase chain reaction

Introduction

Non-A, non-B hepatitis (NANBH)[1] accounts for > 90% of posttransfusion hepatitis cases, with an estimated incidence of 1-10% after blood transfusions in the United States (1). Approximately 50% of infected transfusion recipients develop biochemical evidence of chronic hepatitis, and ~ 20% of recipients with chronic hepatitis subsequently develop histologic evidence of liver cirrhosis (2). Although NANBH was first described in association with blood transfusions, surveillance data from the Centers for Disease Control showed that only 5-10% of patients with NANBH have a history of blood transfusion (3).

A cDNA of the RNA genome of hepatitis C virus (HCV) has recently been cloned from an infected chimpanzee (CH-HCV) which developed NANBH after inoculation with a contaminated human factor VIII concentrate (4). Studies on the genome of HCV revealed positive-stranded RNA of ~ 10,000 nucleotides with distant homology to the Flaviviridae (4). Expression of a recombinant HCV fusion protein in yeast led to the development of immunoassays for antibodies to HCV (anti-HCV) in plasma and serum (5). Seroepidemiologic studies in the United States, Japan, and Europe revealed that 80-100% of patients with chronic posttransfusion NANBH have detectable levels of circulating anti-HCV (5-9). These data support the identification of HCV as the predominant agent of chronic posttransfusion NANBH worldwide (10). However, the frequency of detectable anti-HCV in acute resolving NANBH infections was much lower, ranging from 15 to 60% with a mean delay to seroconversion of almost 6 mo after transfusion (3, 10). These data emphasize the need for sensitive, direct assays for viral markers. In view of the expected low titers of HCV antigens in infected individuals, antigen assays are likely to have inherent limitations of sensitivity. Therefore, we employed reverse transcription (RT) in combination with polymerase chain reaction amplification (RT-PCR) as a sensitive direct assay for the detection of HCV RNA in plasma.

We report here the prevalence of HCV RNA in plasma from asymptomatic, volunteer blood donors with elevated alanine aminotransferase (ALT), and its correlation with the presence of anti-HCV, hepatitis B surface antigen (HBsAg), and antibodies to hepatitis B core antigen (anti-HBc). The titers of HCV cDNA were determined by serial dilution endpoint titration and PCR detection to obtain quantitative information about the virus load in infected individuals. Amplified HCV cDNAs from the three plasma specimens with the highest virus titer were directly sequenced and compared with the CH-HCV cDNA sequence to determine the degree of genome heterogeneity among different HCV isolates.

Methods

Study population. Aliquots of plasma units from 101 asymptomatic, volunteer blood donors, not suitable for transfusion, were obtained from Irwin Memorial Blood Centers, San Francisco, CA. These units were collected between 17 May and 28 September 1989 and were

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1. *Abbreviations used in this paper:* ALT, alanine aminotransferase; anti-HCV, antibodies to HCV; CH-HCV, chimpanzee-derived HCV; HBc, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV and HCV, hepatitis B and C viruses; M-MLV, Moloney murine leukemia virus; NANBH, non-A, non-B hepatitis; PCR, polymerase chain reaction; RIBA, recombinant immunoblot assay; RT, reverse transcription.

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rejected owing to elevated ALT levels ranging from 68 to > 700 IU/liter (reference range from 2 to 54 IU/liter). During this period, Irwin Memorial Blood Centers collected a total of 30,184 donor units, of which a total of 198 were rejected because of elevated ALT. We failed to obtain the remaining 97 ALT-elevated units during this period owing to logistical problems in the collection process. The plasma units were stored at 4°C until use.

Serological tests. All plasma units were tested for HBsAg and anti-HBc with commercially available reagent kits (Auszyme and Corzyme, Abbott Laboratories, North Chicago, IL). Tests for antibodies to HCV were performed using the HCV antibody ELISA test system (Ortho Diagnostic Systems, Raritan, NJ) according to the manufacturer's instructions. The mean absorbance value of two positive controls was 1.860 OD units, of three negative controls 0.033, and the cutoff was set at 0.433 (0.400 units above the mean negative control OD). Specimens positive for anti-HCV by ELISA were tested with the anti-HCV recombinant immunoblot assay (RIBA, Ortho Diagnostic Systems). Specimens reactive with C100-3 and 5-1-1 antigens were scored positive, and specimens reactive with only one antigen were scored indeterminate, according to the manufacturer's instructions.

Preparation of HBV RNA. The complete HBV (adw) genome was cloned into the transcription vector pTZ18U (Bio-Rad Laboratories, Richmond, CA) using standard methods (11, 12). Before in vitro transcription of the HBV template by T7 RNA polymerase the plasmid was linearized with Hind III. After transcription, the HBV DNA template was digested with RNase-free DNase (RQ1 DNase, Promega Biotek, Madison, WI) according to the manufacturer's instructions, followed by sequential extraction of the HBV RNA with phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1) and chromatography on a Sephacryl S300 spun column (Pharmacia-LKB Biotechnology, Piscataway, NJ). The purified RNA was quantitated by spectrophotometry considering 1 OD_{260} to be equivalent to 40 μg/ml of HBV RNA or 6×10^5 molecules/pg. The integrity of the HBV RNA was evaluated by Northern blot hybridization as described by Ausubel et al. (12) and showed predominantly full-length transcripts.

Preparation of HCV RNA and RT into cDNA. Plasma samples (3 ml) were diluted 1:5 in serum dilution buffer (0.1 M NaCl, 0.05 M Tris-HCl pH 8.0, 1 mM EDTA) and the debris was sedimented by centrifugation at 3000 *g* for 30 min at room temperature. The supernatants were transferred to sealable ultracentrifuge tubes and centrifuged for 2 h at 46,000 rpm (model 50 Ti rotor, Beckman Instruments, Inc., Fullerton, CA; RCF_{min} = 87,000 *g*; RCF_{max} = 191,000 *g*) in order to pellet particles of ≥ 46 Svedberg units. Plasma supernatants were discarded and the pellets were solubilized in 0.8 ml of the following buffer (8 M urea, 2% SDS, 0.15 M NaCl, 0.1 M Tris-HCl pH 7.5, 1 mM EDTA) containing 1 μg/ml carrier HBV RNA. The buffer solutions were transferred to 1.7-ml microcentrifuge tubes and extracted three times with phenol/chloroform/isoamyl alcohol; nucleic acids were precipitated from the aqueous phases with 2.5 vol of ethanol. The RNA pellets individually prepared from 101 plasma specimens were resuspended in 0.3 M sodium acetate, pH 5.2. One-fifth of each RNA specimen was used to prepare pools consisting of three or four RNA specimens. After a second precipitation with ethanol, the pooled RNA pellets were resuspended in diethyl pyrocarbonate-treated dH_2O and cDNA was synthesized in a 40-μl vol using random hexamers ($p(dN)_6$, Pharmacia-LKB Biotechnology) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) as described by the manufacturer.

PCR experiments. The cDNA preparations were added to a final volume of 100 μl of amplification mixture containing 2 U of Taq polymerase, 50 pmol of each primer, 200 μM of each dNTP, 10 mM Tris (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, and 200 μg/ml gelatin. Primers for HBV cDNA amplification were PU5(GGGAGGAGATTAGGTTAA) and PU6 (AAGAAGTCAGAAGGCAAA), which amplified an ~ 240-basepair (bp) DNA fragment. The HBV primers annealed to conserved regions upstream and downstream of the precore region and were selected on the basis of DNA sequence alignment for five different human HBV isolates (13). Primers for HCV cDNA were either JR3 (TGCTGACCACTACACAGTGG) and JR4 (GCGTCTGCAAGCAGAAGGAA) or PKL13 (CTGGTCGCATTGGGCATCAA) and PKL14 (CGTGATTGTCTCAATGGTGA) generating a 190- or a 210-bp DNA fragment, respectively. The HCV primers bind to the following nucleotide positions within the published HCV sequence: JR3, 649–668; JR4, 819–838; PKL13, 2871–2890; and PKL14, 3061–3080 (14). The amplification mixtures were overlaid with mineral oil and amplified in a DNA thermal cycler (Perkin-Elmer-Cetus Corp., Norwalk, CT) using a low stringency step-cycle program for 6 cycles (40°C, for 30 s; 72°C for 30 s; 94°C for 30 s), a high stringency step-cycle program for 35 cycles (50°C (for HBV) or 55°C (for HCV) for 30 s; 72°C for 30 s; 94°C for 30 s), followed by a 5-min final extension at 72°C.

Liquid hybridization. This procedure was performed as previously described (15). Individual 30-μl aliquots of amplification reaction mixture were mixed with 10 μl of probe mixture containing 75 mM NaCl, 20 mM EDTA, and 0.2 pmol oligomer probe end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase to a specific activity of ~ 4 μCi/pmol. Probe for amplified HBV DNA was PU7 (GCACCAGCACCATGCAACTT). Probe for JR3-JR4 amplified HCV DNA was JR5.1 (CCACAATGTTCTGGTGGAGG, nucleotide position 725–744) and for PKL13-PKL14 amplified HCV DNA, JR9 (CGGTCATGAGGGCATCGGTT, nucleotide position 2959–2989). After denaturation at 95°C for 5 min, hybridization in solution was carried out for 15 min at 37°C. Hybridized DNA was separated from free probe on an 8% polyacrylamide (acrylamide to bis-acrylamide, 19:1) gel and visualized by autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY). The detection limit was 1 pg of amplified HBV or HCV DNA after 16 h of exposure at −70°C.

Sequencing of amplified DNA. HCV DNA amplified with the primer pair PKL13-PKL14 by the standard PCR procedure was purified by spin dialysis in microconcentrators (Centricon model 100, Amicon Corp. Danvers, MA) (15). Direct sequencing was performed as reported by Wong et al. (16). One amplification primer (2 pmol of PKL13 or PKL14, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase) was annealed to 0.4 pmol of amplified DNA, and sequencing was carried out with Sequenase (United States Biochemicals, Cleveland, OH) according to the manufacturer's instructions.

Results

Serological markers for HBV and HCV in ALT-elevated plasma. 11 of the 101 plasma specimens tested positive for anti-HCV and 4 of the 11 specimens were also positive for anti-HBc. 9 of the 11 anti-HCV ELISA-positive specimens tested positive on the anti-HCV RIBA test, whereas two were indeterminate. Two anti-HCV negative plasmas were positive for anti-HBc, and one of these two was also positive for HBsAg. Table I shows the serological results of the 13 specimens positive for any of the HBV or HCV markers and includes the absorbance values of the HCV antibody ELISA test. The remaining 88 specimens not listed in Table I were negative for anti-HCV, anti-HBc, and HBsAg.

Sensitivity of the RT-PCR amplification procedure. To determine the lower limit of RNA detection in a combined RT-PCR assay, 10-fold serial dilutions of in vitro synthesized HBV RNA were reverse transcribed into HBV cDNA with random primers and M-MLV reverse transcriptase. The cDNA preparations were amplified with the primer pair PU5-PU6 specific for the precore/core gene of HBV. After 41 cycles of amplification the HBV DNA was hybridized in solution to the HBV-specific probe PU7 and then separated by polyacrylamide gel electrophoresis. 10-fold serial dilutions of HBV RNA, from 2×10^6 to 0 molecules, were analyzed. Samples containing an initial quantity of 200 HBV RNA molecules produced hybrid-

Table I. Presence of Serological Markers for HBV and/or HCV in 13 Plasma Units from Asymptomatic Blood Donors with Elevated ALT

Plasma No.	Anti-HBc	HBsAg	Anti-HCV ELISA*	HCV RIBA‡	RT-PCR§
			(A)		
26	+	+	−	NT	−
28	+	−	+(>2.0)	+	+
32	−	−	+(>2.0)	+	++
33	−	−	+(>2.0)	+	++
35	+	−	+(>2.0)	+	+++
36	+	−	+(>2.0)	+	+++
37	−	−	+(>2.0)	IN	+
49	−	−	+(>2.0)	+	+++
60	+	−	−	NT	−
78	−	−	+(0.8)	+	+
86	−	−	+(>2.0)	+	++
88	−	−	+(1.6)	IN	(++)
100	+	−	+(>2.0)	+	++

* Absorbance value: cutoff, 0.433.
‡ +, positive; IN, indeterminate; NT, not tested.
§ −, negative; +, weak positive; ++, strong positive; +++, very strong positive with the primers JR3-JR4 and PKL13-PKL14; (++) positive only with PKL13-PKL-4.

ization signals of the expected size (~ 240 bp). The dilutions theoretically containing 20, 2, and 0 molecules of HBV RNA were negative. All HBV RNA dilutions were also amplified for 41 cycles without the RT step and were negative by liquid hybridization, demonstrating that the signal obtained was not generated from undigested HBV DNA used for in vitro transcription.

Detection of HCV RNA. Pools of three or four RNA specimens, each obtained from 0.6 ml of plasma, were subjected to RT using random primers and then amplified by PCR using either the HCV-specific primer pair JR3-JR4 or PKL13-PKL14. Of the 29 pools generated, three were strongly positive for HCV: pool 11 (containing specimens 31, 32, and 33), pool 12 (containing specimens 34, 35, and 36) and pool D (containing specimens 49, 50, 51, and 52); two pools were weakly positive: pool M (containing specimens 86, 87, 88, and 89), and pool P (containing specimens 97, 98, 99, and 100). In order to identify the source of the HCV RNA, individual RNA specimens (from 0.6 ml of plasma), which had been used to prepare the PCR-positive pools, were similarly reverse transcribed and analyzed by PCR. HCV sequences were detected with both primer pairs in the anti-HCV-positive specimens 32, 33, 35, 36, 49, 86, and 100; specimen 88 was positive only with PKL13-PKL14, and the remaining anti-HCV-negative specimens used in the pools were negative with both primer pairs. Considering the detection limit of the RT-PCR procedure, we assume that the HCV-PCR-positive RNA specimens contain > 200 target molecules and that RNA specimens with fewer targets would be negative. To determine whether the remaining anti-HCV-positive specimens 28, 37, and 78 harbor HCV in lower concentrations, RNA derived from 2.4 ml of plasma was reverse transcribed and amplified; PCR for these specimens was positive with both HCV-specific primer pairs. These results indicate a strong correlation between the presence of circulating antibodies to the recombinant HCV antigen C100-3, as tested by the anti-HCV ELISA, and the presence of HCV RNA in ALT-elevated plasma from asymptomatic blood donors.

Titration of HCV cDNA preparations. Because we found a significant difference in the signal intensity of the RT-PCR assay for the different specimens, we prepared fourfold serial dilutions of individual cDNA preparations (40 μl) obtained from a 0.6-ml aliquot of plasma. The cDNA preparations of specimens 32, 33, 86, 88, and 100 showed no PCR signal when diluted (1:4), suggesting that these specimens harbored $\geq$ 200 molecules of detectable HCV RNA in 0.6 ml of plasma, but < 200 molecules (detection limit) HCV RNA in 0.15 ml of plasma (Fig. 1 *A*). Assuming quantitative recovery of HCV virion RNA by ultracentrifugation and buffer treatment, we calculated that the virus concentration in these specimens is $\geq 3.3 \times 10^2$ and $< 1.3 \times 10^3$ virions/ml of plasma. The cDNA preparations of the strongly positive specimens 35 (Fig. 1 *B*), 36 (Fig. 1 *C*), and 49 (data not shown), on the other hand, had endpoint titers of 40,960, 10,240, and 10,240, respectively. With the same assumptions, we calculated viral titers between ≥ 1.3 and $< 5.5 \times 10^7$ virions/ml for specimen 35 and between $\geq 3.4 \times 10^6$ and $< 1.3 \times 10^7$ virons/ml for specimen 36 and 49. Plasma specimens 28, 37, and 78 have a calculated virus titer between $\geq$ 80 and < 330 virions/ml.

Sequence analysis of HCV cDNA. We sequenced PKL13-PKL14 amplified HCV cDNAs from the high-titer plasmas 35, 36, and 49 to determine the degree of heterogeneity between different human isolates of HCV in this particular region of the genome. Because most populations of RNA viruses do not exist as a single genome species with a unique sequence, but rather as a heterogeneous mixture of related genomes, we per-

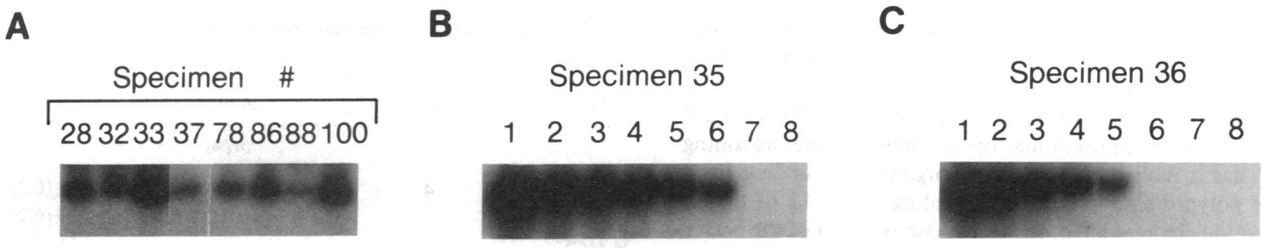

Figure 1. Autoradiogram of PKL13-PKL14–amplified HCV cDNA hybridized to the oligonucleotide probe JR9. (*A*) RNA from an equivalent of 0.6 ml of plasma (specimens 32, 33, 86, 88, and 100), and from an equivalent of 2.4 ml of plasma (specimens 28, 37, and 78) was used. (*B* and *C*) Serial fourfold dilutions of cDNA prepared from RNA from an equivalent 0.6 ml of plasma of (*B*) specimen 35 and (*C*) specimen 36. Lane *1*, 1:40; lane *2*, 1:160; . . . lane *8*, 1:655,360.

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        2895                                                    2945
CH-HCV: GTGGCCTACTA CCGCGGTCTT GACGTGTCCG TCATCCCGAC CAGCGGCGA
35-HCV: GTGGCCTACTA CCGCGGTCTT GACGTGTCCG TCATCCCGAC CAGTGGCGA
36-HCV: GTGGCCTACTA CCGCGGCCTT GACGTGTCGG TCATCCCGAC CAGTGGCGA
49-HCV: GTGGCCTACTA CCGCGGTCTT GACGTGTCTG TCATCCCGGC CAGCGGCGA
                                                        2995
CH-HCV: TGTTGTCGTC GTGGCAACCG ATGCCCTCAT GACCGGCTAT ACCGGCGACT
35-HCV: TGTTGTCGTC GTGGCAACTG ATGCCCTCAT GACCGGCTAT ACCGGCGACT
36-HCV: TGTTGTCGTA GTGGCAACTG ATGCTCTCAT GTCCGGCTTT ACCGGCGACT
49-HCV: TGTTGTCGTA GTGGCAACTG ATGCTCTCAT GACCGGCTTT ACCGGCGATT
                                                        3045
CH-HCV: TCGACTCGGT GATAGACTGC AATACGTGTG TCACCCAGAC AGTCGATTTC
35-HCV: TCGACTCGGT GATAGACTGC AACACGTGTG TCACCCAGAC AGTCGACTTC
36-HCV: TTGACTCGGT GATAGACTGC AACACGTGTG TCACCCAGAC GGTCGACTTT
49-HCV: TCGACTCGGT GATAGACTGC AACACGTGTG TCACCCAGAC GGTCGATTTC
                  3056
CH-HCV: AGCCTTGACC CT
35-HCV: AGCCTTGACC CT
36-HCV: AGCCTTGACC CC
49-HCV: AGCCTTGATC CT
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Figure 2. Nucleotide sequence alignment of the chimpanzee-derived (*CH-HCV*) and three human (35, 36, and 49) HCV isolates. The sequences flanked by the primer pair PKL13-PKL14 are illustrated. Nucleotide numbering is according to the CH-HCV sequence and nucleotides different from CH-HCV are marked (*). The translational reading frame starts with the first nucleotide (2895) in these sequences and the underlined codons represent mutations affecting the predicted amino acid substitutions. These sequence data are available from EMBL/Gen Bank/DDBJ under accession number M55151.

formed direct sequencing of amplified HCV cDNA to obtain the sequence of the most abundant HCV genome present in each of the three high-titer plasmas (17). Sequencing of the cDNA (+) strand was carried out by annealing the upstream amplification primer PKL13 to PKL13-PKL14-amplified HCV cDNA. For sequencing of the cDNA (−) strand, we annealed the downstream amplification primer PKL14 to independently reverse-transcribed and amplified PKL13-PKL14 cDNA. A total of 162 nucleotides (exclusive of primers) could be read on each strand. No nucleotide differences were observed, when (+) strand and corresponding (−) strand of any individual isolate were compared. This excludes the possibility that the observed point mutations are artifacts of RT, PCR, or sequencing. The nucleotide sequences (162 bp) of the HCV isolates from specimens 35, 36, and 49 as well as the published nucleotide sequence of the chimpanzee-derived HCV cDNA are aligned in Fig. 2. The observed point mutations occurred at 17 positions in the 162-nucleotide sequence of the HCV genome. The genetic heterogeneity between the different isolates ranged from 2.5% between CH-HCV and 35-HCV to 8.6% between CH-HCV and 35-HCV (Table II). In all four HCV sequences only one reading frame is continuous. This should therefore be the translational open reading frame, assuming HCV has a flavivirus-like genetic organization coding for a single polyprotein. In this open reading frame 13 of the 17 nucleotide changes affect the third base position of the codon and do not change the amino acid sequence. Four conservative amino acid changes were observed (underlined codons in Fig. 2): two point mutations occurred in the first base position resulting in amino acid changes from threonine (ACC) to alanine (GCC) or serine (TCC), and the other two point mutations occurred in the second position of the codon changing tyrosine (TAT) to phenylalanine (TTT). From the deduced amino acid sequence we calculated a heterogeneity of ≤ 4% between the four HCV isolates. Therefore, the sequences of the chimpanzee-derived and the three human isolates are highly homologous in this region of the HCV genome both at the nucleotide sequence level and at the level of the predicted amino acid sequences.

Discussion

Antibody testing establishes whether an individual has responded immunologically to exposure to viral antigens, but does not distinguish whether the infectious agent was eliminated by the host's immune response or continues to persist in

Table II. Nucleotide Heterogeneity between the Four Sequences from Fig. 2

Virus	35-HCV	36-HCV	49-HCV
		n (%)	
CH-HCV	4 (2.5)	14 (8.6)	10 (6.2)
35-HCV	—	10 (6.2)	10 (6.2)
36-HCV	—	—	11 (6.8)

Data in the table indicate the number of different nucleotides out of 162; values in parentheses express the difference in percent.

the host. Sequential serum samples from prospectively followed patients with HCV-NANBH indicate a prolonged window period for seroconversion (10). This prolonged delay in antibody response suggests that some blood donors capable of transmitting HCV will not be detected by anti-HCV testing. A direct assay for HCV antigens or nucleic acids could be useful for the detection of HCV infection prior to seroconversion. Such an assay might also be useful to distinguish infected from immune individuals. Therefore, we have developed a combined RT-PCR amplification assay for the detection of HCV RNA isolated from plasma. The HCV RNA detected by this assay must be derived from virus particles, not only because of the ultracentrifugation step, but also because of the presence of high levels of RNase activity in plasma (data not shown) making it impossible for RNA to exist freely in plasma.

A strong correlation between the presence of anti-HCV and HCV RNA in plasma of asymptomatic blood donors was observed. All 11 anti-HCV ELISA-positive donors were positive for HCV RNA with our RT-PCR assay using two HCV-specific primer pairs in independent amplifications. The two primer pairs showed congruent results except for plasma 88, which was only positive with PKL13-PKL14. Based on the HCV genome analysis and its comparison to flaviviral genome organization (14, 18), the primer pair PKL13-PKL14 anneals within the putative NS3 gene of HCV. This gene has been shown to be relatively conserved among the flaviviruses. The primer pair JR3-JR4 anneals to a less defined region of the HCV genome, which may correspond to the NS2 region of the flaviviruses, a region known to display very high rates of sequence divergence (18). It is possible that mutations in the JR3-JR4 binding sites in this particular isolate interfered with efficient amplification, thus making minute amounts of HCV RNA undetectable. We also note that our negative RT-PCR results on the 90 anti-HCV-negative specimens derive from assays performed on RNA from an equivalent of 0.6 ml of plasma. These results are not, strictly speaking, comparable to the positive RT-PCR results on the three lowest-titer specimens (Nos. 28, 37, and 78), since the three positive RT-PCR results were obtained only when the assay was performed on RNA from 2.4 ml of plasma. Our data do not rule out the possibility that some of the 90 ALT-elevated anti-HCV-negative specimens we studied might harbor HCV at a titer too low for detection in 0.6 ml with our current RT-PCR protocol. However, the strong correlation of our positive RT-PCR results with the anti-HCV results suggests most or all antibody-positive plasma specimens will contain viral RNA detectable by RT-PCR, and as such are likely to be infectious.

After the PCR results were obtained, the anti-HCV RIBA test became available as a proposed confirmatory test for anti-HCV reactivity. We found that two of the 11 anti-HCV ELISA-positive specimens were indeterminate on the RIBA test. Because all 11 specimens were HCV PCR positive, apparently the manufacturer's definition of an indeterminate RIBA result (reactive with only one HCV antigen) may be too stringent.

Interestingly, in a recent report by Weiner et al. (19) seven out of nine anti-HCV positive chronic NANBH patients had HCV RNA in their livers, but the remaining two were HCV PCR negative. This might reflect biological differences between patients with chronic NANBH and asymptomatic carriers of HCV. However, it is also possible that HCV RNA was present in these two livers, but not detected by the RT-PCR assay employed. Explanations could include: (*a*) the primers used by Weiner et al. (19) were relatively short (16-mers), suggesting less efficient specific amplification of HCV cDNA, and in addition the sense primer contained one mismatch (A-G) on comparison to the published CH-HCV sequence at the extreme 5′ end and another mismatch (T-C) on comparison to the sequences from our three human isolates; or (*b*) amplification was carried out by Weiner et al. (19) for only 30 cycles, even though cDNA amplification is only 35–55% efficient in comparison to 67–90% efficiency for DNA amplification (20, 21).

Because of the absence of specific viral markers in the past, quantitation of viral load in NANB/HCV infections could only be performed by dilutional endpoint infectivity testing in chimpanzees (22), a difficult and expensive procedure. Most investigators have found that human and chimpanzee inocula generally have titers between 10^2 and 10^4 chimpanzee infectious doses/ml, although titers of up to 10^6 have been reported (22). We used the dilutional endpoint titers of HCV cDNA detection by PCR to calculate the concentration of HCV virions and found between 10^2 and 5×10^7 virions/ml in the different plasma specimens. In interpreting these data it must be remembered that RT-PCR is at best semiquantitative, even when employed in careful dilutional endpoint titration studies such as ours. In order to calibrate our HCV RT-PCR assay we used cloned HBV RNA; we note that slight differences in RT efficiency and PCR amplification efficiency could have led to differences in final sensitivity of HBV and HCV detection. Furthermore, in view of the high mutation rate characteristic of RNA viruses, RT-PCR performed on field isolates of HCV is also subject to the problem of sequence variation at the primer binding sites which may lead to differential PCR amplification efficiencies. Therefore, our HCV titration figures must be interpreted with caution; we estimate that their margin of error may be as great as plus or minus an order of magnitude. However, we also note that these figures are consistent with those obtained by chimpanzee inoculation, even though RT-PCR does not measure infectivity and nothing is known yet about the possible existence of defective HCV particles.

For a sensitive, specific, and reliable RT-PCR assay, it is important to select primer binding sites which are conserved among different human HCV isolates. The primer pair PKL13-PKL14 allowed efficient amplification of all HCV cDNA preparations tested so far and therefore we investigated the nucleotide heterogeneity in this particular region of the HCV genome. The sequences of the CH-HCV and the three human HCV isolates are highly homologous, both at the nucleotide level and at the predicted amino acid level, in the region between these two primers.

We conclude that our RT-PCR assay for the detection of HCV sequences in plasma could be useful as a confirmatory test for the diagnosis of HCV infection. It may also provide answers to important questions concerning the natural history of HCV infection, such as frequency and duration of the asymptomatic HCV carrier state.

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