Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis

Nobuyuki Enomoto,1 Masayuki Kurosaki,1 Yujiro Tanaka,1 Fumiaki Marumo1 and Chifumi Sato1,2*

1 Second Department of Internal Medicine and 2 Division of Health Science, Faculty of Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113, Japan

Hepatitis C virus (HCV) populations in vivo consist of heterogeneous mixtures of genetically different but closely related variants defined as a 'quasispecies’. The longitudinal fluctuation of HCV quasispecies populations in chronic hepatitis C has not been elucidated. Serial plasma samples were obtained from four patients with chronic hepatitis C (two patients without any treatment and two patients treated with interferon), and cDNA fragments containing the 5'-terminal region of the E2 gene of HCV were amplified from plasma RNA using PCR. Since conventional cloning of PCR products detects only a small part of the entire population, PCR products of each sample were separated by electrophoresis using single-strand conformation polymorphism (SSCP) analysis, which can distinguish DNA fragments of the same size as different electrophoretic bands depending on their sequence-specific conformation. Separated DNA fragments were recovered from SSCP bands in gels and their nucleotide sequences determined. SSCP electrophoresis separated PCR products into bands with different mobility. Sequence analysis of these bands confirmed that HCV populations in each patient are composed of quasispecies with different E2-hypervariable regions (HVR), which are known to contain antibody epitopes. Different patterns of variation in the HVR of quasispecies were observed in individual patients with different clinical features over time during chronic infection. Following interferon treatment, some quasispecies disappeared during the treatment and reappeared after the end of the treatment, whereas other quasispecies in the same patient remained during the treatment suggesting that the sensitivity to interferon is different among quasispecies.

Introduction

Populations of RNA viruses in vivo consist of heterogeneous mixtures of genetically different but closely related variants called 'quasispecies' (Steinhauer & Holland, 1987; Holland et al., 1992). The heterogeneity of the genome is derived from error-prone replication catalysed by viral RNA-dependent RNA polymerases which lack proofreading activity. A population of infecting virus forming a quasispecies provides the virus with the capacity to adapt easily to new environmental conditions such as immune pressure or antiviral therapy.

Hepatitis C virus (HCV), a recently cloned RNA virus that causes acute and chronic hepatitis leading to liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989), is suggested to have the quasispecies nature throughout its whole genome (Oshima et al., 1991; Murakawa et al., 1992) including the envelope 2 (E2) region containing the hypervariable region (HVR) (Kato et al., 1992a; Okada et al., 1992). HVR is thought to be the neutralizing epitope (Weiner et al., 1992), and several recent studies by us (Enomoto et al., 1993a, b; Kurosaki et al., 1993) and others (Kato et al., 1992b; Okada et al., 1992; Okamoto et al., 1992b) have revealed that HVR sequences of the predominant circulating HCV change during persistent infection or interferon treatment. These results also suggested that selection of escape variants from the HVR quasispecies pool could contribute to persistence of the infection.

However, the longitudinal fluctuation of HCV quasispecies populations in chronic hepatitis C has not been fully elucidated since previous studies on HCV heterogeneity were mainly performed by cloning of PCR products (Oshima et al., 1991; Kato et al., 1992a, b; Murakawa et al., 1992; Okada et al., 1992; Okamoto et al., 1992b) or by direct sequencing of PCR products (Ogata et al., 1991; Enomoto et al., 1993, 1994; Kurosaki et al., 1993). Cloned cDNA molecules do not always represent the true composition of HCV quasispecies despite the laboriousness of the procedure. Direct
sequencing of PCR products easily and reliably identifies the predominant sequence, but minor clones could be overlooked. Therefore, the significance of HCV quasispecies in persistent infection or during interferon treatment is unclear.

Single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989) is a method for detecting sequence differences of ssDNA by non-denaturing PAGE. Partially denatured DNA in the gel matrix has a sequence-specific three-dimensional conformation of characteristic mobility, and even a single nucleotide difference can be identified as a mobility shift. Therefore, complex mixtures of DNA species of the same size, such as a viral quasispecies, can be separated into bands of different mobility. However, for the analysis of viral genomes, there has been only one brief report (Yap et al., 1992b) in which SSCP profiles were utilized to evaluate the sequence heterogeneity among hepatitis B virus isolates.

In the present study, we applied SSCP to analyse serial changes of the heterogeneous structure of HCV subpopulations within isolates in order to clarify the role of HCV quasispecies in persistent infection and in interferon resistance.

**Methods**

**Patients.** Four patients with chronic HCV infection and active hepatitis were studied. They had elevated serum transaminase levels for more than 6 months. The diagnosis of chronic active hepatitis was made by liver biopsy. The patients were all positive for anti-HCV antibody (second generation ELISA; Ortho Diagnostics) and plasma HCV RNA using nested PCR of the 5' non-coding region (Okamoto et al., 1990). The fact that all patients were negative for hepatitis B surface antigen and anti-HBc (hepatitis B virus core protein) there were no other obvious causes of chronic liver disease. Two patients with chronic hepatitis C (patients 1 and 2) were observed for 2 years without any treatment. The other two patients (3 and 4) were treated with 6 million units of interferon-α intramuscularly three times a week for 6 months and 4 months, respectively. Plasma samples were obtained serially and stored at −80 °C until use. The clinical characteristics and the total interferon dose of the patients are summarized in Table 1. The time courses of the alanine aminotransferase (ALT) profile and sampling times in each patient are demonstrated in Fig. 3.

**RNA extraction.** Plasma RNA was extracted with the acid-guanidinium-isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). Briefly, 150 μl of plasma was mixed with 400 μl of the guanidinium buffer (4 M-guanidinium thiocyanate, 25 mM sodium citrate pH 7.9, 0.5% sarkosyl and 1% 2-mercaptoethanol) and extracted with water-saturated phenol. The aqueous phase was extracted once with chloroform. RNA was ethanol-precipitated with 20 μg of glycogen (Boehringer-Mannheim) as a carrier. The obtained RNA was resolved in 6 μl of distilled water and stored at −80 °C until use.

**cDNA synthesis.** Five μl of reverse transcription mixture was adjusted to contain 1 μl of the RNA solution, 50 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories Life Technologies), 10 units of RNase inhibitor (Promega), 50 ng of random hexamer (Takara), 50 μM-Tris-HCl pH 8.3, 75 mM-KCl, 3 mM-MgCl₂, and 10 mM-DTT. The mixture was incubated at 37 °C for 45 min.

**PCR.** Five μl of the cDNA solution was made up to 25 μl of a PCR mixture containing 10 μM-Tris-HCl pH 8.3, 50 mM-KCl, 1.5 mM-MgCl₂, 400 nM of each PCR primer (outer primer), 200 μM of each dNTP, 0.01% gelatin and 0.5 units of Taq polymerase (AmpliTaq, Cetus Corporation) (Saiki et al., 1988). For the amplification of HCV cDNA containing the HVR, PCR consisting of 50 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and polymerization at 72 °C for 1 min was performed. Single-stranded cDNA was amplified by the nested asymmetric PCR with one-hundredth of the first PCR product as a template. Either 5'- or 3'-nested (inner) primer was reduced to 40 nM in the nested asymmetric PCR.

**SSCP analysis of PCR product.** Five μl of the nested PCR product was mixed with 10 μl of a SSCP loading solution (0.05% xylene cyanol, 20 mM-EDTA and 95% formamide) and was subjected to modified non-denaturing PAGE in 0.6 x Tris-borate-EDTA buffer, with 100 V applied for 6 h. The gel was made of 0.5 × MDE gel (Hoefer) to intensify the separation of each band with a specific conformation. The gel was stained with ethidium bromide (1 μg/ml), and the bands were detected and photographed on the u.v. transilluminator (non-isotopic SSCP) (Yap & McGee, 1992a). The same experiments were performed using end-labelled nested primer. Non-limiting nested primer was end-labelled with [γ-32P]ATP using T4 polynucleotide kinase. The SSCP gel was vacuum-dried and exposed to X-ray films (isotopic SSCP). The autoradiograph was compared with the result of ethidium bromide staining.

**Direct sequencing of PCR product.** Separated bands were excised from the SSCP gel under u.v. light. Pieces of the gel were crushed in 100 μl of TE buffer (10 mM-Tris–HCl, 1 mM-EDTA, pH 8.0) and cDNA was eluted by incubation at 60 °C for 3 h. Recovered cDNA was amplified by the asymmetric PCR again and subjected to SSCP analysis to confirm that each band was differently amplified and homogeneous. Residual dNTPs and primers were removed using a spin filtration column (Suprec-02; Takara). Nucleotide sequences of amplified single-

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**Table 1. Clinical characteristics of four patients with chronic hepatitis C**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>History of blood transfusion</th>
<th>Total dose of interferon-α 2a</th>
<th>HCV subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>Male</td>
<td>48</td>
<td>−</td>
<td>Not treated</td>
<td>II</td>
</tr>
<tr>
<td>Patient 2</td>
<td>Female</td>
<td>60</td>
<td>−</td>
<td>Not treated</td>
<td>II</td>
</tr>
<tr>
<td>Patient 3</td>
<td>Female</td>
<td>59</td>
<td>+</td>
<td>516 million units</td>
<td>II</td>
</tr>
<tr>
<td>Patient 4</td>
<td>Female</td>
<td>55</td>
<td>+</td>
<td>270 million units</td>
<td>II</td>
</tr>
</tbody>
</table>
Table 2. Oligonucleotide primers for nested PCR and sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Position (strand)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' Outer (1st PCR)</td>
<td>GCCATTTATCAGGTCACCGCATGGC</td>
<td>1261–1285 (sense)</td>
</tr>
<tr>
<td>3' Outer (1st PCR)</td>
<td>GCCTCGGGGACCCGGGAGGAGTTGAA</td>
<td>1668–1692 (anti-sense)</td>
</tr>
<tr>
<td>5' Inner (2nd PCR)</td>
<td>GCTTGGGATATGATGATGAAGTGGTGC</td>
<td>1284–1309 (sense)</td>
</tr>
<tr>
<td>3' Inner (2nd PCR)</td>
<td>GTGGTGGAGGAGTATTCCATTGCAGTT</td>
<td>1611–1634 (anti-sense)</td>
</tr>
<tr>
<td>5' Sequencing primer</td>
<td>CTATCCCATGTTAGGAGAATGCTAGAAGT</td>
<td>1412–1441 (sense)</td>
</tr>
<tr>
<td>3' Sequencing primer</td>
<td>TTAGGGCGGTGCGGTGAGTTCGCCA</td>
<td>1587–1612 (anti-sense)</td>
</tr>
</tbody>
</table>

* Nucleotide positions are numbered according to the HCV-J sequence.

Oligonucleotide primers for nested PCR and sequencing were synthesized with a DNA synthesizer model 391 (Applied Biosystems Japan) based on the published nucleotide sequence of HCV-J (Kato et al., 1990), which belongs to the major genotype in Japan (Okamoto et al., 1992a). The nucleotide sequences of the primers are shown in Table 2.

Results

Specificity of nested PCR

By the first round of PCR, cDNA fragments of the expected size (432 bp) were amplified. For quasispecies analysis the single-stranded cDNA was further amplified by the second round nested PCR with asymmetric primers. The cDNA of expected size (351 bp) was again amplified with the ssDNA of faster mobility in 2% agarose gel electrophoresis (Gyllensten & Erlich, 1988). These amplified cDNAs were directly sequenced and confirmed as having the nucleotide sequences of the expected HCV genome.

Non-isotopic and isotopic SSCP

PCR products of the second round PCR were separated into isolate-specific bands with different mobility by SSCP analysis. In patients 2 and 3, the radiolabelled PCR products amplified with the end-labelled non-limiting primer of the second PCR were also analysed using the SSCP gel, and an autoradiogram was obtained. The SSCP profiles (patterns and relative intensities of the bands) obtained by either method were essentially the same in ethidium bromide staining and in autoradiography, suggesting that ethidium bromide staining is satisfactory for SSCP analysis. Furthermore, ethidium bromide staining showed a higher resolution and sensitivity for detection of bands than did autoradiography. Therefore, we analysed the SSCP profile in each isolate mainly by ethidium bromide staining with the strand showing better separation of SSCP bands.

Direct sequencing of PCR product

Direct sequencing of a PCR product from each SSCP band showed a single HVR sequence; no mixtures of heterogeneous sequences were observed.

Sequencing of cloned cDNA obtained from patient 3

Fig. 1 shows the nucleotide sequences of 10 clones obtained from the PCR product of patient 3 at time point I. The HVR in the E2 gene is boxed. Horizontal bars indicate sequence identity with clone 1. Nucleotide sequences are numbered at the bottom of the figure according to HCV-J (Kato et al., 1990).

stranded cDNA were determined by the dideoxy nucleotide chain termination method using a modified T7 DNA polymerase (Sequenase version 2.0 kit; United States Biochemical) with sequencing primers. The cDNA of expected size (351 bp) was again amplified with the ssDNA of faster mobility in 2% agarose gel electrophoresis (Gyllensten & Erlich, 1988). These amplified cDNAs were directly sequenced and

Fig. 1. Alignment of nucleotide sequences of 10 cDNA clones obtained from the PCR product of patient 3 at time point I. The HVR sequences are numbered at the bottom of the figure according to HCV-J (Kato et al., 1990).
of bands A to D, with one to four nucleotide differences. No clones with the HVR identical to bands C and D were seen in these 10 clones.

Sensitivity of SSCP analysis

SSCP analysis of mixed clones showed that minor subpopulations with dilutions of up to 0.5 ng:10 ng were visible as discrete SSCP bands (Fig. 2). Therefore, our SSCP analysis can detect minor variants representing at least 5% of the whole population.

Fluctuation of quasispecies population in chronic hepatitis C (patients 1 and 2)

Patient 1 showed marked fluctuation of serum ALT levels ranging from 50 to 150 IU/l during the observation period of about 8 months. On the other hand, serum ALT levels of patient 2 were relatively constant around 100 IU/l for more than 2 years (Fig. 3). In patient 1, HCV quasispecies changed very rapidly (Fig. 4). The major quasispecies (band A) and minor
quasispecies (band B) detected at time point I had 18 nucleotide and 12 amino acid differences in the HVR (Fig. 5). They disappeared completely 4 months later (time point II) and were replaced by other HCV quasispecies (bands C and D). Quasispecies C and D had a similar HVR, with four nucleotide differences and one amino acid difference from each other. Rapid displacement of the predominant HCV by a different quasispecies (band E) was again observed 2 months later (time point III, Fig. 3). Quasispecies E had only three nucleotide and two amino acid differences in the HVR compared to quasispecies B, suggesting it was a direct progeny of quasispecies B. Quasispecies F, which replaced quasispecies E at time point IV, was closely related to quasispecies A since it had only two nucleotide and two amino acid differences.

The fluctuation of the quasispecies in patient 2 was very slow in contrast to patient 1 (Fig. 4). There were two distinct HVR quasispecies, showing eight nucleotide and five amino acid differences in the HVR (Fig. 5). This coexistence continued for the following 1-5 years (time points II and III). Then one quasispecies (band A) disappeared, and the other quasispecies (band B) remained at time point IV.

**Effect of interferon treatment on quasispecies (patients 3 and 4)**

In patient 3, serum ALT levels were normalized immediately after the start of interferon administration and rose again 4 months later. In patient 4, serum ALT levels remained in the abnormal range during the interferon therapy. HCV RNA was detectable in both patients throughout the observation period (Fig. 3).

In patient 3, five bands were present 5 months before interferon treatment (time point I) and at the beginning of interferon treatment (time point II) (Fig. 4). The sequence of each band is shown in Fig. 5. Four of the five bands (A, B, C and D) had a similar HVR, with one to three nucleotide differences from each other. Three of them (A, B and C) had identical deduced amino acid sequences of the HVR, and the remaining quasispecies (band D) had only one amino acid substitution in the HVR. The HVR sequence of band E was quite different.
from that of the other four bands, and it had 18 nucleotide differences out of 81 nucleotides and 11 amino acid differences out of 27 amino acids. One month after the start of interferon treatment (time point III), SSCP profiles of this patient consisted of four bands, and band E had disappeared. This SSCP profile did not change again for 6 months, until the end of the interferon treatment.

Three months after the end of the treatment, the HCV population consisted of bands B, C and D, whereas band A had disappeared.

In patient 4, five distinct bands (A, B, C, D and E) were detected by SSCP analysis 5 months before the start of interferon treatment (time point I), and four distinct bands at the beginning of interferon treatment (time point II) (Fig. 4). The HVR sequence of band C was closely similar to that of band B (one amino acid difference in the HVR), and that of band E was almost identical to band D (two amino acid differences in the HVR) (Fig. 5). One month after the start of interferon treatment (time point III), band A increased in proportion and became predominant after 4 months of interferon treatment (time point IV), whereas bands B, C and D became weak, i.e. just traceable. One month after the cessation of interferon (time point V), bands C and D reappeared and the proportion of band A decreased. Three months after the end of the treatment, the HCV population consisted of bands B, C and D, whereas band A had disappeared.

**Discussion**

In the present study, we demonstrated by SSCP analysis that HCV populations in vivo consist of quasispecies with
differences in the HVR, and that the quasispecies population changed differently in each patient during the persistent infection. Each HCV quasispecies was shown to have a different sensitivity to interferon in the same individual.

Several reports have suggested the existence of HCV quasispecies based on intra-isolate sequence heterogeneities of the 5′ non-coding region (Martell et al., 1992; Murakawa et al., 1992), the NS4 to NS5 region (Oshima et al., 1991) and the HVR (Kato et al., 1992a, b; Okada et al., 1992). However, in these previous studies on the intra-isolate heterogeneity of the HCV genome only two to 10 clones obtained from PCR products were sequenced. Such a small number of clones does not necessarily represent the whole spectrum of quasispecies in vivo without any bias, as shown in this study.

Therefore, we applied SSCP analysis of the nested PCR products to analyse the constitution of HCV quasispecies populations in vivo. SSCP is a method to detect nucleotide differences in ssDNA as mobility shifts caused by conformational changes in a non-denaturing polyacrylamide gel (Orita et al., 1989; Yap & McGee, 1992a). This method can detect a single point mutation and is widely used to screen mutations in various oncogenes (Cottrell et al., 1992). In addition, the nested PCR used in the present study to generate the ssDNA is known to have the same level of specificity as Southern blot hybridization for the detection of a target gene (Cristiano et al., 1991).

Using SSCP analysis, we could discern subpopulations of the HCV genome within each isolate as different bands by single electrophoresis. Fourteen out of 16 HCV isolates in this study were separated into distinguishable bands. Direct sequencing of these bands revealed that these mobility changes were due to the sequence differences of the HVR, indicating that the circulating HCV population consists of heterogeneous mixtures of quasispecies with different HVRs. In theory, DNA species with different sequences might comigrate on SSCP gels, and minor species (under 10% of the population) could be overlooked by direct sequence analysis (Chuang et al., 1993). In the present study, direct sequencing of 18 SSCP bands did not show any heterogeneous mixtures of sequences, confirming that each SSCP band consisted of an almost homogeneous HCV population, and that comigration of different sequences is likely to be a relatively rare phenomenon.

The pattern of variation observed by SSCP analysis was compared with that found for the same sample by sequence analysis of cloned cDNAs. The species and relative amount of sequences detected by SSCP analysis were similar to the result of PCR cloning for patient 3 at time point I. However, the sequences of bands C and D were not observed in 10 clones examined, suggesting that a small number of clones was insufficient to represent the general population. Therefore, SSCP analysis may detect the composition of the virus population more appropriately than cloning of PCR products.

Recent studies (Kato et al., 1992b; Ogata et al., 1991; Weiner et al., 1992) including ours (Enomoto et al., 1993, 1994; Kurosaki et al., 1993) revealed that HVR sequences of circulating HCV change during persistent infection and interferon treatment. The HVR is thought to contain neutralizing epitopes (Weiner et al., 1991), and the changes in HVR sequences are suggested to be escape mutations evading the humoral immune response (Okamoto et al., 1992b; Weiner et al., 1992; Taniguchi et al., 1993). SSCP analysis indicated that these serial changes of the HVR are due to fluctuations in the concentrations of different HVR quasispecies, as seen in patients 1 and 2. In patient 1, quasispecies E at time point III and quasispecies F at time point IV appeared to be the direct progeny of quasispecies A and B, respectively, with two amino acid substitutions in the HVR. They had been predominant 7 months before (time point I), and had become undetectable at time point II. This indicates that quasispecies can survive even if they decrease to below detectable levels, and can later become the progenitors of a subsequent predominant population. It has been shown that only two amino acid substitutions in the HVR can cause escape from the antibody response by changing its antigenicity (Taniguchi et al., 1993). This quasispecies nature of HCV populations with differences in the HVR seems to allow antigenic drift and immune evasion during persistent infection.

The process of fluctuation of HCV quasispecies populations is different in each host, ranging from a relatively slow drift of major populations over a 2 year period (patient 2), to the complete replacement of a major subpopulation by another quasispecies in only a few months (patient 1). The rate of change is probably determined by the intensity of the immunological defence of the host, virus replication rates and mutation frequencies (Holland et al., 1992). The marked fluctuation of the ALT levels in patient 1 may be related to the rapid elimination of hepatocytes infected with earlier quasispecies by immunological reactions in the host.

One of the striking results of the present study is that only some subpopulations of the quasispecies (band E in patient 3 and bands C, D and E in patient 4) disappeared during interferon treatment and reappeared after the end of the treatment, whereas other subpopulations simultaneously present in the same host showed resistance to interferon (bands A to D in patient 3 and band A in patient 4). This suggests that sensitivity to interferon differs among members of the quasispecies.

In a previous study, we reported that the predominant
HVR sequences changed during interferon treatment and that this rapid change of the HVR appeared to be associated with the failure of interferon therapy (Enomoto et al., 1993b). Okada et al. (1992) compared the HVR heterogeneity within isolates before and after interferon treatment by sequencing 10 clones randomly obtained from PCR products. They found that selected HCV clones were detected after interferon treatment, suggesting that some members of the HCV quasispecies may be more resistant to interferon therapy than others in the same host. The present observations demonstrated that such sequential changes of the predominant HCV population are due to the selection of resistant strains during the administration of interferon.

Previously defined factors which were thought to be related to effects of interferon were the patient's age, sex, histology, concentration of HCV RNA in blood and HCV subtype (Kanai et al., 1992; Takada et al., 1992; Yoshioka et al., 1992). The existence of interferon-resistant quasispecies demonstrated in this study suggests that interferon therapy will fail in patients infected with an interferon-resistant HCV quasispecies. The factors identified so far may be the setting for the emergence of interferon-resistant quasispecies.

The mechanism by which HCV quasispecies with a particular HVR appear to show resistance to interferon at one time point in the same host is unclear. HCV detected in patients 3 and 4 was only subtype II based on the sequence of the core region, and coinfection by different subtypes was not observed. Therefore, the differences in the effects of interferon among HCV quasispecies were not due to different HCV subtypes. The effect of interferon may be mediated by the immunological defence of the host, aiming to neutralize epitopes postulated to exist in the HVR. Otherwise, different regions of the HCV genome linked to the HVR may be the true determinant of interferon sensitivity. Further studies are required to elucidate the mechanism of interferon resistance of HCV quasispecies.

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References


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