Hepatitis C virus population dynamics in human lymphocytes and hepatocytes infected in vitro

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We previously found two cell lines (MT-2 and PH5CH) that were susceptible to hepatitis C virus (HCV) infection. Analysis of the infectivity of sera from HCV-positive blood donors for MT-2 and PH5CH cells suggested the cell tropism of HCV. To investigate further the cell tropism of HCV, the dynamics of HCV populations during culture were examined using three MT-2 clones and three PH5CH clones, infected with inoculum 1B-2. To type HCV populations in these infected cells, the HCV hypervariable region 1 (HVR1) in these cloned cells was characterized by sequence analysis and HpaII digestion analysis, which could distinguish three major HVR1 types (I, II and III) derived from the inoculum 1B-2. It was found that genomes containing HVR1 type I became predominant in MT-2 clones, and genomes containing HVR1 type II became predominant in PH5CH clones during culture after inoculation. These results suggest that inoculum 1B-2 contains both lymphotropic and hepatotropic HCV species, which can be distinguished by HVR1 type. To search for cell type-specific sequences in regions other than HVR1, three HCV cDNA clones (3-4 kb of the 5' noncoding region to the nonstructural 2 region) containing HVR1 type I obtained from HCV-infected MT-2C cells, and three HCV cDNA clones containing HVR1 type II obtained from HCV-infected PH5CH7 cells were sequenced. Following a comparison of the sequences, 11 amino acids were identified as candidates for determinants of the cell tropism of HCV.

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, and this persistent virus infection is linked to the development of liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989; Kuo et al., 1989; Ohkoshi et al., 1990; Saito et al., 1990). HCV has a positive-stranded RNA genome of 9-6 kb, including a large open reading frame encoding a polyprotein precursor of about 3000 amino acids, and has markedly similar genome organization to pestiviruses and flaviviruses (Kato et al., 1990 b; Takamizawa et al., 1991; Tanaka et al., 1995). From this polyprotein precursor at least ten viral proteins are proteolytically processed by a host cell signal protease and two distinct viral proteinases (Hijikata et al., 1991 a, 1993 a, b).

Although hepatocytes are the natural target cells for HCV infection, it has been reported that peripheral blood mononuclear cells may also support HCV infection (Gunji et al., 1994; Lerat et al., 1996; Müller et al., 1993; Saito et al., 1996). We recently found relatively high HCV genome titres in the lymph nodes, but not in the sera, from patients with gynaecological cancers, suggesting that the lymph nodes may play an important role in the carrier state and the persistence of HCV infection (Sugiyama et al., 1997 b). In addition, clinical studies have shown a significant association between HCV infection and lymphoproliferative disorders, mixed cryoglobulinaemia (Ferri et al., 1993; Gabrielli et al., 1994), and non-Hodgkin’s lymphoma (Luppi et al., 1996; Silvestri et al., 1996). It was recently proposed that chronic HCV infection
should be considered as a multi-faceted clinical syndrome rather than a simple liver disease (Ferri et al., 1997). These studies suggest that the cell tropism of HCV is a predominant factor in the aetiology of lymphoproliferative disorders.

To date, HCV has been shown to replicate in vitro in human lymphocytes (Cribier et al., 1995; Kato et al., 1995; Mizutani et al., 1996b; Shimizu et al., 1992, 1993; Shimizu & Yoshikura, 1994), human fibroblasts (Zibert et al., 1995), chimpanzee hepatocytes (Lanford et al., 1994) and human hepatocytes (Ikeda et al., 1997, 1998; Ito et al., 1996; Kato et al., 1996; Seipp et al., 1997). Evidence that HCV possesses cell tropism come from the findings that an HCV population with a limited hypervariable region 1 (HVR1) sequence, which is located in the N-terminal region of the second envelope glycoprotein (E2) (Hijikata et al., 1991a; Weiner, 1990), became predominant in cultured cells, despite the complicated quasi-species of the HVR1 in the primary inoculum (Hijikata et al., 1995; Ikeda et al., 1997; Kato et al., 1995; Nakajima et al., 1996; Sugiyama et al., 1997b). However, no systematic studies on the relationship between HCV genome and cell tropism of HCV have been reported to date.

Recently, by cloning the MT-2 [human T-cell leukaemia virus type I (HTLV-I)-infected human T-cell line] and PH5CH (simian virus 40 large T antigen-immortalized non-neoplastic human hepatocyte line) cells, which were susceptible to HCV infection (Kato et al., 1995, 1996), we obtained eight clones (MT-2A to MT-2E, PH5CH1, PH5CH7 and PH5CH8) which could support HCV replication more persistently than the parental MT-2 and PH5CH cells (Ikeda et al., 1998; Mizutani et al., 1996b). To characterize further the cell tropism of HCV, in this study we examined the HCV population dynamics in the culture after virus inoculation, using the cloned MT-2A, MT-2B, MT-2C, PH5CH1, PH5CH7 and PH5CH8 cells. We also sequenced the 5′-terminal 3-4 kb of the HCV RNA genomes obtained from HCV-infected MT-2C and PH5CH7 cells to look for cell type-specific sequences. We found that HCV populations in the infected cells gradually altered with time and converged to two different HCV populations in either the cloned MT-2 or PH5CH cells. Moreover, we identified several amino acid (aa) positions which might be involved in the cell tropism of HCV, in the viral core, the E2 and nonstructural (NS) 2 proteins, based on sequence comparison of HCV genomes derived from MT-2C and PH5CH7 cells, and the HCV-JS genome (Sugiyama et al., 1997b) which were derived from MT-2C cells inoculated with different HCV strains.

Methods

Inoculum. Serum 1B-2 was obtained from an HCV-positive blood donor and was supplied by the Kanagawa Red Cross Blood Center (Yokohama, Japan). The serum alanine aminotransferase level of this blood donor was 19 IU/l. The HCV genotype in serum 1B-2 was determined to be genotype 1b by the method described previously (Kato et al., 1991). HCV genome titre from serum 1B-2 was estimated to be about 10^7 HCV/ml by the RT-nested PCR method using a diluted serum sample (Ikeda et al., 1997).

Cell lines. The non-neoplastic human cell line PH5CH was established by immortalization after transfection with a simian virus 40 large T antigen expression vector, pRSV-TAg (Noguchi & Hirohashi, 1996), and was kindly provided by M. Noguchi and S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). PH5CH cells were maintained as described previously (Noguchi & Hirohashi, 1996). Three PH5CH clones, PH5CH1, PH5CH7 and PH5CH8, which displayed more efficient HCV replication than the parental PH5CH cells, were used in this study (Ikeda et al., 1998). MT-2A, MT-2B and MT-2C cells (Mizutani et al., 1996b), which were cloned from HTLV-I-infected T-cell line MT-2, were also used.

Virus inoculation. Virus inoculation was performed by a previously described method (Ikeda et al., 1997; Kato et al., 1995; Mizutani et al., 1996b) that was modified as follows. A total of 100 μl undiluted serum 1B-2 was added to each of the MT-2 and PH5CH clones (5 × 10^6 cells) suspended in 1 ml fresh culture medium, and cells were incubated for 7 h at 37 °C. After washing three times with 1 ml PBS, one-tenth of the cells was used for RNA preparation, and the remainder was maintained with 10 ml fresh medium at 32 °C (Mizutani et al., 1996a). Complete medium change was carried out at 7 and 14 days post-inoculation (p.i.), and 25–35 % of the cells (more than 10^5) were harvested at 14 and 23 days p.i. for the analysis of HCV RNA.

RT-nested PCR. RNA from cells and culture medium was prepared using ISOGEN and ISOGEN-LS extraction kits (Nippon Gene, Toyama, Japan), respectively. These RNA samples were used as template for the detection of the 5′ noncoding (5′-NC) region of HCV RNA by RT-nested PCR using sense and antisense primers sets, as described previously (Kato et al., 1995; Mizutani et al., 1996a). The second PCR reaction for 5′-NC yielded a 144 bp amplified product. For the detection of HVR1 of HCV RNA, new sense and antisense primers for RT-nested PCR were designed as follows in order to match the nucleotide sequences of HCV RNA genomes from serum 1B-2 (see text). An antisense primer, 145RB2, 5′-GTCGCTCACCACGACAGGC-3′ (corresponding to positions 1864–1885 of HCV-J; Kato et al., 1990b), was used to prime cDNA synthesis. Primers 144B2, 5′-TTGCTCGGATCCACTACCGACAGCCG-3′ (corresponding to positions 3338–3357 of HCV-J; Kato et al., 1990b), and 145RB2, 5′-GTCGCCACGACAGGCTG-3′ (corresponding to positions 1663–1682 of HCV-J; Kato et al., 1990b), were employed in the first round of PCR (35 cycles). Each PCR cycle consisted of annealing at 55 °C for 45 s, primer extension at 72 °C for 2 min and denaturation at 94 °C for 1 min. The internal primers 144A2, 5′-CGAGCCCTTGGATCGATCTACCGACAGCCG-3′, and containing a BanHI recognition site (underlined) in the 5′ region, and a equal mixture of 145RB2, 5′-CAGAGGTGATCCATCCCGAAGCAGGACAGCCGCCG-3′, and 280RA2, 5′-CAGAGGTGATCCATCCCGAAGCAGGACAGCCGCCG-3′ (corresponding to positions 1411–1433 of HCV-J; Kato et al., 1990b) and containing an EcoRI recognition site (underlined in the 5′ region), were used for the second round of PCR (35 cycles). Each PCR cycle consisted of annealing at 55 °C for 45 s, primer extension at 72 °C for 2 min and denaturation at 94 °C for 1 min. The second PCR reaction for HVR1 yielded a 220 bp amplified product. For the amplification of structural region of HCV RNA, the antisense primer 291R, 5′-GGAGAAAGATTGTGTTGCGT-3′ (corresponding to positions 3519–3538 of HCV-J; Kato et al., 1990b), was used to prime cDNA synthesis. Primers 420, 5′-GGCAGACACTCCACCACTAGA-
TCACTC 3' (corresponding to positions 6–30 of HCV-J; Kato et al., 1990b), and 424R, 5' GTCTTTGAGTAAGCCGATGAT 3' (corresponding to positions 3414–3436 of HCV-J; Kato et al., 1990b), were employed in the first round of PCR. An internal primer pair, 421E, 5' CAGAGTGGAGATCTCCTGAGAAGTTTACCTGCTAAGGC 3', and 425E, 5' CAGAGTGGAGATCTCCTGAGAAGTTTACCTGCTAAGGC 3', were extracted with phenol–chloroform and precipitated with ethanol. DNA was digested with EcoRI and the structural region of HCV RNA were cloned into the pTZ19R plasmid vector and into the EcoRI site of the pBR322 plasmid vector, respectively, as described previously (Kato et al., 1996; Mizutani et al., 1996b). Nucleotide sequences were determined by the dideoxy nucleotide chain termination method using an ALF DNA sequencer (Pharmacia).

Restriction enzyme analysis of PCR products. PCR products were extracted with phenol–chloroform and precipitated with ethanol. DNA was digested with HphI, and separated on a 5% agarose gel (NuSieve 3:1, Takara Shuzo) as described previously (Kato et al., 1992a).

Results

HCV infection of clones derived from MT-2 and PH5CH cell lines

Three MT-2 cell-derived clones (MT-2A, MT-2B and MT-2C) and three PH5CH cell-derived clones (PH5CH1, PH5CH7 and PH5CH8) which displayed HCV replication more persistently than the parental MT-2 and PH5CH cells (Ikeda et al., 1998; Kato et al., 1995, 1996; Mizutani et al., 1996b) were used for HCV infection. Cells (5 × 10^6) were cultured at 32 °C for 23 days after inoculation with serum 1B-2 (1 × 10^6 HCV) from an HCV-positive blood donor because we previously found that persistent HCV infection could be achieved by reducing the temperature from 37 °C to 32 °C (Mizutani et al., 1996a). Serum 1B-2 showed good infectivity for these cloned cells because the severe cytolysis observed in parental MT-2 and PH5CH cells within a few days p.i. (Ikeda et al., 1997) did not occur in these cloned cells. Since the doubling time of these cloned cells dropped to 80–100 h in culture at 32 °C, a complete medium change was carried out at 7 and 14 days p.i., and RNAs from 25–35% of the cells at 14 and 23 days p.i. were prepared. To confirm the retention of HCV in the cells, these RNA samples were used for semi-quantitative analysis of the 5'-NC region of HCV RNA using RT-nested PCR (Kato et al., 1996; Mizutani et al., 1996b). Semi-quantitative analysis revealed that intracellular HCV RNA levels were consistently maintained with titres of 4–8 × 10^4 (average number from three independent HCV infection experiments).

Sequence analysis of the surrounding regions of HVR1s of HCV RNAs from serum 1B-2

Sequence analysis of HVR1 is frequently used to monitor the qualitative changes in HCV populations in HCV-replicating cell cultures (Hijikata et al., 1995; Ikeda et al., 1997; Kato et al., 1995, 1996; Nakajima et al., 1996; Sugiyama et al., 1997b) because HVR1 provides a good molecular marker for dis-
Fig. 2. HpaII digestion analysis of 1B-2 HVR1 types. (a) Schematic presentation of HpaII digestion patterns of the PCR product containing HVR1 types I, II and III. (b) Separation of HpaII digests of PCR products amplified from the cDNA clones containing HVR1 species I-1, II-1 and III-1 by 5% agarose gel electrophoresis followed by staining with ethidium bromide. Lanes M1, Hinfl digests of pBR322 as size markers; M2, HaeIII digests of φX174 as size markers.

Fig. 3. HpaII digestion analysis of HVR1 populations from serum 1B-2, MT-2A, MT-2B, MT-2C, PH5CH1, PH5CH7 and PH5CH8 cells inoculated with serum 1B-2. (a) Separation of HpaII-digested HVR1 PCR products by 5% agarose gel electrophoresis. HVR1 PCR products obtained from serum 1B-2, MT-2A, MT-2B and MT-2C cells at 0, 14 and 23 days p.i. were subjected to HpaII digestion. (b) Separation of HpaII-digested HVR1 PCR products by 5% agarose gel electrophoresis. HVR1 PCR products obtained from serum 1B-2, PH5CH1, PH5CH7 and PH5CH8 cells at 0, 14 and 23 days p.i. were subjected to HpaII digestion. (c) Estimation of ratio of types I and II by the serial dilution of HVR1 PCR products. The results of HVR1 PCR products obtained from PH5CH1 and PH5CH8 cells at 14 days p.i. are shown as representative values. I-1 and II-1, which are same as those in (b), were also separated by 5% agarose gel electrophoresis as the standards for HVR1 types I and II, respectively. Lane M, 100 bp DNA ladder as size marker.

 distinguishes between HCV species (Kato et al., 1992a, 1994b; Saito et al., 1996; Sekiya et al., 1994). A general primer set, which has been well matched with the consensus sequences of more than 100 HCV strains registered in the DDBJ/EMBL/GenBank databases, was used for the amplification of HVR1 in our previous studies (Ikeda et al., 1997; Kato et al., 1996). However, this primer set was not predicted to completely match the sequences of HCV genomes derived from serum 1B-2.

To reduce this risk, we first examined the nucleotide sequences of regions of general primers for the amplification of HVR1 using a PCR product containing the structural region (3-4 kb) of HCV RNA from serum 1B-2 as described in Methods. Results revealed that the sense and antisense primers for first-round PCR differed by two nucleotides from all cDNA clones obtained from serum IB-2, and that the antisense primer for second-round PCR also differed at one nucleotide from about half of cDNA clones obtained from serum 1B-2 (data not shown). On the basis of these results, we designed new primers, which completely matched the sequences of HCV RNAs derived from serum 1B-2, and used them for RT-nested PCR as described in Methods.
Table 1. Alterations in 1B-2 HVR1 populations in HCV-infected cells

Ratio of type I and type II in 1B-2 HVR1 populations was estimated by the \textit{HpaII} digestion analysis as shown in Fig. 3.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ratio of type I and type II in 1B-HVR1 populations (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Days p.i. 0 14 23</td>
</tr>
<tr>
<td>MT-2A</td>
<td>50:50 40:60 60:40</td>
</tr>
<tr>
<td>MT-2B</td>
<td>40:60 50:50 75:25</td>
</tr>
<tr>
<td>MT-2C</td>
<td>50:50 50:50 90:10</td>
</tr>
<tr>
<td>PH5CH1</td>
<td>40:60 40:60 0:100</td>
</tr>
<tr>
<td>PH5CH7</td>
<td>50:50 25:75 0:100</td>
</tr>
<tr>
<td>PH5CH8</td>
<td>50:50 25:75 25:75</td>
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Analysis of 1B-2 HVR1 populations by RT-nested PCR using a new primer set

To determine the distribution of quasi-species of HVR1 from the serum 1B-2 inoculum, we sequenced 50 independent HVR1 cDNA clones which were obtained from the products (220 bp) amplified by RT-nested PCR using RNA from serum 1B-2 and the new primer set. A phylogenetic tree constructed by the multi-alignment analysis of sequences of 50 HVR1 cDNA clones using the GENETYX-MAC program, an unweighted pair-grouping method with arithmetic mean (Nei & Gojobori, 1986), revealed that 1B-2 HVR1 populations showed a more complicated quasi-species nature than that observed using the previous primer set (Ikeda \textit{et al}., 1997), and that HVR1 populations were classified into three types (I to III) as shown in Fig. 1(a). The frequencies of 1B-2 HVR1 types were as follows: type I (frequency 40%), II (52%) and III (8%) (Fig. 1b). This result indicated that type II was the predominant HVR1 population in serum 1B-2, in contrast to a previous study in which type I was the major population (frequency 77%) and type II was the minor population (frequency 23%) (Ikeda \textit{et al}., 1997). Type III, which was not detected in the previous study (Ikeda \textit{et al}., 1997), was found in serum 1B-2, although only at a frequency of 8%. These results demonstrated that the new primer set worked more efficiently and specifically than the previous primer set.

\textit{Hpai}l digestion analysis of HVR1 PCR products

While sequence analysis of HVR1 PCR products is frequently used to monitor HVR1 population dynamics, this approach is labour-intensive and time-consuming. To overcome this problem, we used restriction enzyme digestion analysis to detect sequence variations in HCV RNA PCR products (Kato \textit{et al}., 1991; McOmish \textit{et al}., 1993; Nakao \textit{et al}., 1991). On the basis of the nucleotide sequence data of more than 200 HVR1 cDNA clones derived from serum 1B-2 (Fig. 1b; unpublished data), we determined that \textit{Hpai}l digestion could distinguish the three 1B-2 HVR1 types (I, II and III). As shown in Fig. 2(a), \textit{Hpai}l digestion of PCR products containing HVR1 types I and III should produce three fragments (107, 70 and 43 bp for type I, and 116, 70 and 34 bp for type III), while only two fragments (150 and 70 bp) should be obtained from HVR1 type II PCR products with \textit{Hpai}l digestion. The 107 bp type I fragment, 150 bp type II fragment and 116 bp type III fragment could be clearly separated by 5% agarose gel electrophoresis (Fig. 2b).

HVR1 populations in HCV-infected MT-2A, MT-2B, MT-2C, PH5CH1, PH5CH7 and PH5CH8 cells

\textit{Hpai}l digestion analysis of HVR1 PCR products was used to monitor the HVR1 population dynamics in MT-2A, MT-2B, MT-2C, PH5CH1, PH5CH7 and PH5CH8 cells inoculated with serum 1B-2. A mixture of four independent PCR products for the HVR1 region was used for the \textit{Hpai}l digestion analysis to avoid the accidental bias by RT-nested PCR. Fig. 3(a, b) shows the results of \textit{Hpai}l digestion analysis in the cases of MT-2 clones (MT-2A, MT-2B and MT-2C) and PH5CH clones (PH5CH1, PH5CH7 and PH5CH8). The results revealed that HVR1 type I became predominant in MT-2 clones at 23 days p.i., although the ratio of HVR1 types I and II was almost equal until 14 days p.i., equivalent to the original serum 1B-2. In contrast to MT-2 clones, HVR1 type II became predominant in PH5CH clones by 14 days p.i. and HVR1 populations converged to type II at 23 days p.i. (except clone PH5CH8).
Fig. 5. Deduced amino acid sequences of core, E1, E2, p7 and NS2 proteins of HCVs obtained from HCV-infected MT-2C and PH5CH7 cells. The aa positions 1–1008 of clones 15-2, 15-4 and 15-5 containing HVR1 species I-1, and clones 16-6 and 16-8 containing HVR1 species II-1, and clone 16-3 containing HVR1 species II-5, are shown. The aa positions 704–1008 of clone 16-2 containing HVR1 species II-5, and clone 16-9 containing HVR1 species II-1 are also shown. The sequences are indicated by the single letter code. Amino acids differing from the sequence of 15-2 are shown.
We could not detect the 116 bp HVR1 type III-specific band in serum 1B-2, probably because its frequency was only 8%. Since HVR1 type III was not detected in both HCV-infected MT-2 and PH5CH clones, the frequency of this type is likely to be less than 8% in both cell types.

To confirm the results of HpaII digestion analysis, we first performed sequence analysis of HVR1 cDNA clones obtained from MT-2C and PH5CH7 cells at 23 days p.i. All 20 clones obtained from MT-2C and PH5CH7 cells were type I or type II, respectively (Fig. 4). This result was consistent with the HpaII digestion analysis (Fig. 3a, b). In addition, sequence analysis revealed that the HVR1 population in MT-2C and PH5CH7 cells converged to HVR1 species I-1 (80% frequency) and HVR1 species II-1 (75% frequency). To confirm further the results of HpaII digestion analysis, sequence analysis of HVR1 cDNA clones (10 clones each) obtained from PH5CH1 and PH5CH8 cells at 23 days p.i. was also performed. Three distinct HVR1 species, II-1 (6 clones), II-10 (3 clones) and II-21 [1 clone, which differed from II-1 by only one amino acid (Gly) at position 391] were obtained from the PH5CH1 cells, and three distinct HVR1 species, II-1 (8 clones), II-15 [1 clone, which differed from II-1 by only two amino acids (Thr) at positions 384 and 404] were obtained from the PH5CH8 cells. These results were also consistent with the HpaII digestion analysis (Fig. 3b).

To examine the ratio of types I and II, diluted HpaII digests of HVR1 PCR products were subjected to agarose gel electrophoresis, and the intensities of stained bands were compared. Fig. 3(c) shows two examples (PH5CH1 and PH5CH8 cells at 14 days p.i.) of this dilution analysis, in which the ratios of types I and II were 2/3 and 1/3 in PH5CH1 and PH5CH8 cells at 14 days p.i., respectively. The results of this analysis are summarized in Table 1. Type II species became predominant (more than 60%) in PH5CH1, PH5CH7 and PH5CH8 cells after 14 days p.i., and type I became predominant (more than 60%) in MT-2A, MT-2B and MT-2C cells at 23 days p.i., although type I still accounted for less than 50% in MT-2A, MT-2B and MT-2C cells at 14 days p.i. The ratio of types I and II was equivalent in all cloned cells immediately after inoculation. Taken together, these results suggest that HCV possessing HVR1 type I replicates preferentially in MT-2 clones, and that PH5CH clones preferentially support HVR1 type II HCV replication.

Sequence analysis of core, E1, E2 and NS2 coding regions of HCV RNAs from MT-2C and PH5CH7 cells

Although we found the convergence to different HVR1 types in MT-2 clones and PH5CH clones during culture after virus inoculation, sequence comparison of regions other than HVR1 was also required to determine cell type-specific amino acids which may be involved in cell tropism (lymphocyte vs hepatocyte) of HCV, based on our previous observations that sequence relationships in the HVR1 were equivalent to those in the downstream envelope region (Sugiyama et al., 1997b). To determine cell type-specific sequences in the HCV genome, we sequenced six HCV cDNA clones (3-4 kb of 5′-NC region to NS2), which were obtained by RT-nested PCR from HCV-infected MT-2C and PH5CH7 cells. Three cDNA clones (15-2, 15-4 and 15-5) containing the HVR1 type I sequence were obtained from MT-2C cells at 14 days p.i., and the other three cDNA clones (16-3, 16-6 and 16-8) containing the HVR1 type II sequence were obtained from PH5CH7 cells at 14 days p.i. The nucleotide sequences of the three cDNA clones from MT-2C cells and the three PH5CH7 clones showed 99.6–99.7% identity and 98.1–98.8% identity, and the deduced amino acid sequences of these cDNA clones showed sequence variation of only 0.7–0.8% and 1.0–1.6%, respectively.

Based on this sequence analysis, we found that sequence relationships of the HVR1 (type I vs type II) were equivalent to those of the core region to NS2 region sequenced (Fig. 5). However, only cDNA clone 16-6 showed unusual features of deduced amino acid sequences. cDNA clone 16-6 had strong identity with clones 16-3 and 16-8 (HVR1 type II) up to aa position 674, but between aa positions 741-951 this clone had an almost identical amino acid sequence to cDNA clones 15-2, 15-4 and 15-5 (HVR1 type I). After aa position 968, cDNA clone 16-6 again showed a type II sequence (Fig. 5). It is not clear whether cDNA clone 16-6 was produced by the recombination between two HCV RNAs or HCV cDNAs containing HVR1 types I and II during RT-nested PCR, or whether it was present as a chimeric HCV RNA in the PH5CH7 cells. The sequencing of two additional cDNA clones derived from PH5CH7 cells (16-2, HVR1 type II-5; 16-9, HVR1 II-1) showed that the clones were also similar to cDNA clones 16-3 and 16-8 (Fig. 5), indicating that cDNA clone 16-6 is a minor species (probably with a frequency of less than 20%) in the PH5CH7 cells. The 36 Cys residues in the sequenced region were completely conserved in all cDNA clones examined, and 14 Asn glycosylation sites (Asn-X-Thr/Ser) were also well-conserved in all cDNA clones. The positions of the Cys residues and Asn-glycosylation sites were well matched to the HCV-1b genotype consensus sequences. There was no extra Cys residue or potential Asn-glycosylation site in any of the cDNA clones sequenced.

The nucleotide sequences of the 5′-NC region (nucleotide positions 58–329 of HCV-J) (Kato et al., 1990b) of all cDNA clones showed close identity, with no apparent differences between the MT-2C and PH5CH7 cell-derived clones (data not shown). However, the deduced consensus amino acid sequences between MT-2C and PH5CH7 cell-derived clones showed 3.7% sequence variations (37 out of 1008 amino acids), although 14 of these 37 changes were located in HVR1. The other 23 amino acid differences were located in the core (1 aa), E1 (3 aa), E2 (10 aa), p7 (1 aa) and NS2 (8 aa). The frequency of amino acid changes in each region was 0.5% (core), 1.5% (E1), 3.0% (E2, except HVR1), 1.6% (p7) and 4.0% (NS2).
Discussion

In this study, we examined the cell tropism of HCV using HCV-susceptible clonal cells (MT-2A, MT-2B and MT-2C derived from human T-lymphocytes, and PH5CH1, PH5CH7 and PH5CH8 derived from human hepatocytes). HVR1 was used as a molecular marker to distinguish HCV quasi-species, as used in several previous reports (Hijikata et al., 1995; Ikeda et al., 1997; Kato et al., 1992a, 1994b, 1995, 1996; Nakajima et al., 1996; Saito et al., 1996; Sekiya et al., 1994; Sugiyama et al., 1997b). By sequence analysis and HpaII digestion analysis of HVR1 PCR products, we found that HCV species possessing HVR1 type I became predominant in MT-2A, MT-2B and MT-2C cells, and HCV species possessing HVR1 type II became predominant in PH5CH1, PH5CH7 and PH5CH8 cells, although serum 1B-2 which was used as an inoculum in this study showed a typical quasi-species nature, which consisted of HCV species possessing the three HVR1 types I, II and III. These results suggest that different quasi-species may have different tissue tropisms, and support similar observations made recently by using chimpanzees infected with HCV-positive plasma H77 (Shimizu et al., 1997).

Comparison of the deduced amino acid sequences of HCV cDNA clones (5′-terminal 3-4 kb) obtained from MT-2C and PH5CH7 cells inoculated with serum 1B-2 revealed that 37 amino acids commonly differed between cDNA clones containing HVR1 types I and II. To further narrow down candidate amino acids which may be involved in cell tropism, we compared 37 amino acid positions with those deduced from the HCV-JS genome (Sugiyama et al., 1997b), which was obtained as a convergent HCV species from MT-2C cells inoculated with the other HCV-positive serum, 1B-1. Eleven out of 37 amino acids from MT-2C cell-derived clones were conserved in HCV-JS, and were located in the core (aa 70), E2 [aa 399 (HVR1), 454 and 464], p7 (aa 765) and NS2 (aa 824, 837, 853, 887, 951 and 968) (Fig. 6). From these data, these 11 amino acids are considered to be the candidates for determinants of the cell tropism of HCV, although no direct evidence is presented which actually demonstrates the tropism of selected HCV variants.

We previously pointed out structural similarities with regard to sequence diversity or variability (Kato et al., 1990b, 1992b, 1994b), and isolate-specific immunodominant B-cell epitopes (Kato et al., 1993, 1994a; Sekiya et al., 1994) between HVR1 of the E2 envelope protein of HCV and the third variable region (V3 loop) of gp120 of human immunodeficiency virus type 1 (HIV-1). Since the V3 loop of HIV is known to be a key determinant of viral cell tropism (Hwang et al., 1991; Shioda et al., 1994; Takeuchi et al., 1991), the results of this study suggest that HVR1 itself could control the cell tropism of HCV. However, it was difficult to identify the specific amino acids involved in cell tropism, because HVR1 type I differed from type II at 14 out of 27 aa. As shown in Fig. 6, comparison with the HVR1 of HCV-JS revealed that only position 399 (Leu) was same as clone 1B-2 HVR1 (type I). Since the frequencies of Leu (type I in this study) and Phe (type II in this study) at position 399 of HVR1s (more than 400 HCV isolates) registered in the DDBJ/EMBL/GenBank databases was 41% and 38%, respectively, this position might be an important determinant of the tropism of HCV on cloned MT-2 and PH5CH cells. To clarify this point, further investigation using MT-2 and PH5CH cloned cells inoculated with other HCV-positive sera will be required.

An alternative possibility is that the cell tropism of HCV is determined by regions other than HVR1, since several reports demonstrated that the 5′-NC of poliovirus (Kawamura et al., 1989) or human hepatitis A virus (Day et al., 1992) regulated cell type-specific virus replication, and that envelope regions other than the V3 loop of HIV could also control the cell tropism of HIV (Carrillo et al., 1993; Koito et al., 1995). However, it is unlikely that an internal ribosome entry site within the 5′-NC region of the genome is involved in the tropism of HCV in MT-2C and PH5CH7 cells, because the nucleotide sequences of the 5′-NC region of cDNA clones obtained from MT-2C and PH5CH7 cells were the same.

Although, at the present time, we can not directly
determine the amino acids which control the cell tropism of HCV, positions 70 (Arg/Gln), 464 (Lys/Glu) and 951 (Asp/Asn) of the 11 amino acids in this study are of particular interest, because an amino acid substitution in these positions may cause changes in the net charge of the viral protein (core, E2 or NS2), and sequence variations in these positions are also observed among the 25 full-length HCV genomes registered in the DDBJ/EMBL/GenBank databases.

To determine directly which of these amino acid positions are involved in the cell tropism of HCV, new experimental systems such as the HCV proliferation system using infectious HCV cDNA clones, which have been recently used in tissue culture (Dash et al., 1997), the lymph nodes (Sugiyama et al., 1997) and in tissue culture (Dash et al., 1997), will be needed. In addition, since we recently found relatively high HCV genome titres in the lymph nodes (Sugiyama et al., 1997), comparison of HCV genomes from lymph nodes and liver tissues might also help clarify the cell tropism of HCV. Furthermore, sequence analysis of the NS3 region through to the 3′ terminus of HCV genomes from infected cells will also need to fully identify all major determinants of cell tropism of HCV.

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