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Replication of Hepatitis C Virus in Cultured Non-neoplastic Human Hepatocytes

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We established a replication system for hepatitis C virus (HCV) using the PH5CH non-neoplastic human hepatocyte line that had been immortalized with simian virus 40 large T antigen. In cells inoculated with sera derived from two HCV-positive blood donors, positive-stranded HCV RNA was detected up to 30 days postinoculation (p.i.). Semi-quantitative analysis of HCV RNA revealed that HCV multiplied during the period of culture. Sequence analysis of the HCV hypervariable region 1 (HVR1) in both cases indicated that HVR1 populations from the cells at 8 days p.i. were apparently different from those of the original inocula. HVR1 populations in infected cells became homogeneous or just a few species were selected over time. These results suggest that HCV is replicating in the human hepatocyte PH5CH cells. This culture system will be useful for detailed studies of the biological effects of HCV in human hepatocytes.

Key words: Hepatitis C virus — Human hepatocyte — HCV replication system — Hypervariable region 1

Most hepatitis C virus (HCV) infection causes chronic hepatitis; this persistent viral infection is linked to the development of liver cirrhosis and hepatocellular carcinoma.[1-4] Comprehensive genetic analyses of HCV[5-10] have enabled the establishment of a diagnostic system for HCV infection.[2,10] However, the mechanisms of viral replication in the targeted cells, and the pathogenesis of hepatic diseases are still unclear. The mechanism of persistent viral infection is also poorly understood, but this chronic viral infection may be facilitated by frequent mutations in the hypervariable region 1 (HVR1) that enable the virus to escape the host immunosurveillance system.[11,12] A major reason why these questions remain unsolved is the lack of a good experimental HCV replication system, although several trials using human T cells,[13] human fibroblasts,[14] human peripheral blood mononuclear cells[15] and chimpanzee primary hepatocytes[16] have been reported.

To address this problem, we have attempted to establish an *in vitro* HCV replication system using cultured human cell lines, and we recently found that a human T-cell leukemia virus type I infected cell line, MT-2, could support the replication of HCV.[17] Furthermore, in cloned MT-2C cells that supported viral replication more efficiently, antisense oligonucleotides complementary to the sequences of the core region of HCV genome could inhibit viral replication.[18] To date, a human hepatocyte line which showed good susceptibility to HCV infection has not been established.[17] Since hepatocytes are thought to be the natural target cells for HCV infection, making an HCV replication system using human hepatocytes is important for future studies. Therefore, we further examined the susceptibility of several human hepatocyte lines, and found that a recently established non-neoplastic hepatocyte line, PH5CH, showed good susceptibility to HCV infection. The PH5CH cell line was established by the immortalization of normal human hepatocytes following transfection with a simian virus 40 (SV40) large T antigen expression vector, pRSV-TAg.[19] PH5CH cells express hepatocyte differentiation markers, cytokeratin, albumin, and SV40 large T antigen.[19]

Inoculation of virus was performed by a previous method[17] that was modified as follows. Fifty microliters of HCV-positive undiluted human serum 1B-1, which has been described previously,[17] or human serum 1B-3 containing 10^7 HCV genomes (HCV-1b) per ml was added to PH5CH cells (1×10^5) suspended in 0.1 ml of fresh culture medium as previously described[19] and incubated for 20 h at 37°C. Cells were then washed five times with 1 ml of phosphate-buffered saline and 1 ml of fresh culture medium was added. At various times during the culture period, aliquots of culture medium (0.25 ml) and cells ($2–3\times10^5$) were collected and RNAs were extracted from these specimens for the detection of HCV RNA. RNA samples were used for the detection of the 5′-noncoding (5′-NC) region or HVR1 of HCV RNA by RT-nested polymerase chain reaction (PCR) as previously described.[17]

As shown in Fig. 1A, positive-stranded HCV RNA from the cells and culture medium after infection with inoculum 1B-1 was detected up to at least 12 days postinoculation (p.i.) in cells and 8 days p.i. in culture

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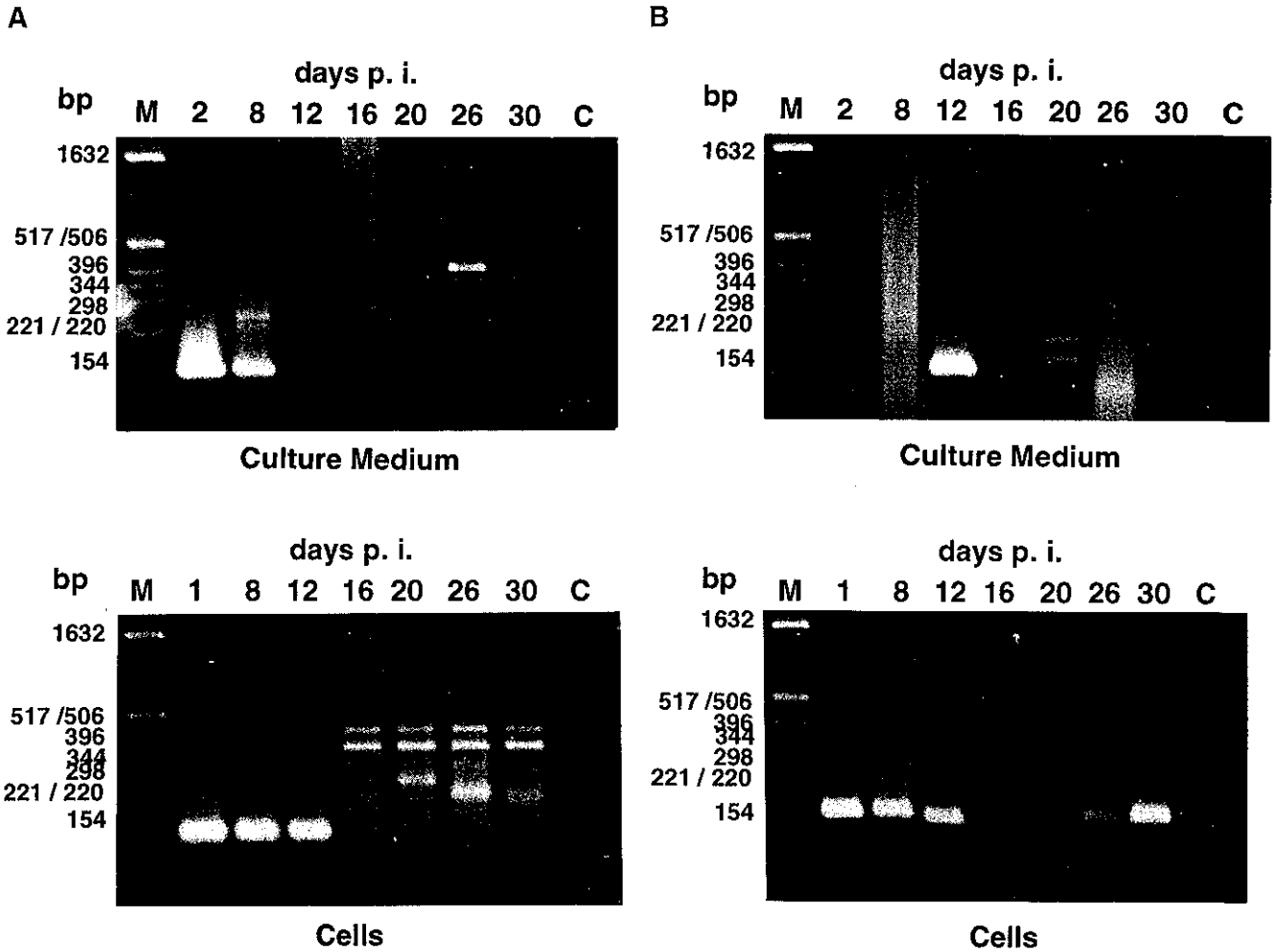

Fig. 1. Detection of HCV RNA by RT-nested PCR in samples from PH5CH cells infected with inoculum 1B-1 or IB-3. The 5′-NC region of positive-stranded HCV RNA was detected in samples from culture medium and cells infected with inocula 1B-1 (A) and 1B-3 (B). One-fourth (0.25 ml) of the culture medium and half of the cells were used for the detection of HCV RNA at each sampling point. RT-nested PCR products (144 bp) were detected by staining with ethidium bromide after separation by 3% agarose gel electrophoresis. PCR products between 200 and 500 bp were not hybridized with the HCV sequence of the 5′-NC region. Lane M, *Hinf* I digests of pBR322 as size markers; Lane C, without RNA sample. Numbers indicate days postinoculation (p.i.).

medium. This result indicates that PH5CH cells are the most susceptible human hepatocyte line to HCV infection that we have examined.[17] Previously, the hepatocyte line Li23 was shown to retain HCV in cells up to 6 days p.i., while HCV RNA in HCV-infected MT-2 non-hepatic cells was detected up to 15 days p.i.[17]

PH5CH cells were more susceptible to infection with inoculum 1B-3 than inoculum 1B-1, although the HCV genome titers of both inocula were almost the same. As shown in Fig. 1B, positive-stranded HCV RNA was consistently detected in the cells over a culture period of 30 days after infection with inoculum 1B-3, although the HCV RNA levels in the cells between 16 to 26 days p.i. were low. The increase of HCV RNA levels in these cells at days 30 p.i. indicated that HCV was replicating in this culture system. The appearance of HCV RNA in the culture medium at 12 days p.i. is further evidence for replication of HCV RNA in these cells (Fig. 1B). We confirmed that the susceptibility of PH5CH cells to HCV infection and the replication of HCV RNA in PH5CH cells were reproducible.

To examine the changes in the amounts of HCV RNA following infection in greater detail, a semi-quantitative analysis was performed.[20] The level of PCR products of

Table I. Semi-quantitative Analysis of HCV Derived from Cells Infected with Inoculum 1B-1 or IB-3

A. *In vitro*-synthesized positive-stranded HCV RNA

Cycles				
15	++	+	−	−
20	++	++	+	−
25	++	++	+	+
30	++	++	++	+
35	++	++	++	++
Copy number of HCV RNA	10^4	10^3	10^2	10^1

B. PH5CH cells (inoculum 1B-1)

Cycles	Days p.i.				
	1	2[a]	8	8[a]	12
15	+	+	−	−	−
20	++	++	+	−	+
25	++	++	+	+	+
30	++	++	+	+	++
35	++	++	++	++	++
Estimated number of HCV RNA	10^3[b] (1/5)[c]	10^3 (1/20)	5×10^1 (1/10)	10^1 (1/20)	10^2 (1/10)
Total amount of HCV	5×10^3	2×10^4	5×10^2	2×10^2	10^3

C. PH5CH cells (inoculum 1B-3)

Cycles	Days p.i.							
	1	8	12	12[a]	16	20	26	30
15	+	−	−	−	−	−	−	−
20	++	+	+	+	−	−	−	+
25	++	+	++	++	−	−	−	+
30	++	++	++	++	−	−	−	++
35	++	++	++	++	+	+	+	++
Estimated number of HCV RNA	10^3[b] (1/5)[c]	10^2 (1/10)	5×10^2 (1/10)	5×10^2 (1/20)	$<10^1$ (1/10)	$<10^1$ (1/10)	$<10^1$ (1/10)	10^2 (1/10)
Total amount of HCV	5×10^3	10^3	5×10^3	10^4	$<10^2$	$<10^2$	$<10^2$	10^3

a) Culture medium
b) Numbers of HCV RNA were estimated by comparison with the pattern of amplification in 2nd PCR using different amounts of *in vitro*-synthesized HCV RNA as shown in (A).
c) The amount of RNA sample used for RT-nested PCR.

the 5′-NC region of HCV RNA was monitored after every five cycles in the second round of PCR. At first, RT-nested PCR using *in vitro*-synthesized HCV RNA encoding the 5′-NC region[21] was performed to obtain a positive standard, and to determine the pattern of gradual amplification according to the copy number of initial HCV RNA as shown in Table IA. This revealed that a significant amplification product was obtained from an original sample containing more than 10 copies of HCV RNA, and that the cycle-dependent amplified pattern was reproducible. By comparison with the pattern obtained for *in vitro*-synthesized HCV RNA, the amount of HCV after inoculation with inoculum 1B-1 or 1B-3 was estimated (Table I, B and C). The results revealed that, with both inocula, about 5×10^3 HCV from the original inoculum (5×10^5 HCV) had been adsorbed on or had invaded the PH5CH cells at 1 day p.i. Intracellular HCV decreased to less than 20% of the initial levels at 8 days p.i. However, since more than 2×10^4 HCV were observed in the culture medium of 1B-1 infected cells up to 8 days p.i., HCV might be able to multiply in this cell culture system for a period of a few days. Alternatively, HCV in the culture medium at 2 days p.i. might be virus released from the cells that had adsorbed HCV. However, this is unlikely, because no HCV was detected in culture medium at 2 days p.i. when inoculum 1B-3 was used. When inoculum 1B-3 was tested, HCV RNA was detected in the culture medium at 12 days p.i. at an estimated level of 10^4 HCV. In addition, at 30 days p.i. HCV increased again to a level of 10^3. These results suggest that replication and multiplication of HCV had occurred in the culture following inoculation.

To investigate further the HCV replication in these cells, we examined HVR1 populations of HCV as previously described.[17] HVR1 of HCV derived at 8 days p.i. from PH5CH cells inoculated with 1B-1 or 1B-3, was amplified by RT-nested PCR and sequenced. Sequences were compared with those of HCV in the original inoculum. In the second round PCR, we used a newly designed reverse primer 280RA [5′-CTGTTGATGTGCCAGCTGCC-3′; corresponding to positions 1581 to 1600 of HCV-J[5]] to replace the primer 280R [5′-GTTGATGT-

Table II. Deduced Amino Acid Sequences of HVR1 Populations Obtained from Inoculum 1B-1 and from PH5CH Cells at 8 Days p.i.

HVR1 sequence	Species	Numbers of clones
Inoculum 1B-1		
HTHVTGGVQAYTTHGFTSLFRRGASQT	I -1 (A-2)	10
T----------HGAY-LA---NV-PH-K	II-1	5
----------S	I -2 (A-1)	2
N--------AGRNAYRI-----SF-P--N	III-1 (B-5)	2
N--------AGRNAYRI-----TI-PA-N	III-3	2
V--------AGRSAYRI--I-NL-P--N	III-6 (C-1)	2
N--------V-RNAYRI-TFLNP-PA-N	IV-4	2
-A----------	I -3	1
T----------	I -4	1
----------SF-P--N	I -5	1
T----------HGAY-LA---SF-P--N	II-2	1
---------A-RNAYRI--I-SF-P--N	III-2	1
N--------AGRNAYRI-----TIRPA-N	III-4	1
V--------AGRSAYRI-----NV-PH-K	III-5	1
N--------V-RNAYR--TFLNP-PA-K	IV-1	1
N--------V-RNAYR--TFLNP-PA-N	IV-2 (B-3)	1
N--------V-RNAYRI-TFLNP-SA-N	IV-3	1
D--------V-RNAYRI-TFLNP-PA-N	IV-5	1
N--------AGRNAYRI-TFLNP--A-N	IV-6	1
N---M----V-RSAYRI--FLSP--A-N	IV-7 (B-1)	1
N---M----V-RSAYRI---LSP--A-N	IV-8	1
V--------V-RNAYRI---LSP--A-N	IV-9	1
Cell 8 days p.i.		
N--------AGRNAYRI-----SF-P--N	III-1	5
----------	I -1	2
----------S	I -2	1
I----------HGAY-LA----------	II-3[a)]	1
N--------AGRNAYRI--F-TI-PA-N	III-7[a)]	1

HVR1 populations were assigned by multi-alignment analysis using the unweighted pair-grouping method[23)] in the GENETYX-MAC program, and each HVR1 species was named according to the position in the phylogenetic tree obtained (data not shown). The parentheses show the previous names from Ref. 17. The number on the right side indicates the actual number of plasmid clones obtained and sequenced.

a) Novel species obtained from the infected cells.

GCCAGCTGCCGTTGGTG-3′; positions 1574 to 1598 of HCV-J[5)]] previously described,[17)] because primer 280RA has a higher degree of homology to sequences of HCV strains registered in the DDBJ/EMBL/GenBank data bases. PCR products were cloned into the pTZ19R plasmid vector as previously described.[22)] Nucleotide sequences were determined by the dideoxy nucleotide chain termination method using an A.L.F. DNA sequencer (Pharmacia, Uppsala, Sweden).

Table II shows the HVR1 populations derived from the original inoculum 1B-1 and PH5CH cells at 8 days p.i. A complex pattern of quasi-species of HVR1 populations was obtained from this inoculum. Using the multi-alignment analysis of 40 independent HVR1 cDNA clones by the GENETYX-MAC program (unweighted pair-grouping method[23)]), four subgroups (I to IV) and 22 distinct species were identified. All HVR1 species were renamed I-1 to IV-9, as shown in Table II, and a number of these species corresponded to HVR1 species detected in a previous study.[17)] The HVR1 populations obtained by using primer 280RA were more complex than those obtained by primer 280R. Five distinct HVR1 species, III-1 (5 clones), I-1 (2 clones), I-2 (1 clone), II-3 (1 clone) and III-7 (1 clone), were obtained from the cells at 8 days p.i. (Table II). The frequency of the species III-1 had increased to 50% in the infected cells, from a 5% frequency in the original inoculum 1B-1. This suggested that the species III-1 of HCV selectively proliferated in PH5CH cells. A similar phenomenon was observed in HCV-infected MT-2 cells[17)] and a T cell line, HPB-Ma,[24)]

Table III. Deduced Amino Acid Sequences of HVR1 Populations Obtained from Inoculum 1B-3 and from PH5CH Cells at 8 Days p.i.

HVR1 sequence	Species	Numbers of clones
Inoculum 1B-3		
G Y H V T G G T Q A R T T Q G L V S L F T R G P S Q K	I -1	6
– S P – V V –	II -1	4
– T R –	I -2	2
– T –	I -3	1
– – R –	I -4	1
– T – – – – – G A S – – – – – – – – – – – – – – – –	III-1	1
Cell 8 days p.i.		
– –	I -1	10

HVR1 populations were assigned by the same method as in Table II.

although HVR1 species obtained from PH5CH cells did not become a homogeneous population, as observed in the case of MT-2 cells.[17]

Sequence analysis of HVR1 derived from PH5CH cells infected with the inoculum 1B-3 gave a simpler result than that in the case of inoculum 1B-1 (Table III). Analysis of 15 independent cDNA clones revealed three subgroups (I to III), and 6 distinct species were obtained from this inoculum. As shown in Table III, HVR1 populations from the cells at 8 days p.i. became homogeneous as species I-1, although species I-1 amounted to only 40% in the original inoculum 1B-3. This result suggests that HCV species I-1 was replicating in these cells, and is consistent with observations in MT-2 cells.[17] In the sequence analyses of HVR1 populations in both cases, no HVR1 cDNA clones containing terminal codons or deletions were obtained. In conclusion, these results strongly suggest that HCV is able to replicate and multiply in the human hepatocyte line, PH5CH. However, the mechanism of the selection of HVR1 species during the viral replication is still not clear, although it is possible that the selection of HCV in the inoculation depends on the presence of specific antibodies against each HVR1 species, as proposed previously.[11, 12] Further analysis is needed to clarify this point. The HCV replication and multiplication system using human immortalized non-neoplastic hepatocytes obtained in this study will be useful for the development of anti-viral reagents including neutralizing antibodies, and for the analysis of genetic alterations in virally infected human hepatocytes.

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