Nucleotide Sequence Diversity of Hypervariable Region 1 of Hepatitis C Virus in Japanese Hemophiliacs With Chronic Hepatitis C and Patients With Chronic Posttransfusion Hepatitis C

By Hidenori Toyoda, Yoshishide Fukuda, Yasuo Koyama, Isao Nakano, Moritoshi Kinoshita, Toru Hadama, Junki Takamatsu, and Tetsuo Hayakawa

Hemophilic patients with chronic hepatitis C might be exposed to and become infected with multiple hepatitis C virus (HCV) strains by means of frequent use of blood products, even if they are infected with a single subtype of HCV. To test this hypothesis, we analyzed the genetic diversity of hypervariable region 1 (HVR1) of HCV in chronically infected hemophiliacs and in patients with chronic posttransfusion hepatitis with a single HCV inoculation. The diversity of nucleotide sequences in HVR1 of serum HCV RNA was compared between 21 hemophiliacs infected with a single HCV subtype and 16 patients with posttransfusion HCV infection. The number of HCV quasispecies was determined by fluorescence single-strand conformation polymorphism (SSCP) analysis. Direct sequencing was performed to determine the diversity in HVR1. The number of HCV quasispecies in the

HEPATITIS C VIRUS (HCV) was molecularly cloned in 1989 and subsequently implicated in the majority of cases of posttransfusion hepatitis. With further study, Farci et al. and Prince et al. have indicated the lack of protective immunity against reinfection with HCV, and superinfection with HCV of different subtypes has been reported in humans and chimpanzees.

Hemophiliac patients may have been frequently exposed to HCV because of blood-product requirements, and consequently, the prevalence of HCV infection in Japanese hemophiliacs is greater than that in healthy blood donors. Hemophiliacs with chronic hepatitis C therefore can be assumed to have received multiple inocula of HCV, and some have been shown to be infected with mixed subtypes. Furthermore, even patients with a single HCV subtype may still have had multiple exposures to HCV. We compared the sequence diversity of hypervariable region 1 (HVR1) in the E2/NS1 gene of serum HCV particles in hemophiliacs versus patients with chronic posttransfusion hepatitis who had been confirmed to have incurred only a single HCV inoculation.

SUBJECTS AND METHODS

Patients. Twenty-three Japanese patients with hemophilia or other coagulation disorders and documented HCV infection and 17 Japanese patients with chronic posttransfusion hepatitis C were studied. Hemophilic patients were selected from 195 HCV-infected hemophiliacs under follow-up study at Nagoya University Hospital as outpatients, and met the following criteria: (1) liver function had been proved to be normal before the first use of blood product (coagulation factor), and (2) they had a history of use of more than 1,000 U blood product at least once per week for longer than 3 years before 1989, although the total dose was unknown. Patients with chronic posttransfusion hepatitis C were selected from 72 patients with chronic hepatitis C with a history of blood transfusion under follow-up evaluation at the same hospital as the outpatients, and met the following criteria: (1) liver function had been proved to be normal before the blood transfusion, (2) they had a history of acute hepatitis following the blood transfusion, and (3) they had no history of risk for reexposure to HCV, such as repeated blood transfusions, surgery, drug abuse, acupuncture, or tattoo, after blood transfusion.

Patients in both groups were all infected with a single HCV subtype—patients infected with mixed HCV subtypes were excluded. Asymptomatic HCV carriers with serum alanine aminotransferase within the normal range (<40 IU/L) and patients with clinically evaluated cirrhosis were also excluded. The presence of HCV was proved by both HCV antibody assay (second-generation; Dinabot, Tokyo, Japan) and HCV RNA detection by the nested reverse transcription polymerase chain reaction (PCR) method. HCV virus (HBV) surface antigen and HBV DNA were negative in all patients of both groups. They were all without a history of antiviral therapy such as interferon administration. Their clinical profiles are shown in Table 1. The female patient in the hemophiliac group had a fibrinogenemia. Fifteen patients had received blood products, manufactured both in Japan and in the United States, and the other eight had received only blood products from Japan. Eleven hemophiliac patients were also infected with human immunodeficiency virus (HIV), but none had developed AIDS/ARC and all had a CD4+ T-lymphocyte count within normal limits. The HCV subtype was identified using the method of Okamoto et al. and the line-probe assay (INNO-LIPA HCV; Immunogenetics N.V., Zwijnaarde, Belgium), respectively, and classified according to nomenclature reported by Simmonds et al. There was no significant difference in the composition of subtypes. Patients in the hemophilia group were significantly younger than patients in the posttransfusion hepatitis group, but there were no significant differences in the interval from transfusion or first use of blood concentrates or in disease duration. There were also no differences between groups regarding other clinical characteristics such as biochemical data.

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**NUCLEOTIDE DIVERSITY AND MULTIPLE INFECTIONS**

**Table 1. Clinical Profiles**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hemophiliacs With HCV Infection</th>
<th>Patients With Chronic Posttransfusion Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>Male:female</td>
<td>22:1</td>
<td>11:6</td>
</tr>
<tr>
<td>Age (mean ± SD)§</td>
<td>27.8 ± 9.1</td>
<td>60.4 ± 12.4</td>
</tr>
<tr>
<td>Median duration of HCV carriage. yr (range)*</td>
<td>22.5 (11-40)</td>
<td>18.6 (4-40)</td>
</tr>
<tr>
<td>Median disease duration, yr (range)*</td>
<td>7.0 (1-17)</td>
<td>4.7 (0.2-28)</td>
</tr>
<tr>
<td>Biochemistry (mean ± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>70.2 ± 47.0</td>
<td>68.6 ± 51.3</td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>50.5 ± 26.7</td>
<td>59.7 ± 37.9</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>4.29 ± 0.48</td>
<td>4.26 ± 0.37</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>3.34 ± 0.69</td>
<td>3.45 ± 0.42</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>134.4 ± 77.7</td>
<td>177.4 ± 55.2</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.81 ± 0.58</td>
<td>0.75 ± 0.24</td>
</tr>
<tr>
<td>Serology (on admission)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HCV† (second-generation)</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>HCV RNA* (nested PCR)</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>HBs antigen*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HBs antibody*</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Subtype</td>
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<td></td>
</tr>
<tr>
<td>1a</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>2a</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2b</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Histology†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic persistent hepatitis</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

*Since the first elevation of alanine aminotransferase above the normal limit.
† Liver biopsy specimens were obtained after selection of the patients. They were taken after patients had given informed consent.
§ P = .0035.
¶ P = <.0001.

**HCV RNA extraction.** RNA was extracted from serum HCV by the acid-phenol extraction method. It was extracted from a 100-μL aliquot of serum from a patient and then subjected to reverse transcription.

**Quantitation of HCV.** Quantitation of HCV RNA was performed by both competitive PCR methods and branched DNA (bDNA) probe assay. In patients whose HCV RNA was undetectable by bDNA probe assay, which could not detect less than 0.5 × 10⁶ Eq/mL, HCV RNA concentrations by bDNA probe assay were considered equal to 0.5 × 10⁶ Eq/mL.

**Oligonucleotide primers for PCR amplification of HVR1.** HVR1 of HCV was PCR-amplified using a newly developed primer set. This primer set was designed by analyzing 128 variations of reports of the HCV RNA genome so that the sequences of the primers were greater than 80% homologous to every described isolate. The primer sequences of the region were as follows: HVR1 (sense primer for the first PCR), TGGGACACATGATGACTACCGT; HVR2 (sense primer for the second PCR), TACTACTCCAATGCTGGGCCAGAC-TGGGC; HVR3 (antisense primer for the second PCR), CATGTTGGCACCTGTTGG; and HVR4 (antisense primer for the first PCR and reverse transcription), CGGTGCTGTATATGATGCAACCTG.

These primers were obtained with an LKB Gene Assembler Plus DNA Synthesizer (Pharmacia, Uppsala, Sweden) using a protocol described previously. The HVR2 primer was labeled with fluorescein isothiocyanate, and the HVR3 primer was labeled with biotin.

**Reverse transcription and PCR amplification.** Reverse transcription from HCV RNA to cDNA was performed using a protocol described previously. All nested PCR reactions were performed in a thermal cycler (Lobocycler 40; Stratagene, La Jolla, CA) and Nippon Genetics (Tokyo, Japan) over 29 cycles with 1 μmol/L primers and a Gene Amp kit (Perkin-Elmer, Foster City, CA) under conditions recommended by the kit supplier. Each PCR cycle consisted of denaturation at 94°C for 1 minute, annealing at 56°C for 1.5 minutes, and extension at 72°C for 1.5 minutes.

To determine the degree of sequence variation of the HCV-amplified sequences and the diversity of quasispecies, we performed an assay based on PCR amplification of viral sequences.

**Fluorescence single-strand conformation polymorphism (SSCP) analysis.** The number of quasispecies was determined by a fluorescence SSCP analysis. PCR products were denatured for 5 minutes at 95°C in formamide dye (Pharmacia) and electrophoresed in 7% acrylamide gel (acylamide: N, N'-methylenebisacrylamide, 99.1) using the ALF II DNA Sequencer (Pharmacia). The conditions for electrophoresis were as described in the technical manual supplied by Pharmacia. Detection of HCV HVR1 clone (quasispecies) was performed using the Fragment Manager (Pharmacia) software system.

**Direct DNA sequencing.** PCR products were denatured by alkaliizing, and then biotin-labeled fragments were recovered with streptavidin-coated magnetic beads (Dyna, Oslo, Norway) using a protocol described previously. The sequencing reactions were performed using an auto-read sequencing kit (Pharmacia) under the conditions recommended by the kit supplier, with a protocol based on the dideoxy-mediated chain-termination method. The reaction products were electrophoresed in 6% acrylamide gel (Ready Mix Gel, Pharmacia) using the ALF II DNA Sequencer. Identification of the mutation site was performed using Alignment software (Fujitsu, Tokyo, Japan).

Entire clones were analyzed directly, and the sites at which nonconserved nucleotides were located among HVR1 of HCV from each individual were detected by autosequencing. We sequenced 75 nucleotides of HVR1, and the percentage of sites at which nucleotides were not conserved in the entire population was calculated as an indication of diversity.

**Statistical analysis.** Statistical analyses were performed using the χ² test and Mann-Whitney U test where appropriate. Pearson’s test was used in the correlation analysis. A P level less than .05 was considered statistically significant.

**RESULTS**

**Comparison between HCV-infected hemophiliacs and patients with chronic posttransfusion hepatitis C.** HCV RNA concentration tended to be lower in hemophiliacs than in posttransfusion chronic hepatitis patients when measured by a competitive PCR method (4.7 ± 1.3 log(copies)/mL in hemophiliacs vs 5.4 ± 1.2 log(copies)/mL in posttransfusion hepatitis patients, P = .0514) and when measured by bDNA probe assay (4.1 ± 3.8 × 10⁶ Eq/mL in hemophiliacs vs 6.2 ± 9.4 × 10⁶ Eq/mL in posttransfusion hepatitis patients, P = .9449), but it was not statistically significant. The distribution of patients in each group according to HCV RNA concentration is shown in Table 2.

In 21 of 23 hemophiliacs with chronic hepatitis C and 16 of 17 posttransfusion hepatitis patients, HVR1 was amplified by PCR. Both groups were similar regarding the number of viral quasispecies identified by SSCP analysis: 5.2 ± 2.0
Table 2. Distribution of Patients in Each Group According to HCV RNA Concentration

<table>
<thead>
<tr>
<th></th>
<th>Competitive PCR (copies/mL)</th>
<th>bDNA Probe Assay (× 10^6 Eq/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10^4</td>
<td>10^4 ≤ ≤ 10^6</td>
</tr>
<tr>
<td>Hemophiliacs with HCV infection</td>
<td>4/23 (17.4%)</td>
<td>14/23 (66.9%)</td>
</tr>
<tr>
<td>Chronic posttransfusion hepatitis patients</td>
<td>1/17 (5.9%)</td>
<td>9/17 (52.9%)</td>
</tr>
</tbody>
</table>

clones in hemophiliacs and 4.0 ± 2.3 clones in posttransfusion hepatitis patients (P = .0943; Fig 1).

Analysis by direct sequencing showed that the rate of sequences of nonhomology among quasispecies studied from a given patient (diversity) was 13.0% ± 7.4% (9.8 ± 5.5 of 75 nucleotides) in hemophiliacs and 2.7% ± 2.8% (2.0 ± 2.1 of 75 nucleotides) in posttransfusion hepatitis patients, the difference being statistically significant (P < .0001; Fig 2). In addition, the degree of diversity tended to increase with the duration of HCV infection in the posttransfusion hepatitis group (r = .6187, P = .0106), but not in the hemophiliac group (r = .0525, P = .8210).

Comparison among HCV-infected hemophiliacs. In HCV-infected hemophiliacs, the number of viral quasispecies identified by SSCP analysis was similar between patients who had received blood products both from Japan and from the United States (13 patients) and patients who had received only Japanese-made blood products (eight patients): 4.9 ± 2.6 clones in the former and 5.9 ± 2.1 clones in the latter (P = .2773). The diversity in both groups of patients was also similar: 12.7% ± 7.7% (9.5 ± 5.8 of 75 nucleotides)

Fig 1. Number of populations in hemophiliacs and posttransfusion hepatitis patients measured by SSCP analysis. No significant difference was observed between these 2 groups, which indicated the existence of the same number of quasispecies in the serum of a given patient. (○) Patients with coinfection of HIV; (□) patients without coinfection of HIV.

Fig 2. Diversity of HVR1 in E2/NS1 gene in hemophiliacs and posttransfusion hepatitis patients analyzed using direct sequencing. The diversity of hemophiliacs with HCV infection was significantly larger than for patients with posttransfusion chronic hepatitis C, which suggests the coexistence of HCV strains from multiple origins. (○) Patients with coinfection of HIV; (□) patients without coinfection of HIV.
in patients who had received blood products both from Japan and from the United States and 13.5% ± 7.4% (10.1 ± 5.5 of 75 nucleotides) in patients who had received only Japanese-made blood products (P = .8848).

In addition, the number of viral quasispecies and the diversity of patients coinfected with HIV in HCV-infected hemophiliacs (11 patients) were 4.7 ± 2.1 clones and 13.9% ± 7.7% (10.5 ± 5.8 of 75 nucleotides), and those of patients infected only with HCV (10 patients) were 5.8 ± 1.9 clones and 12.0% ± 7.3% (9.0 ± 5.5 of 75 nucleotides). There were no differences between these two groups with regard to both the number of viral quasispecies and the diversity (P = .2050 and P = .5035, respectively).

**DISCUSSION**

Populations of RNA virus particles from a given patient consist of heterogeneous mixtures of genetically different but closely related variants called quasispecies.29,30 This heterogeneity has been attributed to the high error frequency in RNA replication.29,31,32 The genome of HCV itself has also been characterized by marked sequence heterogeneity33-35 and is suggested to have the quasispecies nature throughout the entire genome.36-38 HCV quasispecies have recently been demonstrated to be relevant to persistent infection,39 clinical features,40,41 viral titer,41 disease progression,41,42 and response to interferon therapy.43-45

Previous studies of HCV heterogeneity have generally consisted of cloning PCR-amplified sequence products,36,37,43,46-48 and some clones of which were subject to direct sequencing.48,52 However, cloned cDNA molecules do not always represent the true composition of HCV quasispecies.

By the methods used in this study, all possible quasispecies from individual patients were analyzed by direct sequencing, and therefore, diversity within the HVR is more reflective of the entire population of quasispecies.

We compared the diversity of HVR1 between hemophiliacs with chronic hepatitis C and posttransfusion hepatitis C patients. Because hemophiliacs have a frequent use of blood products, we considered them the model cases that have a greater possibility of multiple HCV inocula than other patients with chronic hepatitis C. And we selected chronic posttransfusion hepatitis C patients who had no episode of a risk of reexposure to HCV after blood transfusion as the model cases for a single HCV inoculation.

Ogata et al46 sequenced the H strain of two HCV isolates from a single patient obtained during both the acute and chronic phase of posttransfusion hepatitis C, an interval of 13 years of continuous viral replication between July 12, 1977 (acute phase) and August 1, 1990 (chronic phase). It was found that 4.6% of the nucleotides in the NS1 coding region were mutant (44 of 960 nucleotides). Okamoto et al46 sequenced two HCV-J isolates taken from experimentally infected chimpanzees acutely and after an 8.2-year interval, and detected a mutant frequency of 2.2% (23 of 1,038 nucleotides) within the E2 region. The results of these previous studies are compatible with the sequence variability of isolates from patients with posttransfusion chronic hepatitis observed in our study, the mean interval from transfusion being 18.9 years. Spontaneous mutation incurred during HCV replication therefore appears to be the likely mechanism. The significant correlation between the degree of diversity and the duration of HCV infection in the posttransfusion hepatitis group would support this.

On the other hand, the 13.0% sequence variability of hemophiliacs with HCV infection is well above the background spontaneous mutation frequency, even when the increased interval since first receiving blood concentrates, 22.4 years, is considered. These patients were presumably repeatedly exposed to HCV of various strains. The hemophiliacs’ great sequence variability in the HVR in the E2/NS1 gene of HCV isolates, despite the same number of quasispecies as observed in posttransfusion chronic hepatitis C patients, suggests the coexistence of single HCV subtypes resulting from multiple inoculations, as well as spontaneous mutation. With regard to the number of HCV quasispecies, they were similar in both groups. HCV strains for which specific antibodies were produced were reported to disappear from serum,35 and this may suppress the increase in the number of quasispecies by the multiple inocula of HCV.

Kao et al4 reported a superinfection of genotype III (subtype 2a) in a patient with persistent HCV infection of genotype II (subtype 1b), and observed the disappearance of HCV of genotype III from serum and the promotion of the genetic variation of HCV of genotype II. In our study, it could also be suggested that the great sequence variability in the HVR of hemophiliacs might be caused by the promotion of genetic variation by means of repeated inoculation and disappearance of HCV of different subtypes.

Also, the tendency for a lower HCV RNA concentration in hemophiliacs with chronic hepatitis C versus chronic posttransfusion hepatitis C patients, especially as measured by competitive PCR, might be explained by the frequent immune response associated with repeated inoculation of HCV of different or even the same subtypes, which might be considered to promote the genetic variation.

In conclusion, it appears that hemophiliacs with chronic HCV infection have particles with considerable sequence variation in the HVR1 of the E2/NS1 gene, due to spontaneous mutation and to acquisition of new infecting strains. These results indicate the possibility of the coexistence of a single HCV subtype from another origin. The effect of multiple infection on the diversity of the HVR should be considered in analyses of nucleotide diversity in individual patients.

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