No Significant Changes in Levels of Hepatitis C Virus (HCV) RNA by Competitive Polymerase Chain Reaction in Blood Samples from Patients with Chronic HCV Infection

ERIKO YOSHIMURA, BS, JUN HAYASHI, MD, PhD, KUMIKO UENO, MD, YASUHIRO KISHIHARA, MD, KOZABURO YAMAJI, MD, YOSHITAO ETOH, BS, and SEIZABURO KASHIWAGI, MD, PhD

To determine if levels of hepatitis C virus (HCV) RNA change over a several-year period, we quantified the amount of HCV RNA by competitive polymerase chain reaction. The population studied included 44 residents of a rural area with chronic HCV infection, 39 had chronic hepatitis C and 37 were patients on hemodialysis. All these Japanese patients had HCV RNA of genotype II. Blood samples were collected once a year from 1992 to 1995. From 1993 to 1995 between the groups, there was no significant difference in change of HCV RNA levels of 44 residents with chronic HCV infection, with and without liver dysfunction, nor was there any change in the 31 hemodialysis patients from 1992 to 1995. The HCV RNA levels in the 25 with chronic hepatitis who did not respond to interferon-α during 1992–1993 returned to pretreatment levels after the cessation of interferon treatment. In two of six hemodialysis patients who were infected with HCV during this observation period, HCV RNA was eliminated within one year, and the remaining four became HCV carriers. HCV RNA levels in the latter rose rapidly after infection and were sustained at a high level throughout the study period. Thus, HCV RNA level did not change remarkably during a three-year period, a finding which supports that it does not correlate with deterioration of liver damage and aging of HCV carriers.

**KEY WORDS:** chronic hepatitis C; hepatitis C virus RNA levels; competitive polymerase chain reaction; interferon; hemodialysis.
our comparison of the HCV RNA levels of patients at various stages of liver disease, based on genotype; the HCV RNA level strongly depends on HCV RNA genotype (11). Correlations between HCV RNA levels and the duration of HCV infection are also controversial. Because our study and those of other workers were cross-sectional surveys and may have included bias, a cohort study was necessary for resolution of the discrepancy.

To observe changes in HCV RNA levels in patients with chronic HCV infection, we used competitive PCR and examined serial serum samples from residents of a rural area of Japan with chronic HCV infection, patients with chronic hepatitis C, and hemodialysis patients with chronic HCV infection.

**MATERIALS AND METHODS**

**Population Studied.** The 106 Japanese patients with chronic HCV infection whom we studied included 44 residents of "H village" of Fukuoka Prefecture, Japan, an endemic area of HCV infection (12), 39 with chronic hepatitis seen at Kyushu University Hospital, and 37 hemodialysis patients treated at three units in Fukuoka (13, 14). All were positive for antibody to HCV, had HCV RNA of genotype II, and were negative for both hepatitis B surface antigen and antibody to human immunodeficiency virus.

Of the 44 residents, 15 had normal levels of serum alanine aminotransferase (ALT) for at least eight years and the remaining 29 had elevated levels of ALT for at least eight years. Of 39 patients with chronic hepatitis C, 25 were treated with natural interferon-α for six months (in 13 patients HCV RNA was eliminated by the end of treatment but relapse occurred within six months, and in the other 12, HCV RNA was never eliminated during treatment). The remaining 14 were not treated with either antiviral or immunosuppressive agents. No subject was alcoholic, a drug addict, or had evidence of autoimmune liver disease.

Serum samples were taken during 1992 to 1995 and stored at −20°C and were tested after the first thawing. The level of HCV RNA was measured at the end of interferon treatment and at 6- and 12-month follow-ups in the 25 patients treated with interferon and once a year in the remaining 81 patients with chronic HCV infection.

To clarify the influence of repeated freezing and thawing on serum samples stored for quantitation of the HCV RNA level, we measured the HCV RNA level in some serum samples that had been frozen and thawed 1, 5, 10, and 20 times, by both competitive PCR and branched DNA probe assay.

**Assay Methods.** Conventional liver function tests were done using a multiple autoanalyzer. A level of ALT above 36 IU/liter was regarded as abnormal. Anti-HCV (HCV EIA II, Abbott Laboratories, North Chicago, Illinois) was examined using enzyme-linked immunosorbent assay. HCV RNA was detected by two-stage PCR, using primers from the 5'-noncoding region of the HCV genome (1): 5'-CTGTGAGGAACACTGTCCTTT-3' (sense; nt 28–47) and 5'-CCTCAGCGTCTAGCAGT-3' (antisense; nt 229–248) in the first stage and 5'-TTCACGCA-GAAGGCTCTAG-3' (antisense; nt 171–190) in the second stage.

The HCV genotype was determined by two-stage PCR using universal and type-specific primers from the putative C gene of the HCV genome, according to the method of Okamoto et al (16).

Competitive RT-PCR assay was performed as follows (17). For synthesis of complementary DNA and its amplification, we prepared a set of “outer” and “inner” primers located in the 5'-noncoding region of the HCV genome. To synthesize HCV cDNA, we incubated a 100-μl reaction mixture containing an unknown concentration of HCV RNA from 50 μl of serum, a known concentration of mutant HCV RNA, 1 μmol/liter of each of the “outer” primers, KC liter 50 mmol/liter, Tris HCl 20 mmol/liter (pH 8.4), MgCl 2.5 mmol, deoxyribonucleoside triphosphate 200 μmol/liter, ribonuclease inhibitor 1 unit/μl (Takara Shuzo, Kyoto, Japan), and 15 units of reverse transcriptase (Takara Shuzo) at 42°C for 20 min.

After addition of 2.5 units of recombinant Thermus aquatics DNA polymerase (Ampli Taq DNA polymerase, Perkin-Elmer Cetus, Norwalk, Connecticut), the reaction mixture was overlaid with 100 μl mineral oil. After 1 min of denaturation at 95°C, the substrate cDNA was heated at 95°C for 1 min (denaturation), cooled to 50°C for 1 min (annealing), and heated to 70°C for 1 min (elongation). These steps were repeated for 30 cycles. Two microliters of the first-round PCR product were transferred to the second-round 98-μl reaction mixture containing 1 μmol/liter of each of the “inner” primers. The second-round PCR was performed under the same regimen as that of the first-round. After the final step of the second amplification, 8 μl of each sample was mixed with 1 μl of NaCl (500 mmol/liter) and eight units of EcoRI (Takara Shuzo), and the mixture applied to an 8% acrylamide gel. The RT-PCR product of HCV RNA obtained from serum was observed at 93 bp, by ethidium bromide staining. The RT-PCR product of mutant RNA was digested with EcoRI and was observed at the 56 bp and 37 bp.

The branched DNA assay was performed according to the manufacturer's instructions (Quantiplex HCV-RNA, Chiron Corporation, Emeryville, California) (2, 3). Brieﬂy, duplicate 50-μl serum samples were added to wells of a 96-well plate. Lysis and inhibition of RNases were carried out by adding a buffer containing proteinase K and detergent. The buffer also contained two sets of oligonucleotide probes: one set mediates capture of the target nucleic acid to capture probes on the microwell and another set binds the target to amplify molecules. After an overnight incubation at 63°C and washing, multiple synthetic branched DNA molecules were added. Conjugated alkaline phosphatase probes were then annealed to the immobilized complex, resulting in signal amplification when the chemiluminescent substrate (dioxetane) was added. The visible light output was measured using a luminometer. Quantification of HCV RNA was determined from a standard curve. The range of linear relationship provided by the branched DNA assay is 0.35 to 57 Meq/ml.

**Statistical Analysis.** Wilcoxon signed rank test and t test were used to determine the P value. P ≤ 0.05 was considered statistically significant.