Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus

T. Ito, J. Mukaigawa, J. Zuo, Y. Hirabayashi, K. Mitamura and K. Yasui*

1 Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu-shi, Tokyo 183, 2 The 2nd Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

To investigate the viral replication cycle and genomic heterogeneity of hepatitis C virus (HCV), we established an HCV cultivation system by using a primary hepatocyte culture from patients with chronic hepatitis C. Liver tissue was obtained by needle biopsy or surgery, then hepatocytes were isolated by collagenase digestion. After several weeks, we determined the HCV RNA titre of the cultured cells and supernatant by a competitive polymerase chain reaction (PCR) method. A significant amount of HCV RNA was observed in the cells and supernatant during cultivation. Negative-strand RNA, regarded as a marker of viral replication, could be detected by a strand-specific reverse transcription PCR method and the HCV core protein could be detected by immunofluorescence microscopy. Many HCV particles released into the supernatant were infectious. In addition, we compared the nucleotide sequences in the E2/NS1 region of pre- and post-cultivation hepatocytes for 8 weeks. At the beginning of the culture period, three major HCV types containing two subtypes were isolated. Following cultivation, the same types were isolated from the cultured hepatocytes in the same ratio as prior to cultivation. We could detect the same clones in this patient's serum, but in vivo we observed genetic variability over a 6 month interval. One clone detected throughout the 6 month period mutated extensively in the hypervariable region. These results indicated that HCV can replicate in cultured hepatocytes, and that infectious virions are released into the supernatant. This cultivation system should facilitate the study of HCV genomic heterogeneity, infection and replication.

Introduction

Hepatitis C virus (HCV) is now recognized as the principal agent of parenterally transmitted non-A, non-B hepatitis. HCV is a 9.4 kb single-stranded positive-sense RNA virus encoding a large polyprotein (Choo et al., 1989; Kato et al., 1990) and has structural genes located at the 5' end of the genome preceding the non-structural protein genes. In the codons corresponding to the N terminus of the E2/NS1 region, in particular, there are two hypervariable regions (HVR) [HVR1 (27 amino acids) and HVR2 (7 amino acids)] with marked sequence diversity (Hijikata et al., 1991; Weiner et al., 1991). This region has been shown to have a high rate of mutation in HCV strains recovered from the same chronically infected patient (Kato et al., 1992; Ogata et al., 1991; Weiner et al., 1992). HCV is also thought to have a neutralization epitope in the E2/NS1 region (Taniguchi et al., 1993; Weiner et al., 1992). Mutations in this region may generate quasispecies variation and subsequent escape from immune surveillance.

Although our understanding of the molecular biology of HCV has progressed rapidly, little is known about its general biological characteristics because in vivo studies have been limited to chimpanzees infected with HCV. Development of efficient in vitro culture systems for HCV or readily available in vivo models are priorities for the study of HCV. HCV replication has been described from studies of liver tissue and peripheral blood mononuclear cells (PBMC) from infected patients (Willems et al., 1994; Zignego et al., 1992). In vitro culture systems for HCV replication have been developed from human T and B cell lines (Bertolini et al., 1993; Shimizu et al., 1993), human fetal liver cells (Iacovacci et al., 1993) and chimpanzee hepatocytes (Lanford et al., 1994). A recent report showed that HCV could replicate in a human hepatoma cell line, Huh7, after transfection with HCV RNA from a putative full-length cDNA clone (Yoo et
al., 1995). Efficient long-term viral replication, however, has not been described in any system. Possibly HCV is stringently hepatotropic being restricted to replicate and release only in differentiated hepatocytes, which may have HCV receptors on the cell surface and specific functions that support viral growth.

In this study, we established an in vitro culture system for HCV by cultivation of human hepatocytes from patients with chronic hepatitis C. We were able to demonstrate viral replication by assaying HCV RNA both from cultured cells and from culture supernatant and by specific detection of the negative-strand viral RNA, a marker for replication (Shimizu et al., 1993; Takehara et al., 1992; Willems et al., 1994). HCV core protein was demonstrated in cultured hepatocytes by immunofluorescence microscopy. Furthermore, we confirmed the infectivity of the virions released into the supernatant from cultured hepatocytes by subsequent passage of HCV to uninfected human hepatocytes.

We then determined the nucleotide sequence of the E2/NS1 region from cultured hepatocytes at 8 weeks and compared the nucleotide and amino acid sequences of this HVR1 containing region in pre- and post-cultivation hepatocytes. We isolated three major viral clones containing two subtypes from pre-cultivation hepatocytes. We continued to study viral sequences from this patient for 6 months and observed the changing nature of the genome of these HCV clones in contrast to the evolution of these same clones in vitro, where immune selection pressure does not exist.

Methods

Patients. We collected four liver biopsy tissues (cases 1, 2, 3 and 4) and three resected liver tissues (cases 5, 6 and 7) from seven patients (ranging in age from 34–68 years) with chronic hepatitis C who were admitted to Showa University Hospital for evaluation and treatment of chronic hepatitis C. All the cases were positive for anti-HCV antibody (Boehringer Mannheim), 20 μM-DETT, 0.5 μM of each dNTP (Boehringer Mannheim), 20 pmol of the 1st sense PCR primer, an appropriate volume of reverse transcriptase buffer (5 x) and DEPC-treated water. The cDNA synthesis was carried out at 37 °C for 40 min.

The first PCR mixture contained 1.25 U Taq DNA polymerase (AmpliTaq; Takara, Japan), 20 pmol of the 1st sense primer, an appropriate volume of PCR buffer (10 x) and DEPC-treated water was added to the cDNA solution. The thermoster (Techne) was programmed for 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. For the second amplification, 2.5 μl were removed from the first reaction and added to a similar reaction mixture supplemented with 0.2 mM-dNTP and 20 pmol of the 2nd sense and antisense primers. The PCR was carried out as described above for the first amplification.

To quantify the HCV RNA in a sample, we prepared four tubes containing four couples of serial diluted samples and internal control (tube 1, undiluted samples only; tube 2; 10-fold diluted samples with 105 copies of internal control cDNA; tube 3; 100-fold diluted samples with 104 copies of internal control; tube 4; 1000-fold diluted samples with 103 copies of internal control) and determined the HCV RNA content of samples judging from the comparison of two cDNA signals. This method enabled us to determine the HCV RNA content within a range of 105–106 copies of HCV RNA. We assayed one part of the total RNA of samples and calculated the total HCV RNA content per well. The method was found to be highly sensitive and specific for the detection of HCV RNA in cultured hepatocytes.

Negative-strand HCV RNA in cultured hepatocytes (strand-specific RT-PCR). For detection of negative-sense HCV RNA strands, we used a tagged RT-PCR method that specifically detected the negative-strand HCV RNA (Lanford et al., 1994). For detection of the negative-strand RNA, cDNA synthesis was performed with an oligonucleotide primer
containing a tag sequence (in bold) unrelated to HCV at its 5' end and the outer sense primer 5NC-1, (5' TCAAGTAGCGAATAAACTC-CACCATAGATCCTCC 3', Tag-5NC-1) and was performed as described above. The cDNA synthesis was stopped by heating at 99 °C for 1 h. The samples were treated with 50 μg/ml RNaseA (Boehringer Mannheim) at 37 °C for 30 min in order to digest the RNA and thereby avoid possible reverse transcription and subsequent amplification of positive-sense RNA strands by Taq polymerase (Willems et al., 1994). As a negative control, we performed cDNA synthesis without using RNA extracted from liver tissue homogenates containing high quantities of HCV to detect the non-specific reaction caused by self-priming of positive-strand RNA (Lanford et al., 1994). The 1st PCR was carried out as described using the sense primer of the tag sequence alone (5' TCAAGTAGCGAATAAACTC 3', Tag) and the 5NC-1R primer, then the 2nd PCR was performed using the Tag and the 5NC-2R primers.

Immunofluorescence microscopy. HCV-infected hepatocytes obtained from a non-cancerous portion of resected liver (case 7) were grown for 14 days on collagen-coated chamber slides (Lab-Tek; Nunc). Following removal of the culture medium, the cells were briefly dipped once or twice into PBS. The chamber slides then were fixed in acetone for 5 min at -20 °C and air dried. Indirect immunofluorescence antibody analysis was carried out with an anti-HCV core monoclonal antibody; this monoclonal antibody was obtained from a mouse that had been immunized with highly purified core protein, which was isolated from Escherichia coli that expressed the HCV core gene. The secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel). We prepared COS7 cells transfected with cDNA corresponding to the HCV core region as a positive control. The cDNA clones were expressed in COS7 cells by using an SRα promoter (Takebe et al., 1988). COS7 cells were transfected with the plasmid DNA in the presence of lipofectin (Gibco BRL).

Fig. 1. The samples in (a) and (c) each contained 10⁶ copies of synthetic HCV RNA, samples (b) and (d) contained 10⁵ copies. (a, b) Ethidium bromide staining of SalI-digested RT-PCR products. The RT-PCR product of wild-type HCV RNA was 211 bp. SalI digestion of the RT-PCR product of the mutant HCV-RNA yielded a 163 bp product and a 48 bp product. Lane 1 contained 10⁶ copies of mutant RNA; lane 2, 10⁵ copies; lane 3, 10⁴ copies; lane 4, 10³ copies; lane 5, 10⁰ copies; lane 6, 10⁻⁰ copies. The two RNA samples appeared to contain 10⁴ copies of HCV-RNA and 10⁵ copies by a competitive RT-PCR method. (c, d) The PCR product of wild-type HCV cDNA was 145 bp long. The PCR product of the mutant HCV cDNA was 130 bp. Lane 1 contained undiluted samples without mutant cDNA; lane 2, 10-fold diluted samples and 10⁻⁰ copies of mutant; lane 3, 100-fold diluted samples and 10⁵ copies of mutant; lane 4, 1000-fold diluted samples and 10⁴ copies of mutant. The RNA samples appeared to contain 10⁴ copies (c) and 10⁵ copies (d) by a competitive PCR method.

Results
Sensitivity of this competitive PCR assay
To confirm the sensitivity of our competitive PCR assay, we quantified a known concentration of a synthetic HCV RNA both by using a competitive PCR system and by using a competitive RT-PCR system established by other groups (Kaneko et al., 1992; Hagiwara et al., 1993). In the competitive RT–PCR assay, we used serially diluted HCV RNA mutated in its 5' NC region for an internal control. The synthetic HCV RNA and the internal control were kindly provided by M. Seki (Mitsubishi Chemical, Japan) and synthesized by in vitro transcription of the T7-based plasmids pt7NI-19 linearized with BamHI, which contained nucleotides 1–2006 of the HCV genome and its mutant with a SalI site inserted in its 5' NC region (Seki & Honda, 1995). We quantified 10⁶ copies of the synthetic RNA as 10⁵ copies using both systems and we quantified 10⁵ copies of the RNA as 10⁴ copies by our system and as 10² copies by the competitive RT-PCR (Fig. 1). The other titres of the synthetic RNA were almost the same titres using by these two systems.

We added 10⁶ copies of synthetic RNA to HCV-negative cultured hepatocytes and to culture media alone for 4 weeks and quantified the HCV RNA titre by using our system. The titre of HCV RNA extracted from cultured cells was 10⁵ copies and from culture medium was 10⁴ copies. These results indicated that naked single-
stranded RNA was extensively digested by RNase in the culture medium and especially in cultured cells during treatments. So our system may underestimate the amount of RNA in the sample.

**Replication of HCV in cultured hepatocytes**

In each hepatocyte culture system from cases 1–5, HCV RNA was detected both in cells and supernatant throughout the culture period, as measured by competitive PCR (Fig. 2a, b). The medium of each hepatocyte culture was changed at 5 to 7 day intervals. In three cases (cases 1, 3 and 4) obtained from patients with chronic hepatitis, the PCR value remained stationary both in the cells and in the supernatant for 2 to 4 weeks at a time. In two cases (case 2 and 5) from patients with liver cirrhosis, the level of HCV RNA in the cells decreased for 4 weeks, but HCV was continuously released at consistently high titre for 8 weeks. In addition, we assayed the titre of the HCV RNA in culture medium during the shorter interval. We also cultured hepatocytes obtained from an HCV-infected patient (case 6) with liver cirrhosis as described and totally changed the culture medium on day 7. After that the medium was...
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HCV has a single-strand RNA genome with positive polarity. Viral replication is indicated by detection of negative-strand RNA. Therefore, we determined the negative-strand RNA in cultured hepatocytes for 4 weeks (cases 1, 2 and 5) during which the HCV RNA level in the cells and in the culture supernatant remained high. Following 28 days of culture, the negative-strand RNA was observed in cultured hepatocytes by ethidium bromide staining of RT-PCR products (Fig. 3). No HCV RNA was observed in the control HCV RNA extracted from HCV-infected liver tissues as determined by cDNA synthesis in the absence of primers.

When examined by immunofluorescence microscopy for HCV core protein by using an anti-HCV core monoclonal antibody, a granular staining pattern was observed in the cytoplasm of both the cultured hepatocytes at 14 days (Fig. 4b) and the positive control (Fig. 4a). The anti-HCV core monoclonal antibody used for this test could react with the authentic virion core protein, because the antibody could capture HCV virion cores containing RNA by the capture PCR method (Shindo et al., 1994; J. Zuo, K. Yasui et al., unpublished data).

**Infectivity of the released HCV in the culture supernatant**

To determine the infectivity of HCV particles released into the culture supernatant, we inoculated the culture supernatant in an HCV-negative hepatocyte culture and assayed for negative-strand HCV RNA. We harvested culture supernatant with $3 \times 10^5$ copies of HCV RNA/100 μl from a primary hepatocyte culture (case 5). HCV-negative hepatocytes were taken from two patients (cases 8 and 9) with metastatic liver tumours who did not have anti-HCV serum antibodies or HCV RNA. Following isolation, hepatocytes were seeded at a concentration of $5 \times 10^4$ cells/well in 200 μl of maintenance medium supplemented with the culture supernatant containing HCV and incubated over night at 37 °C. Following incubation, the medium was replaced with normal medium. On days 3 and 7 after initiating the culture, we replaced the medium and looked for the presence of negative-strand HCV RNA by the strand-specific RT-PCR as described. Fig. 5(a) shows that positive-strand RNA was detected in cultured hepatocytes on days 3 and 7; negative-strand RNA was not detected on day 3, but was seen on day 7 in each case. The titre of positive-strand RNA (case 8) increased from $10^2$ copies/well on day 3 to $10^3$ copies/well on day 7.

HCV RNA in the culture media was detected on day 7 (case 9) and its PCR titre was $5 \times 10^2$ copies/well. When we inoculated with HCV in culture medium to the another HCV-negative hepatocyte culture as previously
described, the intracellular HCV RNA was also detected after inoculation.

The HCV-negative hepatocyte culture (case 8) was infected with HCV released from the HCV hepatocyte culture. After infection, we determined whether or not a correlation existed between HCV-replicating cells and albumin-producing cells. Fourteen days after the infection, we examined a double staining of indirect immunofluorescent antibody using anti-HCV core mouse monoclonal antibodies and an anti-human albumin rabbit polyclonal antibody (Cappel) mixture. An FITC-conjugated anti-mouse IgG (Cappel) and a Texas red-conjugated anti-rabbit IgG (Cappel) mixture were used as second antibody. The HCV core protein was detected mainly in the cultured hepatocytes which expressed human albumin (Fig. 5b).

To analyse the nature of the released HCV particles, we layered 100 μl of hepatocyte culture supernatant (case 5; day 7) onto a 10 to 40% continuous sodium iotalamate (Angio Conray; Daiichi-seiyaku, Japan) gradient in 20 mM-Tris–HCl pH 8.0 and 0.15 M-NaCl. Subsequently we fractionated the gradient after ultracentrifugation at 121000 g in a Beckman SW41 Ti rotor for 16 h at 4 °C. HCV RNA titres in each fraction were assayed by competitive PCR. High HCV RNA titres were found at 1.16 to 1.20 g/ml with the HCV RNA peak was in 1.18 g/ml.

In addition, infectious culture medium was inactivated by ultraviolet (UV) irradiation. The HCV RNA content of HCV-negative hepatocytes inoculated with the infectious culture media was 10³ copies/well without inactivation and 10¹ copies/well with the UV inactivation 7 days after inoculation.

**HCV quasispecies in infected patients: comparison of genomic fluctuation between in vitro and in vivo viral life cycle**

We found three major types containing two subtypes of HCV clones classified by the 1150–1419 nucleotide dendrogram in the E2/NS1 region in the original hepatocytes and in serum from the same patient (Fig. 6a). Likewise, the HCV clones were classified according to the deduced amino acid sequences in this region. Type A, B and C clones had different sequences through this region with nucleotide identities of 88 to 94% (Fig. 6b). Type B1 and B2 clones had the same amino acid sequence in the non-HVR (410–474), but HVR1 sequences were different. Type B2 clone was not found in

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Fig. 5. (a) RT-PCR products from HCV-negative hepatocyte cultures infected with culture medium containing HCV virions (cases 8 and 9). For the detection of positive-sense RNA, the 5NC-1R primer was used for cDNA synthesis (lanes 1 and 2) and for the detection of negative RNA strands, the Tag-5NC-1 primer was used (lanes 3 and 4). On days 3 (lanes 1 and 3) and 7 (lanes 2 and 4) after initiating the culture, both positive and negative RNA strands were analysed in cultured hepatocytes. (b) Double staining of cultured hepatocytes (case 8) 14 days after the infection using anti-human albumin polyclonal antibodies (i) and a mixture of anti-HCV core monoclonal antibodies (ii). Scale bar represents 20 μm.
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(a) Three major types containing subtypes of HCV clones were classified by nucleotide dendrogram of the coding region of amino acids 384 to 473 of E2/NS1 and detected in hepatocytes before initiating culture and in the serum of patient on day 0 (case 2). (b) The nucleotide sequence of the four types of HCV.

Fig. 6. (a) Three major types containing subtypes of HCV clones were classified by nucleotide dendrogram of the coding region of amino acids 384 to 473 of E2/NS1 and detected in hepatocytes before initiating culture and in the serum of patient on day 0 (case 2). (b) The nucleotide sequence of the four types of HCV.

HCV clone population variations are shown in Fig 7. Two major (types A and B1) and one minor quasispecies (type C) were detected before initiating the culture. The two major quasispecies were also detected in the same population ratio following a 56 day culture. In the patient’s serum, one major species (type A) and two other quasispecies (types B1 and B2) were detected on day 0. But 1 month later, the type A clone had decreased while the B1 clone increased and became the major clone, becoming as much as 70% of the population. A type D clone was detected de novo in serum after the first month. Six months after beginning the culture, the type A and
B2 clones disappeared completely. The type C clone, which was detected in hepatocytes on day 0 in only as many as 5% of the total clones, eventually became a major clone in the serum.

Comparison between in vitro and in vivo HCV genomic mutations in the E2/NS1 region

In patient's serum (case 2), we consistently detected one clone (type B1) throughout the 6 month interval. We determined the frequency of genomic mutations in the E2/NS1 region containing HVR1 (Fig. 6a) by sequencing of this clone. Genomic mutations were observed mainly in HVR1. On day 0, we detected the B1 clone without variation both in cells and in serum.

In vitro, in the cultured hepatocytes, nucleotide changes in HVR1 occurred in 4 of 11 clones at a frequency of 1–4%, and resulted in few amino acid replacements (Fig. 8a, b). In the infected patient, on the other hand, even by one month nucleotide mutations occurred in 5 of 9 clones with a frequency of 1 to 4%. By 6 months 5 of 6 clones showed a mutation frequency of 3 to 18% in association with many amino acid replacements (Fig. 8c, d). Clearly, in vivo amino acid mutations accumulated in the HVR1 region over the 6 month interval. Furthermore, we analysed the A clone in the same manner as the B1 clone. In cells on day 0, 6 of 8 clones had the same sequences defined as type A and another two clones had two nucleotide changes in the HVR1. In the cultured hepatocytes on day 56, 6 of 8 clones were the same as type A clones and the others had one nucleotide change in the HVR1. In the infected patient, we could detect only one clone of type A after 1 month and found no clones of type A after 6 months. In genome of the clone detected after 1 month, one nucleotide change was observed with an amino acid replacement.

In the culture supernatant on day 7, we detected the same A1 clone as in cells on day 0. At this time 10 of 12 clones had the same sequence as the original clone and 2 of 12 clones had one nucleotide change.

Discussion

HCV preferentially infects and replicates in human or chimpanzee hepatocytes. Differentiated hepatocytes have specialized characteristics such as production of specific proteins and lipoproteins. A human T cell line can support HCV replication in vitro for several weeks (Shimizu et al., 1993, 1994a, b) as can a primary chimpanzee hepatocyte culture system (Lanford et al., 1994). However, HCV culture in human hepatocytes in vitro has not been reported. Because of the need for a human hepatocyte culture system in understanding the molecular biology of HCV infection and replication and in unravelling how genetic variants evolve in hepatocytes, we developed an in vitro human hepatocyte culture and demonstrated that it could support HCV replication.

The present study revealed that: (i) HCV replicated in vitro in this culture system. A high titre of HCV particles were released from infected hepatocytes into the culture medium and were infectious to other hepatocytes and (ii) most HCV clones remained genetically stable in this in vitro system as compared with natural infection in the whole organism.
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To demonstrate viral replication in in vitro culture systems, to assay not only for positive-sense HCV RNA but also for negative-sense RNA is generally recommended (Iacovacci et al., 1993; Lanford et al., 1994; Shimizu et al., 1993). In our culture system, both positive- and negative-sense RNA strands were detected in cultured hepatocytes and the titre of HCV RNA remained static during the 4 week culture interval. On harvesting cultured hepatocytes, we suspended the cells by trypsin treatment. Free HCV particles on the surface of hepatocytes can be removed by this treatment. We assumed, therefore, that hardly any contamination by extracellular HCV occurred in our system. Furthermore, HCV released into the culture medium was detected throughout the culture period and the viral RNA titre rapidly increased to a certain extent after the medium exchange. These results showed that HCV replicated in cultured hepatocytes and that virions were constantly released into the supernatant. The released virions were infectious to human hepatocytes, so virions must have been structurally complete to be able to interact with the viral receptor on the hepatocyte surface. In infected cells, HCV is thought to synthesize negative RNA strands during the initial step of replication after uncoating. Fig. 5(a) showed that HCV started to replicate in cultured hepatocytes directly after infection. UV treatment partially destroyed viral infectivity of uninfected hepatocytes.
The buoyant density of flavivirus particles is 1.18-1.20 g/ml in sucrose (Brinton, 1986) and HCV virions in patients' sera were recently reported to exist at a density of 1.14-1.16 g/ml when using immuno-electron microscopy to titrate the virus in the gradient fractions (Kaito et al., 1994). Our data indicated that the buoyant density of HCV virions released from hepatocytes into supernatant ranges from 1.16 to 1.20 g/ml, the peak fraction was at 1.18 g/ml. These data were in the same range as the previous reports and show that the density of released HCV particles from hepatocytes is the same as that of other flaviviruses. Some papers have reported that HCV in patients' sera sedimented in lighter fractions (Hijikata et al., 1993) and bound to lipoproteins. The density of HCV particles in serum was consistent with the density of virions released from infected hepatocytes and intracellular virions from immunologically disrupted hepatocytes. The latter virions may be complexed with other molecules such as lipoprotein or immature particles like those observed in flavivirus-infected cells. We analysed the centrifugation profile of intracellular HCV. Homogenized liver tissue from patient case 5 was layered on the same gradient described previously and centrifuged. We detected HCV from liver tissue in the light fraction (data not shown).

Two cases in which viral titres decreased after 4 week intervals were obtained from patients with liver cirrhosis (LC), however chronic active hepatitis (CAH) cases showed a continuous level of HCV RNA titre during infection. We isolated fewer hepatocytes from LC liver specimens than from CAH hepatocytes suggesting that some infected cells in culture might be injured by trypsinization when harvested. The stability of viral titres in these culture supernatants from day 7 to day 28 showed that HCV replicated in the surviving cells for the duration. Negative-strand RNA was also detected in these cells on day 28.

Detection of the HCV core protein in cultured hepatocytes by immunofluorescence microscopy indicated that HCV RNA was translated and HCV proteins were produced in cultured hepatocytes. The frequency of core protein-positive cells in the population was less than 10% (data not shown). The cultured hepatocytes, which were most similar to native liver, appeared to be more susceptible to HCV infection based on the results of the double staining experiment with anti-HCV core and anti-human albumin. But positive staining of HCV core protein was observed in some hepatocytes whose albumin expression was negative, so further study is required to analyse the correlation between HCV core protein expression and the cellular protein synthesis of host cells.

HCV is thought to constitute a new genus of the Flaviviridae family judging from the organization of the genome and the amino acid sequence of the polyprotein (Kato et al., 1990; Okamoto et al., 1992). Flaviviruses have some epitopes for viral neutralization in the envelope (E) region (Kimura-Kuroda & Yasui, 1988; Lobigs et al., 1987). Anti-E antibodies in patient serum may play an important role in viral neutralization, because HCV also has E spike proteins. Chimpanzees immunized with both putative envelope glycoproteins, E1 and E2 (copurified from transfected mammalian cells) were protected against an infectious challenge with HCV (Choo et al., 1994). The N terminus of the E2/NS1 region contains two hypervariable regions (HVR) with marked sequence diversity (Hijikata et al., 1991; Weiner et al., 1991). These regions have a high rate of mutation over time in HCV strains recovered from the same chronically infected patient (Kato et al., 1992; Ogata et al., 1991; Weiner et al., 1992). This domain is thought to be a target for the human immune response (Taniguchi et al., 1993; Weiner et al., 1992). If the mutation rate in key epitopes outpaces the rate of host immune response against those same epitopes, then the virus may be able to persist in the host.

HCV populations in vivo consist of quasispecies that differ within the HVR and these populations evolve differently in each patient during the natural history of persistent infection (Enomoto et al., 1994). In the present study, the patient in case 2 had HCV quasispecies clones both in hepatocytes and in serum before initiating hepatocyte culture. We therefore monitored the quasispecies population both in vitro without immune pressure and in vivo. In the in vitro culture system, no variations within the population were observed between pre- and post-cultivation, but dynamic changes were observed in the patient's serum. In addition, genomic mutation in the HVR1 was observed in one serum clone, type B1, which was always present during the 6 month interval both in the in vitro culture system and in vivo. Mutations in the serum clone accumulated predominantly in the HVR1 and the number of the original clones without mutations decreased over the 6 month interval. On the other hand, in vitro, nucleotide mutations accompanied by amino acid replacements were observed in a few clones, but most clones had no mutation after 2 months in culture. The same result was observed with type A clones.

From these results, it appears that genetic evolution of HCV quasispecies may be influenced by host immune pressure, and that replication of each viral clone is reasonably stable in the absence of immune pressure. As in all RNA viruses, spontaneous mutations in the HCV genome due to misreading during replication will occur at a background frequency. Therefore, HCV is expected to mutate even in vitro. Under immune pressure, in natural infection, mutants in the HVR can escape from the host immune response and gain a selective advantage until specific antibodies or a cell-mediated immune
response to the mutant protein appear. Both quasi-species variation and the development of escape mutants may enable HCV to infect persistently.

The mechanisms of HCV infection, replication, assembly, and neutralization can now be studied in detail in vitro. This system should also prove useful for investigating the genesis of spontaneous genomic heterogeneity in vitro without immune pressure, which can be compared and contrasted with natural infection.

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