Antiviral Research xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Antiviral Research

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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ARTICLE INFO

Article history:
Received 8 April 2009
Received in revised form 19 October 2009
Accepted 18 December 2009

Keywords:
Hepatitis C virus
Replication
Ribavirin
Drug resistance

ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

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1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents (Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN-α were purchased from MP Biomedicals (Eschwege, Germany) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

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0166-3542/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.antiviral.2009.12.008

Please cite this article in press as: Hmwe, S.S., et al., Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin. Antiviral Res. (2010), doi:10.1016/j.antiviral.2009.12.008

Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position[a]	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCCAG	7220–7200	Antisense

[a] Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO_2 atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super ScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS34AB region and JF2AS for NS5AB region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep[R] Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH/Luc-P1969S, -JFH/Luc-V2405A and -JFH/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 μg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975uFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC_{50} (50% effective concentration) values of 15–225 μM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 μM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon

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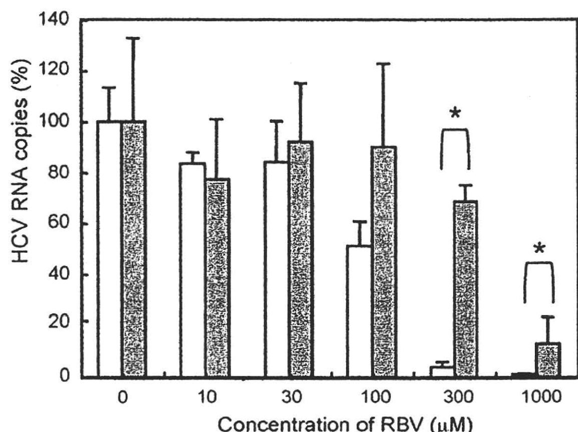

Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV: gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. $^{*}p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM, respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concentrations of RBV. EC_{50} values were calculated to be 93 and 449 μM, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN-α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

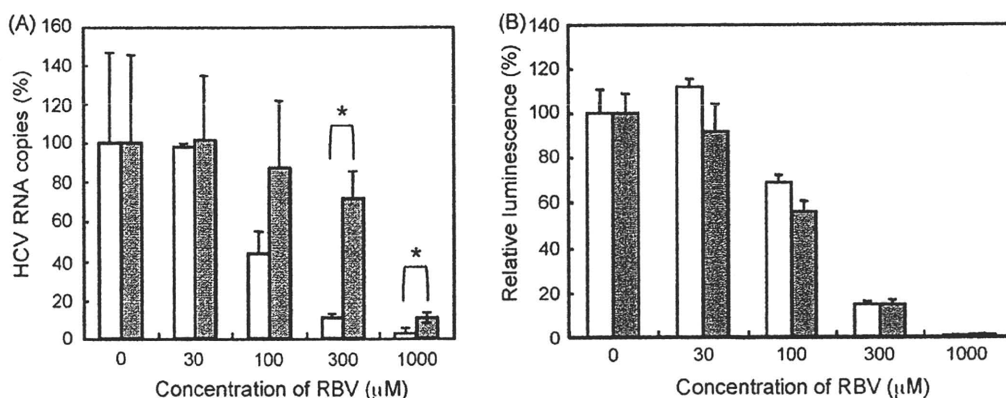

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. $^{*}p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN-α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

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Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})	No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9[b]
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7[c]
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2[c]
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

[a] Values are means ± standard deviations.
[b] $p<0.05$ relative to No-treatment by the unpaired t-test.
[c] $p<0.01$ relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. *Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility*

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μM RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported

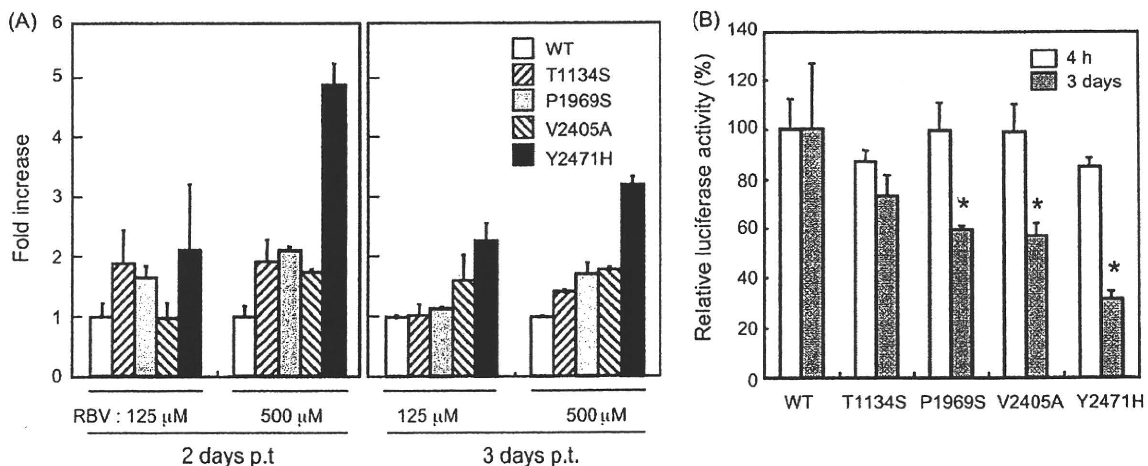

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. *$p<0.05$ against WT.

that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to "error catastrophe", (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction in vivo resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

Acknowledgments

We thank M. Matsuda, S. Yoshizaki, M. Ikeda, T. Shimoji, M. Kaga and M. Sasaki for their technical assistance. This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology, and by Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, Japan and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan. S.S.H. is the recipient of a Research Resident Fellowship from Viral Hepatitis Research Foundation of Japan.

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Please cite this article in press as: Hmwe, S.S., et al., Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin. Antiviral Res. (2010), doi:10.1016/j.antiviral.2009.12.008

JOURNAL OF VIROLOGY, July 2007, p. 7208–7219 Vol. 81, No. 13
0022-538X/07/$08.00+0 doi:10.1128/JVI.01774-06
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In Vivo Study of the HC-TN Strain of Hepatitis C Virus Recovered from a Patient with Fulminant Hepatitis: RNA Transcripts of a Molecular Clone (pHC-TN) Are Infectious in Chimpanzees but Not in Huh7.5 Cells[▽]

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Received 16 August 2006/Accepted 28 March 2007

Both viral and host factors are thought to influence the pathogenesis of hepatitis C virus (HCV) infection. We studied strain HC-TN (genotype 1a), which caused fulminant hepatic failure in a patient and, subsequently, severe hepatitis in a chimpanzee (CH1422), to analyze the relationship between disease severity, host immune response, viral evolution, and outcome. A second chimpanzee (CH1581) was infected from CH1422 plasma, and a third chimpanzee (CH1579) was infected from RNA transcripts of a consensus cDNA of HC-TN (pHC-TN). RNA transcripts of pHC-TN did not replicate in Huh7.5 cells, which were recently found to be susceptible to infection with another fulminant HCV strain (JFH1). The courses of viremia were similar in the three animals. However, CH1581 and CH1579 developed a less severe acute hepatitis than CH1422. CH1579 and CH1422 resolved the infection, whereas CH1581 became persistently infected. CH1579 and CH1581, despite their differing outcomes, both developed significant intrahepatic cellular immune responses, but not antibodies to the envelope glycoproteins or neutralizing antibodies, during the acute infection. We analyzed the polyprotein sequences of virus recovered at five and nine time points from CH1579 and CH1581, respectively, during the first year of follow-up. High mutation rates and high proportions of nonsynonymous mutations suggested immune pressure and positive selection in both animals. Changes were not selected until after the initial decrease in virus titers and after the development of immune responses and hepatitis. Subsequently, however, mutations emerged repeatedly in both animals. Overall, our results indicate that disease severity and outcome of acute HCV infection depend primarily on the host response.

Acute hepatitis C is often asymptomatic. However, the disease burden of hepatitis C virus (HCV) is very significant, since about 170 million people worldwide are chronically infected, leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in a significant proportion of infected individuals. HCV is now the leading cause of liver transplantation (35). The chimpanzee is the only animal model in which to study the natural history of HCV (4). Experimental infections permit collection of frequent samples, including liver tissue samples, before infection and during the acute phase and thus permit studies of early virological and immunological events that define the outcome. Furthermore, by inoculating chimpanzees intrahepatically with RNA transcripts of infectious HCV clones it is possible to study monoclonal virus infections (20, 45–47), in which virus interaction with the host is not initially influenced by a viral quasispecies. Also, in the study of the association between HCV and pathogenesis, infection from a molecular clone eliminates the possibility that an observed phenotype is caused by a coinfecting agent.

Fulminant hepatitis caused by hepatotropic viruses is a rare but potentially fatal condition. Initially, HCV was not recognized as an etiological agent of fulminant hepatitis (43). However, a significant number of Japanese patients with fulminant hepatitis had evidence of HCV infection (30, 44). Subsequently, the temporal relationship between transfusion-acquired HCV (genotype 1b) infection and development of fulminant hepatitis was described (12). Certain HCV strains, including strain HC-TN, recovered from a patient with fulminant hepatitis, appear to be associated with the development of severe hepatitis (13, 19). To study the relationship between HC-TN and disease phenotype, we transmitted this strain to chimpanzees and constructed an infectious clone to investigate monoclonal infection in a transfected chimpanzee.

The host and viral factors that determine the outcome of primary HCV infection are poorly understood. The host re-

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▽ Published ahead of print on 4 April 2007.

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solves less than 30% of infections. Viral clearance is associated with vigorous cellular immune responses (10, 24, 39), but persistence may be associated with viral escape from such T-cell responses (11). Others found that the development of antibodies to the envelope 2 (E2) hypervariable region 1 (HVR1), which contains a neutralization epitope, was associated with clearance (1, 50). Finally, genetic heterogeneity, in particular in HVR1, might predict the outcome of acute HCV (14).

Previously, we used chimpanzees to study the virological and immunological correlates of disease and outcome of acute HCV infection (16, 37, 38). Animals with viral clearance or with transient clearance followed by persistence at low titers had significant intrahepatic $CD4^+$ and $CD8^+$ T-cell responses, as well as induction of gamma interferon and gamma interferon-induced genes in the liver. Thus, the initial control of HCV is mediated by intrahepatic cellular immune responses. However, it is still unclear why animals with significant intrahepatic responses can have different outcomes. In the present study, we found that two chimpanzees infected with the HC-TN strain had comparable courses of viremia and vigorous host cellular immune responses. However, the infection resolved in only one animal. A detailed sequence analysis of viruses recovered from these animals was undertaken to determine the potential role of virus evolution in the outcome of acute HCV.

MATERIALS AND METHODS

Source of HCV strain HC-TN. HCV strain HC-TN was from a patient who developed fulminant hepatic failure twice after liver transplantation for cryptogenic liver cirrhosis (13); she apparently became infected with HCV after receiving red blood cells before transplantation. A chimpanzee (CH1422) was inoculated with 100 μl of serum obtained 5 days before the first liver transplantation (see Fig. 1A) (13). (The data in Fig. 1A were adapted from Farci et al. [13], but the qualitative and quantitative HCV RNA tests were performed in this study.) A pool of HC-TN virus was made from plasmapheresis units collected from CH1422 during weeks 4 to 6 postinoculation.

Amplification, cloning, and sequence analysis. RNA extracted from 100-μl aliquots of the HC-TN pool with a TRIzol LS system (Life Technologies, Gaithersburg, MD) was denatured at 65°C for 2 min. HCV cDNA was synthesized at 42°C for 1 h with Superscript II reverse transcriptase (Life Technologies) and specific reverse primers. The cDNA was treated with RNase H and RNase T1 (Life Technologies) (46). To clone the entire open reading frame (ORF), we performed a one-round long PCR using an Advantage PCR polymerase mix (Clontech, Palo Alto, CA) (46). Gel-purified amplicons were A tailed with *Taq* DNA polymerase (Life Technologies) at 72°C for 1 h and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). DH5α-competent cells (Life Technologies) were transformed and selected on LB agar plates containing 100 μg/ml ampicillin (Stratagene, La Jolla, CA) and amplified in LB liquid cultures at 30°C (46). A region spanning from nonstructural 5B (NS5B) to the conserved region of the 3′ untranslated region (UTR) was amplified by nested PCR with an Advantage 2 PCR polymerase mix and cloned as described previously (46). Final DNA preparations were sequenced using standard procedures.

The 5′ terminus was amplified from serum by 5′ rapid amplification of cDNA ends (RACE) with dC or dA tailing (Life Technologies) and three antisense C primers (615R [5′-CGCAACCCTCATTGCCATAG-3′] for reverse transcription [RT], 519R [5′-CTCGAGGTTGCGACCGCTCGGAAG-3′] for the first PCR, and 433R+Kpn-I [5′-CGGGGTACCACGATCTGACCGCCACCCGGG AAC-3′] for the second PCR). To determine the 3′-terminal sequence, the 5′ end of the negative-strand HCV RNA extracted from liver homogenate obtained from CH1581 was amplified by 5′ RACE with dC tailing and specific primers (−351R [5′-TGGTTCACGGCTGGCTACAG-3′] for RT, −334R [5′-CAGCG GGGGAGACATTTATCACAG-3′] for the first PCR, and −315R [5′-CACAG CGTGTCTCATGCCCGGCCC-3′] for the second PCR). The PCR products were cloned into pCR2.1-TOPO (Invitrogen).

To determine the consensus sequence of the entire ORF of HC-TN recovered from chimpanzees, we used two procedures. In serum samples with titers of $>10^5$ IU/ml, we performed long RT-PCR followed by nested PCR with genotype 1a-specific primers of 10 fragments (46). In samples with titers of $<10^5$ IU/ml, we performed RT-nested PCR, with *Taq* Gold DNA polymerase (Perkin Elmer, Wellesley, MA) (5), of 19 fragments by use of 1a-specific primers. The numbers of observed synonymous substitutions (ds) and nonsynonymous substitutions (dn) and the ratios of synonymous to nonsynonymous substitutions (ds/dn) were calculated using the Syn-SCAN program (http://hivdb6.stanford.edu/synscan/synscancgi).

Full-length consensus cDNA clone of HC-TN. pHC-TN was constructed by standard molecular techniques using three clones that contained the ORF, one clone that contained the variable and poly(U-UC) regions of the 3′ UTR, and pCV-H77C (46). Large-scale preparation of a single clone was performed with a QIAGEN (Valencia, CA) Endofree maxi kit (46). The final DNA had the expected sequence.

Experimental infection of chimpanzees. The housing and care of chimpanzees were in compliance with relevant guidelines and requirements (32). CH1581 was inoculated intravenously with dilutions of the CH1422 pool. CH1579 was inoculated intrahepatically by a percutaneous procedure (47) with RNA transcribed by T7 RNA polymerase (Promega, Madison, WI) from 20 μg of XbaI-digested pHC-TN (46). Serum samples were collected once or twice weekly and tested for HCV RNA (Monitor 2.0; Roche Diagnostics, Indianapolis, IN), HCV antibodies (ELISA 2.0; Abbott, Chicago, IL), and alanine aminotransferase (ALT) (Anilytics, Gaithersburg, MD). Monitor-negative samples were tested by a more sensitive RT-nested PCR (5). Samples obtained by weekly liver biopsies were examined for necroinflammatory changes (7).

We tested for anti-E1 by use of an enzyme-linked immunosorbent assay (ELISA) with recombinant E1 protein (amino acids [aa] 192 to 329) expressed from strain H77 (2, 29) and for anti-E2 by use of an ELISA with recombinant E2 protein (aa 388 to 664) of strain H, provided by I. K. Mushahwar (Abbott) (25, 29). Antibodies against E2 HVR1 were assayed with an ELISA using a biotinylated HC-TN-specific peptide (aa 384 to 410) (29). The percent neutralization in postinfection sera, compared with that in the preinoculation sample, was determined with a retroviral HCV pseudovirus assay using ppH77(1a) (provided by Francois-Loic Cosset, Ecole Normale Superieure de Lyon, Lyon, France), as described in detail previously (29).

The details of protocols used to detect cellular immune responses were published previously (38, 39). Peripheral blood mononuclear cells (PBMC) were isolated from 40 ml of blood. Liver-infiltrating lymphocytes were isolated from liver tissue obtained by needle biopsy. Cell suspensions were incubated with magnetic beads coupled to anti-CD4 or anti-CD8, and bound $CD4^+$ or $CD8^+$ T cells were isolated using a magnetic particle concentrator and next expanded for 2 weeks. PBMC or polyclonally expanded $CD4^+$ T cells were tested for HCV-specific proliferative capacity after 6 days of culture with HCV-1 proteins (C22, C33-c, c100, and NS5), provided by M. Houghton (Chiron, Emeryville, CA). ^{3}H[thymidine] was added for 16 h, and the mean levels of thymidine incorporation in the HCV protein-stimulated and control cultures were used to calculate the stimulation indexes (SI); values of >2.0 were considered positive. Polyclonally expanded $CD8^+$ T cells were tested by intracellular gamma interferon staining after 5 h of stimulation with autologous Epstein-Barr virus-immortalized B-cell lines that were infected with recombinant HCV H77-encoding vaccinia viruses vHCV(1–1488) or vHCV(827–3011) together with VTF7, provided by C. M. Rice (Rockefeller University, New York, NY), or with VTF7 alone. The frequency of HCV-specific $CD8^+$ T cells was defined as the percentage of $CD8^+$ T cells that produced gamma interferon in response to stimulation by B-cell lines coinfected by vHCV and VTF7 after subtraction of the gamma interferon-positive, $CD8^+$ T cells detected after stimulation in the absence of vHCV.

Transfection of Huh7.5 cells with RNA transcripts from pHC-TN. Huh7.5 cells, provided by C. M. Rice (Rockefeller University, New York, NY), were maintained in growth medium consisting of complete Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 50 IU/ml penicillin G, and 50 μg/ml streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 incubator. RNA was transcribed, as described above, from pJFH1 and pHC-TN digested with XbaI; the pJFH1 plasmid was provided by Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan).

Transfection was performed using a DMRIE-C reagent (Invitrogen) in six-well plates (4×10^5 Huh7.5 cells/well). Briefly, cells were washed with 2 ml of Opti-MEM I medium (Gibco). Eight microliters of DMRIE-C reagent was first diluted in 1 ml of Opti-MEM I medium before the addition of a transcription mixture containing approximately 3 μg of RNA transcripts (based on gel analysis). Finally, the complexed RNA was incubated with the washed Huh7.5 cells at 37°C for 4 h and the medium was replaced with complete growth medium. For immunofluorescence staining with mouse anti-HCV core protein monoclonal antibody (B2) (Anogen, Mississauga, Ontario, Canada), the Huh7.5 cells were trypsinized, transferred to eight-well chamber slides, and incubated at 37°C

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FIG. 1. Course of infection with HCV strain HC-TN in (A) CH1422 (first-passage polyclonal infection), (B) CH1581 (second-passage quasipolyclonal infection), and (C) CH1579 (pHC-TN monoclonal infection). Serum samples collected once or twice weekly were tested for HCV RNA by an in-house RT-nested PCR with 5′ UTR primers and/or by use of a Roche Monitor 2.0 test. Red rectangle, positive by RT-nested PCR and/or by Monitor; white rectangle, negative by RT-nested PCR in two independent assays. The orange dots represent HCV Monitor titers; samples below the detection limit of 600 IU/ml (indicated by the dotted line) are shown as not detected (ND). Seroconversion in the second-generation ELISA is represented by a green horizontal bar. Yellow-shaded area, serum ALT. For liver histology, necroinflammatory changes of liver biopsy samples are graded 0 (normal), 1 (mild), 2 (mild to moderate), 3 (moderate to severe), or 4 (severe).

overnight. The cells were washed twice with phosphate-buffered saline (PBS) and fixed and permeabilized with acetone for 3 min. Twenty-five microliters of a 1/200 dilution (in 5% bovine serum albumin in PBS) of the HCV anticore antibody was added to each grid and incubated at room temperature for 20 min. After a wash with PBS, a 1/100 dilution of the secondary antibody, anti-mouse immunoglobulin G (heavy plus light chains) fluorescein isothiocyanate-conjugated antibody (Pierce), was added to each grid and incubated at room temperature for 3 min. A drop of VectaShield containing DAPI (4′,6′-diamidino-2-phenylindole) was added to each grid of cells to stain nuclei. Slides were read with an Axioscope 2 Plus fluorescence microscope (Zeiss).

Nucleotide sequence accession number. The nucleotide sequence of pHC-TN (an infectious clone of strain HC-TN) has been deposited in the GenBank database under the accession number EF621489.

RESULTS

We studied a genotype 1a strain (HC-TN) of HCV recovered from a patient who developed fulminant hepatic failure (13). Following transmission to CH1422, it caused severe hepatitis, with an ALT peak of 744 IU/liter and with an unusually pronounced necroinflammatory activity in liver biopsy samples (Fig. 1A). Serum HCV titers reached 10^5 to $10^{5.5}$ IU/ml during weeks 4 to 9, followed by a dramatic decrease and clearance during week 20. We did not have any remaining sera from the patient used to inoculate CH1422. Thus, to further investigate the phenotype of the HC-TN strain, we performed a second passage, this time to CH1581 (Fig. 1B). Furthermore, we constructed a full-length consensus cDNA clone, the infectivity of which was confirmed in CH1579 (Fig. 1C), to study the phenotype of monoclonal infection with this particular HCV strain.

Genetic analysis of strain HC-TN. We analyzed the HC-TN sequence from the CH1422 plasma pool. To determine the consensus ORF, we directly sequenced amplicons obtained by long RT-PCR followed by PCR of 10 overlapping fragments. In addition, we analyzed three clones obtained from the long-RT-PCR amplicons. The ORF consisted of 9,033 nt encoding 3,011 aa. The genome population in CH1422 was virtually homogeneous, since heterogeneity was found among the three

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TABLE 1. Differences in nucleotide and predicted amino acid sequences between HC-TN and other genotype 1a strains

Genomic region	nt position[a]	No. (%) of nt differences				aa position[a]	No. (%) of aa differences			
		H77C	HCV-1	HC-J1	HCV-UK		H77C	HCV-1	HC-J1	HCV-UK
5′ UTR	1–341	1 (0.3)	0 (0.0)	2 (0.6)	2 (0.6)					
Core	342–914	7 (1.2)	13 (2.3)	11 (1.9)	15 (2.6)	1–191	1 (0.5)	3 (1.6)	3 (1.6)	3 (1.6)
E1	915–1490	24 (4.2)	34 (5.9)	43 (7.5)	39 (6.8)	192–383	6 (3.1)	9 (4.7)	11 (5.7)	9 (4.7)
E2	1491–2579	70 (6.4)	76 (7.0)	81 (7.4)	150 (13.8)	384–746	25 (6.9)	28 (7.7)	28 (7.7)	48 (13.2)
HVR1	1491–1571	12 (14.8)	13 (16.0)	15 (18.5)	32 (39.5)	384–410	7 (25.9)	9 (33.3)	8 (29.6)	15 (55.6)
p7	2580–2768	9 (4.8)	10 (5.3)	11 (5.8)	22 (11.6)	747–809	2 (3.2)	3 (4.8)	4 (6.3)	5 (7.9)
NS2	2769–3419	36 (5.5)	42 (6.5)	47 (7.2)	65 (10.0)	810–1026	7 (3.2)	8 (3.7)	9 (4.1)	18 (8.3)
NS3	3420–5312	86 (4.5)	90 (4.8)	109 (5.8)	143 (7.6)	1027–1657	12 (1.9)	14 (2.2)	16 (2.5)	18 (2.9)
NS4A	5313–5476	8 (4.8)	9 (5.5)	9 (5.5)	17 (10.4)	1658–1711	1 (1.9)	1 (1.9)	1 (1.9)	3 (5.6)
NS4B	5477–6257	32 (4.1)	27 (3.5)	44 (5.6)	67 (8.6)	1712–1972	6 (2.3)	4 (1.5)	9 (3.4)	11 (4.2)
NS5A	6258–7600	67 (5.0)	74 (5.5)	86 (6.4)	108 (8.0)	1973–2420	23 (5.1)	21 (4.7)	25 (5.6)	30 (6.7)
NS5B	7601–9374	53 (3.0)	62 (3.5)	73 (4.1)	101 (5.7)	2421–3011	5 (0.8)	9 (1.5)	8 (1.4)	19 (3.2)
ORF	342–9374	392 (4.3)	437 (4.8)	514 (5.7)	727 (8.0)	1–3011	88 (2.9)	100 (3.3)	114 (3.8)	164 (5.4)
3′ UTR	9375–9599									
Variable region	9375–9417	1 (2.3)	0 (0.0)	3 (7.0)	NA[b]					
Conserved region	9499–9599	0 (0.0)	NA	NA	NA					

[a] The nucleotide and predicted amino acid positions correspond to those of pHC-TN.
[b] NA, not available.

clones at only 26 (0.29%) nucleotide and 20 (0.66%) amino acid positions. Also, the clones had identical sequences within HVR1. The consensus sequence deduced from the ORF clones was identical to that obtained by direct sequencing. It differed from those of other 1a strains (9, 23, 33, 46) by 4.3% to 8.0% and by 2.9% to 5.4% at the nucleotide and amino acid levels, respectively (Table 1). A tree analysis of the polyprotein sequence of representative HCV isolates (9, 23, 31, 33, 34, 45–47) showed that HC-TN was most closely related to the prototype strains HCV-1 and H77 (Fig. 2). Since the polyprotein cleavage sites were highly conserved among the 1a strains, the HC-TN gene products are predicted to be the same as those of strain H77 (17, 26).

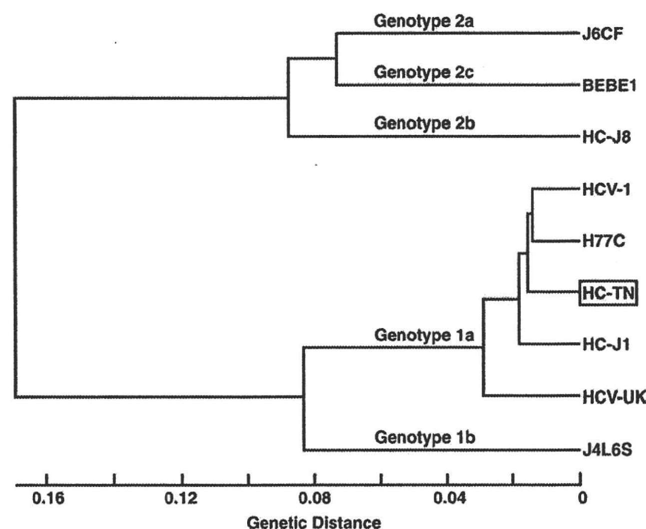

FIG. 2. Tree analysis of predicted polyprotein sequences of HC-TN (boxed) and other HCV strains. The multiple sequence alignment and tree analysis were performed with GeneWorks (6).

To analyze the 5′ UTR of HC-TN, we performed 5′ RACE and sequenced 10 and 3 clones (nucleotides [nt] 1 to 409) obtained following dC and dA tailing, respectively. All clones had identical 5′-terminal sequences, and the remainder of the 5′ UTR was highly conserved. The HC-TN 5′ UTR sequence was identical to that of HCV-1 (18). To analyze the 3′ UTR, we first sequenced 12 clones of PCR products, which included the variable and the poly(U-UC) regions. The variable region consisted of 43 nt (nt 9375 to 9417), including two in-frame termination codons. All clones had identical sequences except at position 9391 (11 C, 1 T). The poly(U/UC) regions varied in length (76 to 148 nt), entirely due to variation of the poly(U) regions (41 to 113 nt). The poly(UC) regions had the same length (35 nt), and the sequences (5′-CUUUUUCCCUCUU UUUCUUCUCUUUUUCCUUCUUU-3′) were identical in all 12 clones except at position 3 (11 U, 1 C). Furthermore, we found that this region had the same sequence in the five clones from negative-strand RNA extracted from CH1581 liver. The sequence of the conserved region was determined by 5′ RACE (dC tailing) on the negative-strand RNA extracted from CH1581 liver homogenate collected at week 8. Five clones (nt 9410 to the 3′ terminus) analyzed had the same 3′-terminal sequences, and the consensus sequence (101 nt) was identical to that of strain H77 (21).

Infectivity titration of HC-TN plasma pool. The pool collected from CH1422 during weeks 4 to 6 had an HCV genome titer of $\sim 10^5$ IU/ml (Monitor 2.0, $10^{5.3}$ IU/ml; Versant HCV RNA b-DNA 3.0 [Bayer, Tarrytown, NY], $10^{5.0}$ IU/ml). Its infectivity titer was determined by reverse titration in CH1581. The 10^{-6} dilution was noninfectious. However, HCV was transmitted to CH1581 by inoculation of 1 ml of a 10^{-5} dilution (Fig. 1B), indicating an infectivity titer of $\sim 10^5$ chimpanzee infectious doses/ml. We analyzed the ORF of HCV recovered from the serum of CH1581 at week 8. Differences between the CH1581 sequence and the consensus sequence of

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A

Chimpanzee 1581: Course of Infection with Strain HC-TN

HCV-RNA

Liver Histology (0-4)

Acute phase plasma pool, CH1422

10^{-5} Dilution

ALT (IU/L)

HCV Titer (log_{10}IU/ml) Monitor 2.0

Evolution of HC-TN

Serum Neutralizing Antibodies

Neutralization (%)

Peripheral CD4+ T cell response

Stimulation Index

C22
C33-c
C100
NS5

Intrahepatic CD4+ T cell response

Stimulation Index

Intrahepatic CD8+ T cell response

% IFN-γ +/CD8+ T cells

vv 1-1488
vv 827-3011

Week Post Inoculation

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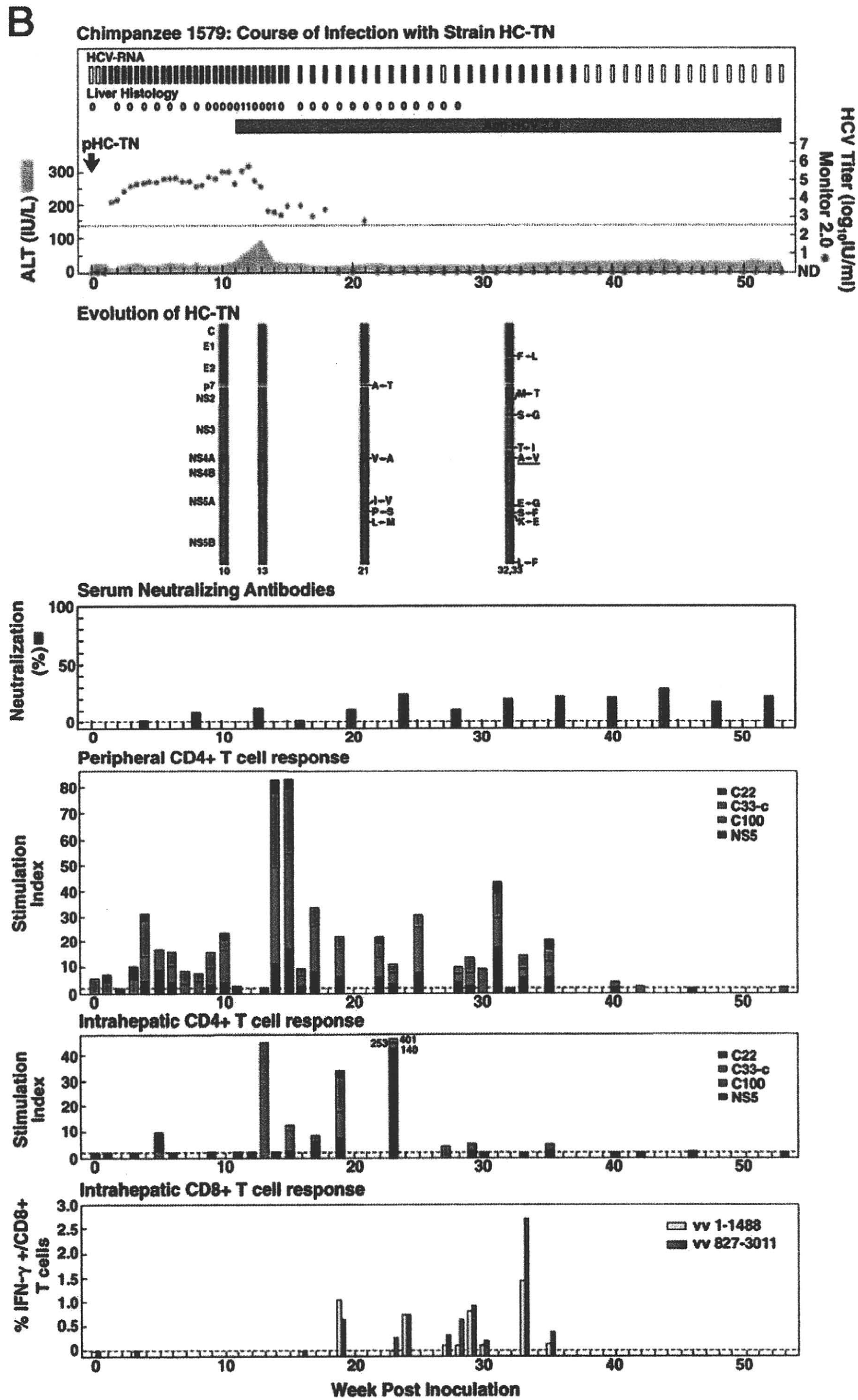

FIG. 3. Course of infection, viral evolution, and immune responses in (A) CH1581 and (B) CH1579, which were infected with HCV strain HC-TN (quasipolyclonal and monoclonal infections, respectively). See the legend for Fig. 1 for the details of the course of infection. Representing the evolution of HC-TN, the HC-TN genome is shown as a vertical bar with the core (C) at the top and NS5B at the bottom. Solid black lines with capital letters indicate new amino acid changes that were identified when a sequence was compared with the sequence obtained at the previous time point. Underlined capital letters indicate mutations that had occurred by one time point but had changed back to the original sequence by the next time point analyzed. Solid black lines without capital letters represent amino acid changes that persisted. The week the sequence was analyzed is indicated at the bottom of each genome. For neutralizing antibodies, the percent neutralization of retroviral pseudovirus particles bearing the HCV envelope proteins (>50% was considered significant) is shown. The peripheral and intrahepatic $CD4^+$ T-cell responses to core (red), NS3 (orange), NS3-NS4 (green), and NS5 (blue) are shown as specific SI. A specific SI of >2 was considered significant. At weeks tested in which the SI was ≤2 against all four antigens, the negative result is indicated by a black bar (with a value of 2). The intrahepatic $CD8^+$ T-cell response is represented as the percentage of intrahepatic $CD8^+$ T cells that produced gamma interferon (IFN-γ) after stimulation with transiently expressed HCV proteins, as described in Materials and Methods. vv 1–1488, vaccinia virus vHCV(1–1488); vv 827–3011, vaccinia virus vHCV(827–3011).

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the CH1422 virus used as the inoculum were found at only two nucleotide positions (A1535G and G6531A) and resulted in one NS5A amino acid change (A2064T). Both substitutions were also found at week 1.

In CH1581, HCV RNA titers peaked at ~10^6 IU/ml during weeks 5 to 10, followed by a 3-$\log_{10}$ decrease in viremia titers during weeks 10 to 15 (Fig. 3A). The titers were frequently below the detection limit of the Monitor test ($10^{2.8}$ IU/ml) during weeks 15 to 37. Furthermore, a sensitive RT-nested PCR test was negative at weeks 16, 24, 29, and 30, and during weeks 38 to 52, the HCV titers remained at 10^3 to 10^4 IU/ml. The second-generation ELISA became positive at week 10. However, the animal did not develop anti-E1, anti-E2, or anti-HVR1 until after 45 weeks of follow-up (29). Furthermore, the chimpanzee did not develop significant (≥50% neutralization) neutralizing antibodies during the first year of follow-up (29) (Fig. 3A). The chimpanzee developed acute hepatitis, with a peak ALT level of 296 IU/liter (week 10). Mild necroinflammatory changes were detected in liver biopsy samples at weeks 10, 22, and 24, as well as during the persistent phase of infection.

RNA transcripts from a consensus clone (pHC-TN) are infectious in vivo but not in vitro. In pHC-TN, the core sequence of the T7 promoter and the consensus sequence of HC-TN (9,599 nt) were contained in the NotI/XbaI-digested pGEM-9Zf vector. It contained a 5′ UTR of 341 nt, an ORF of 9,033 nt, and a 3′ UTR with a variable region of 43 nt followed by an 81-nt poly(U/UC) tract (46 U, 35 UC) and a 3′-terminal conserved sequence of 101 nt. RNA transcripts of pHC-TN were percutaneously injected into the liver of CH1579. For unknown reasons, the first in vivo transfection failed. However, following a subsequent transfection 4 weeks later with new RNA transcripts, the animal became infected (Fig. 3B). The HCV ORF sequence recovered from week 10 sera was identical with that of pHC-TN. The HCV titers reached peak levels of 10^5 to $10^{5.5}$ IU/ml during weeks 4 to 13, followed by a significant decrease. The quantitative Monitor test was negative during weeks 22 to 53. However, the qualitative RT-nested PCR test remained positive until week 37. Thus, this chimpanzee had prolonged acute resolving HCV infection. The animal became positive in the second-generation ELISA at week 11 but did not develop anti-E1, anti-E2, or anti-HVR1 antibodies. Furthermore, the animal did not develop neutralizing antibodies (29) (Fig. 3B). It had elevated ALT only during weeks 12 to 13 (peak, 90 IU/liter) and mild histological changes in liver biopsy samples during weeks 11 to 18.

A molecular clone of strain JFH1, also recovered from a patient with fulminant hepatitis C, was recently found to be infectious in Huh7 and Huh7.5 cells (41, 49). We transfected Huh7.5 cells with RNA transcripts from pJFH1 and pHC-TN (Fig. 4). We also included JFH1 chimeras, which contain core through NS2 from HC-TN (data not shown). Clear evidence of replication was observed with JFH1 and TN/JFH1 chimeras, but there was no evidence of HCV replication with HC-TN. The fact that replication of the TN/JFH1 chimeras could be detected by staining for core proved that the anticore used for immunofluorescence staining could readily detect the HC-TN strain; therefore, the lack of staining in cells transfected with pHC-TN indicated that this virus could not replicate in these cells. Following one transfection with RNA transcripts of pHC-TN, the culture was monitored for more than 20 weeks with no evidence of HCV replication.

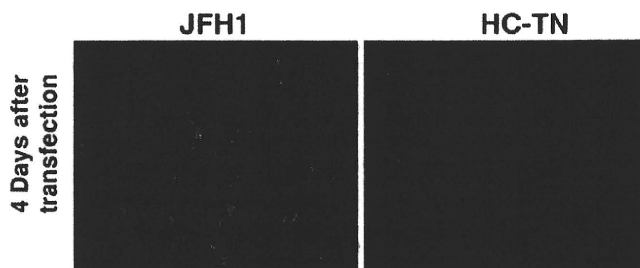

FIG. 4. Testing for replication of pHC-TN in vitro. Huh7.5 cells were transfected with RNA transcripts of pJFH1 and pHC-TN. Immunofluorescence staining was performed with an HCV core-specific mouse monoclonal antibody.

T-cell responses in HC-TN-infected chimpanzees irrespective of the final outcome. Analyses of the cellular immune responses were performed with CH1581 and CH1579 (Fig. 3). Cells had not been collected from CH1422. $CD4^+$ T cells from PBMC or polyclonally expanded from liver specimens were tested for HCV-specific proliferative responses with HCV-1 proteins (C22, C33-c, c100, and NS5). Polyclonally expanded liver $CD8^+$ T cells were tested for intracellular gamma interferon staining after coculture with autologous B cells expressing the entire H77 polyprotein from vaccinia viruses.

In CH1581, HCV-multispecific peripheral and intrahepatic $CD4^+$ T-cell responses were detected in the liver beginning at weeks 7 and 8, respectively (Fig. 3A) (38). These responses decreased during the period with low-titer viremia (weeks 22 to 31). Intrahepatic $CD8^+$ T-cell responses were detected at week 9 and in the available samples tested thereafter, including samples tested during the period with low-titer viremia (Fig. 3A) (38). In CH1579, the peripheral HCV-multispecific $CD4^+$ responses appeared earlier and were more vigorous than the intrahepatic $CD4^+$ responses (Fig. 3B). In fact, multispecific $CD4^+$ responses against core, NS3, NS4, and NS5 proteins were detected during weeks 1 to 35. In contrast, intrahepatic multispecific $CD4^+$ T-cell responses against core, NS3, NS4, and NS5 proteins were detected primarily during the initial decrease of viremia titers during weeks 13 to 23. The strongest intrahepatic $CD4^+$ response observed in the present study occurred at week 23 in CH1579. Weaker intrahepatic $CD4^+$ responses were observed during the following period with low-titer viremia. Vigorous intrahepatic $CD8^+$ T-cell responses were observed during the low-titer-viremia period that preceded viral clearance (Fig. 3B). We tried but failed to detect a reproducible $CD8^+$ T-cell response against selected epitopes in either animal, owing to the apparent low frequency of that response in the periphery (data not shown). Overall, we found that both animals had vigorous HCV-specific T-cell responses during the dramatic decrease in HCV titers and that these responses were sustained while viremia was present.

Repeated emergence of new virus variants during the host immune response. We sequenced the entire ORF of viruses recovered from the three chimpanzees, each at multiple time points (Tables 2, 3, and 4). For CH1422, we analyzed the HCV sequence from the pool taken at peak viremia titers (weeks 4 to 6) as well as that from a serum sample taken at week 19,

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TABLE 2. Nucleotide and amino acid substitutions observed in chimpanzees infected with HC-TN

Chimpanzee	Time point (wk)	Mutation rate (10^3)/site/yr		No. of ds	No. of dn	ds/dn ratio
		nt	aa			
CH1422[a]	19	23.63	17.27	61	17	11.1
CH1581[b]	8	0	0	0	0	NA[d]
	11	0	0	0	0	NA
	14	11.51	23.03	2	4	1.52
	18	14.39	34.53	2	8	0.76
	27	6.40	11.51	3	7	1.30
	32	3.45	6.91	1	2	1.52
	36	24.47	25.91	11	6	5.58
	45	3.38	9.59	1	5	0.61
	52	4.11	9.87	1	4	0.76
	52[c]	3.65	6.64	13	20	1.98
CH1579[b]	10	0	0	0	0	NA
	13	0	0	0	0	NA
	21	4.32	10.79	1	5	0.61
	32	5.76	14.13	2	9	0.68
	32[c]	2.34	6.48	1	12	0.25

[a] The week 19 sequence was compared to the HCV sequence obtained from an acute-phase plasma pool of CH1422.
[b] At each time point, the HC-TN sequence was compared with that obtained at the previous time point.
[c] The HC-TN sequence obtained at week 52 from CH1581 and that obtained at week 32 from CH1579 were also compared with that of the inoculum.
[d] NA, not applicable.

after the virus became transiently undetectable at weeks 16 and 17 (Fig. 1A). We detected 78 nucleotide and 17 amino acid substitutions at week 19. The relatively high mutation rate and ds/dn ratio compared with those of CH1581 and CH1579 (Table 2) suggested that the virus that reemerged represented the selection of a preexisting minor variant. The amino acid changes were located in core (G187V), E2 (I438V and S453P), p7 (L765V and L790F), NS2 (V873I, V879I, and K927N), NS3 (L1504P), NS4B (A1832T), NS5A (K2016R, Q2095R, E2228G, L2340P, and K2414E), and NS5B (H2483Y and T2810I).

During the first 11 weeks, prior to the initial decrease in virus titers, the sequence for CH1581 remained unchanged, but at each subsequent week tested, new variants emerged (Tables 2 and 3; Fig. 3A). The mutation rates observed to occur between two subsequent time points thereafter ranged from 3.38×10^{-3} to 24.47×10^{-3} and 6.91×10^{-3} to 34.53×10^{-3} substitutions/site/year at the nucleotide and amino acid levels, respectively, and the ds/dn ratios were relatively low during the first year. Twenty amino acid changes were maintained by week 52. Only one of these, K2414E, was detected also in CH1422. The first four changes, including a change in p7, occurred at week 14. At week 18, five additional changes occurred in NS2, NS4B, NS5A, and NS5B. It is noteworthy that three changes observed at week 14 within the nonstructural proteins, in which the original sequence was present as a minor species, had reverted to the initial sequence at week 18. To rule out that these changes observed only at week 14 did

TABLE 3. Evolution of HC-TN polyprotein in CH1581

Wk[a]	Amino acid sequence[b]																									
	E2			p7		NS2				NS3			NS4B			NS5A									NS5B	
	401S	433L	461P	767S	793M	834Y	837R	841W	861I	1405A	1406K	1583F	1746V	1747A	1751Q	2064A	2116V	2118S	2220D	2223D	2227I	2278S	2341P	2358S	2414K	2664D	3005I
1																T											
8	S	L	P	S	M	Y	R	W	I	A	K	F	V	A	Q	T	V	S	D/g	D	I	S	P	S	K	D	I
11	S	L	P	S	M	Y	R	W	I	A	K	F	V	A	Q	T	V	S	D/G	D	I	S	P	S	K	D	I
13						Y/h	R/H	W/r	I				V	A	Q	T	V	S	G/d*	D	I	S					
14	S	L	P	S	V*	Y/h	R/H	W	I	A	K	F	V	A/t	H/q*	A/t*	V	S	G/d*	D	I	S	P	S	K/E	D	I
18	S	L	P	S	V*	Y	L*	W	I	A	K	F	V	T/a*	Q/h	T/a	V	S	D/g	D	I	P*	P	S	E*	G*	I
26						Y/h	L*	W	V/i*								V	P*	D	N*	I	P*					
27	S	L	S*	S	T*	H/y*	L*	W	V/i*	A	K	F	V	T*	Q	T	V	P*	D	N*	I	P*	P	S	E*	G*	I
31										T/a*	K																
32	S	L/F	S*	S	T*	H/Y*	L*	W/R	V*	T*	K	F	V	T*	Q	T	V	P*	D	D/n	I	P*	P	S	E*	G*	I
36	S	F*	S*	S/p	T*	Y	L*	R*	V*	A	K	F	V/i	T*	Q	T	V	P*	D	D	V/i*	P*	P	S	E*	G*	T*
45	G/s*	F*	S*	P/s*	T*	Y	L*	R*	V*	A/v/t/i	K/R	L/f*	I/v*	T*	Q	T	V/i	P/S*	D	D	V*	P*	P	P/s*	E*	G*	T*
51																						P*	S/p*	P*	E*		
52	G*	F*	S*	P*	T*	Y	L*	R*	V*	A/t	R/k*	L*	V/i	T*	Q	T/a	I/v*	P*	D	D	V*	P*	S/p*	P*	E*	G*	T*

[a] The entire ORF sequence of HC-TN was determined at weeks 8, 11, 14, 18, 27, 32, 36, 45, and 52. Only subdomains were analyzed at weeks 1, 13, 26, 31, and 51.
[b] Amino acid positions correspond to those of pHC-TN. The consensus sequence of viruses recovered from a CH1422 acute-phase plasma pool is shown at the top. Dominant sequences recovered from the chimpanzee are shown in capital letters. Minor sequences recovered from the chimpanzee are shown in lowercase letters. Dominant amino acid changes are marked with an asterisk. Note that two consecutive changes occurred at aa 793.

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TABLE 4. Evolution of HC-TN polyprotein in CH1579

Wk[a]	Amino acid sequence[b]												
	E2	p7	NS2	NS3		NS4A	NS5A					NS5B	
	403F	789A	1018M	1148S	1563T	1700V	2252I	2263E	2341P	2374S	2414K	2456L	3008L
10	F	A	M	S	T	V	I	E	P	S	K	L	L
13	F	A	M	S	T	V	I	E	P	S	K	L	L
20			M			A/v*							
21	F/l	T*	M/v	S	T	A/v*	V/i*	E/g	S*	S/f	K/T/n	M/l*	L/f
31				G*	I/t*								
32	L*	T*	T*	G*	I/t*	V	V*	G*	S*	F*	E*	M*	F*
33	L*	T*	T/M*	G*	I/t*	V	V*	G*	S*	F*	E*	M*	F*

[a] The entire ORF sequence of HC-TN was determined at weeks 10, 13, 21, 32, and 33. Only subdomains were analyzed at weeks 20 and 31.

[b] Amino acid positions correspond to those of pHC-TN. The sequence of pHC-TN is shown at the top. Dominant sequences recovered from the chimpanzee are shown in capital letters. Minor sequences recovered from the chimpanzee are shown in lowercase letters. Dominant amino acid changes are marked with an asterisk.

not represent PCR or sequencing errors, we reamplified and sequenced the genome regions with these mutations from the week 14 sample, as well as from a week 13 sample (Table 3). All three changes were confirmed at week 14, and one change was present also at week 13. A virus with six additional mutations, in E2 (outside HVR1), p7, NS2, and NS5A, emerged at week 27. This included a new change at the p7 position that had occurred at week 14. During weeks 32 to 52, changes were observed at each time point analyzed. They were located within E2, p7, NS2, NS3, NS5A, and NS5B. They included a single HVR1 change at week 45.

Changes were not found in CH1579 during the first 13 weeks of follow-up (Table 4; Fig. 3B). The mutation rates observed thereafter were high, and the ds/dn ratios were low (Table 2). Twelve amino acid changes, located in E2 (within HVR1), p7, NS2, NS3, NS5A, and NS5B, had emerged by week 32, and the identical sequence was also present at week 33. A single mutation in NS5A (K2414E) was also identified in CH1422 and CH1581. Another NS5A mutation (P2341S) was found to occur in CH1581. Since CH1579 was infected from RNA transcripts of an infectious clone, these mutations could not have originated from the original source virus but evolved de novo in this animal. Four mutations, located in p7, NS5A, and NS5B, existed already at week 21; one mutation detected in NS4A changed to the original sequence at week 32. However, we found that this mutation was also present at week 20, confirming that it was not an artifact.

DISCUSSION

It is not known whether infection with particular HCV strains is associated with severe forms of acute hepatitis C. We previously reported that infection with strain HC-TN (genotype 1a) was associated with fulminant hepatic failure and, following transmission to a chimpanzee, caused unusually severe acute hepatitis (13). In the present study, however, two additional chimpanzees infected with strain HC-TN developed typical acute hepatitis, with peak ALT levels of 296 IU/liter and 90 IU/liter and minimal necroinflammatory changes in liver biopsy samples (Fig. 1). The ALT values were similar to the mean peak ALT of 215 ± 122 IU/liter (mean ± standard deviation) observed in >30 chimpanzees acutely infected with other genotype 1 strains (13). Thus, we could not confirm that strain HC-TN was more virulent than other strains in chimpanzees.

Virulence depends upon a complex interplay between the virus and the host and may be influenced by the dose of infecting virus, route of entry, and virus sequence, as well as by the immune status of the host. Virus dose or transmission route could have influenced the liver disease in the HC-TN-infected chimpanzees. However, Feinstone et al. (15) reported that there was no correlation between the infectious HCV dose of the inoculum and the peak ALT among experimentally infected chimpanzees. Furthermore, the course of infection did not differ in animals infected from RNA transcripts and from intravenous inoculation (8, 27, 28, 46).

Single nucleotide or amino acid changes in a virus genome can result in different levels of virulence, as reported, for example, for an amino acid change in the VP4 region of poliovirus (3). We demonstrated that the ORF sequence of virus recovered at peak viremia from CH1579, with mild hepatitis, was identical to the virus sequence recovered from CH1422, with severe hepatitis. It is possible that the sequences of the poly(U/UC) tract of the 3′ UTR, which vary in length and composition among different HCV isolates, differed among the viruses infecting the animals. However, the poly(UC) region of the RNA transcripts used to initiate infection in CH1579 was an exact match with the sequence recovered from CH1422. In fact, the infectious clone of strain HC-TN represents the first true consensus clone of HCV since it did not contain any nucleotide changes, perhaps with the possible exception of the length of the poly(U) stretch of the 3′ UTR.

The virus infecting the chimpanzee with severe hepatitis might have had a higher degree of heterogeneity (quasispecies) than those found in the chimpanzees infected with the lowest possible infectious dose and with the monoclonal virus. However, sequence analysis suggested that the virus recovered from the animal with severe hepatitis was very homogeneous. The courses of viral replication during the early acute phase of infection were very similar in the three animals, suggesting that the viruses infecting the chimpanzees had similar replication capacities. It is noteworthy that exposure to low doses of HCV or RNA transcripts from molecular clones, which did not result in detectable infection, were reported to have primed the host immune response in chimpanzees (22, 36). CH1422, with severe hepatitis, did not have such prior exposure, whereas CH1581 and CH1579, with typical hepatitis, both had such prior exposure. However, the intrahepatic T-cell responses in

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both of these animals appeared only after about 2 months of active infection. Yet, in CH1579 we did detect a weak peripheral T-cell response in the preinoculation samples.

Host immune responses are thought to determine the outcome of HCV infection. We recently demonstrated that neutralizing antibodies do not appear to play a role in the control of acute HCV in chimpanzees since they do not develop in animals with resolving infection (2, 29). CH1579 did not develop envelope or neutralizing antibodies even though the HC-TN infection resolved (29). A significant peripheral $CD4^+$ T-cell response occurred much earlier in the animal that cleared the infection (CH1579) than in the animal that became chronically infected (CH1581), suggesting that early priming of the T-cell response may be important to the outcome. A significant response was detected at baseline in CH1579 and thus prior to inoculation, maybe reflecting priming through previous inoculations (see above). Both CH1581 and CH1579 developed HCV-specific intrahepatic antiviral $CD4^+$ and $CD8^+$ T-cell responses; the animal (CH1581) with persistence actually appeared to have an earlier appearance of these responses and in general the strength of these responses was greater than those detected in the animal (CH1579) with acute resolving infection (Fig. 3). However, CH1579 had extraordinarily strong CD4 and CD8 responses at weeks 23 and 33, respectively. It is possible that the intrahepatic $CD8^+$ T-cell response, which was more vigorous during the low-titer period in CH1579 than during the corresponding period in CH1581, was efficient enough to eliminate the virus before escape mutants could establish a robust infection in CH1579, which was not the case with CH1581 (see below). The peripheral $CD4^+$ T-cell response waned soon after viral clearance in CH1579, as did the intrahepatic $CD4^+$ and $CD8^+$ T-cell responses, but they all persisted in the chronically infected animal (CH1581), suggesting that persistence of these responses requires continuous antigen stimulation. It was recently reported that HCV-infected chimpanzees with acute resolving infection had an earlier initial decrease in virus titers than animals that developed a persistent infection (27). However, we found the opposite with CH1581 and CH1579 (Fig. 1).

We wondered whether differences in virus evolution in response to the host cellular immune response could explain the different outcomes for CH1579 and CH1581. The emergence of escape mutations in T-cell-targeted epitopes has been documented previously for HCV-infected chimpanzees (11, 42). However, these mutations were not analyzed in the context of coexisting mutations, since only small segments of the genome were sequenced. The cellular immune response against HCV is frequently targeted against multiple epitopes, and escape from a single epitope might not lead to persistence. We analyzed the entire polyprotein sequence of consecutive samples during the acute infection and correlated changes directly with the host humoral and cellular immune responses. In addition, we limited the possibility of selection of preexisting variants in the chimpanzees studied, since CH1581 was inoculated with the lowest possible infectious dose of polyclonal virus and CH1579 was transfected with RNA transcripts from an infectious clone and thus initially had a monoclonal infection. We did not detect any mutations in viruses recovered from CH1581 and CH1579 during the first 11 and 13 weeks of follow-up, respectively. In contrast, during the next 7 and 8 weeks of follow-up we detected six and five amino acid changes, respectively. Thus, despite a high rate of replication and an error-prone RNA-dependent RNA polymerase, mutations were not selected until the initial decrease in HCV titers. The accumulation of minor variants might occur during initial replication, and these variants could be selected by means of host immune pressure or replicative advantages, perhaps as second-site changes compensating for decreased replication fitness caused by other changes. Finally, changes might represent random coselected mutations. Our study does not demonstrate the specific mechanism for the development of mutations but rather a close temporal association with host cellular immune responses. We attempted to analyze the potential escape mechanism, but unfortunately the stored T cells could not be recovered sufficiently to perform the analysis.

Major et al. (28) studied the evolution of monoclonal H77 virus, another genotype 1a strain, in two chimpanzees that became persistently infected. Overall, the mutation rates observed for these animals were lower than those for the HC-TN-infected animals. Both animals developed mutations in p7; one of these mutations (M793V) was observed to occur also in the HC-TN-infected animal that became persistently infected. There was only one other common mutation in the two studies, L2456M, which occurred in the HC-TN-infected animal that cleared HCV. Finally, it should be noted that a similar pattern of development of mutations was observed in chimpanzees infected with monoclonal genotype 1b viruses (7, 40).

Recently, it was found that RNA transcripts from the full-length JFH1 genome (genotype 2a) produced viruses in human liver hepatoma cell lines (41, 49). The JFH1 strain was isolated from a patient with fulminant hepatitis, and it has been a question as to whether that fact was related to the unique ability to grow in cell culture. In contrast, wild-type, full-length HC-TN did not replicate in Huh7.5 cells even though it too was isolated from a patient with fulminant hepatitis. The same was reported previously for strain H77, but it was recently reported that a cell-culture-adapted H77 genome could produce viruses in Huh7.5 cells (48). Given that strains H77 and HC-TN belong to the same HCV subtype and are relatively closely related (Fig. 2), it is possible that these adaptive mutations would also permit replication of the HC-TN strain. Further studies are required to develop a cell culture system for the HC-TN strain.

In conclusion, we have developed an infectious clone of the HC-TN strain. The HC-TN sequence was infectious in vivo, but like other infectious clones of HCV genotype 1, this wild-type sequence was not infectious in Huh7-derived cells. Our in vivo study of the HC-TN strain demonstrates that virulence of HCV depends primarily upon host responses and not the particular virus strain. The cellular immune response against HCV precedes the initial decrease in virus titer and the development of acute hepatitis. The cellular immune responses did not appear to predict the final outcome of the infection, although differences in timing and magnitude of these responses might have played a role. The emergence of new virus variants, in the absence of neutralizing antibodies, is temporally associated with host cellular immune responses. This might play an important role in viral persistence. However, the accumulation of such variants does not assure viral persistence.

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ACKNOWLEDGMENTS

We thank Marisa St. Claire and Max Shapiro (Bioqual, Inc., Rockville, MD) for animal care and Ronald E. Engle (Hepatitis Viruses Section, National Institutes of Health) and Carola Steiger (Department of Molecular and Experimental Medicine, The Scripps Research Institute) for technical assistance. We thank Isa K. Mushahwar, Charles M. Rice, Takaji Wakita, Michael Houghton, and François-Loïc Cosset for providing reagents.

These studies were supported in part by NIH grant CA76403 (F.V.C.). R.T. was supported by a fellowship from the Cancer Research Institute, New York, and DFG grant TH 719/2-2 (Emmy Noether Program). This research was supported by the Intramural Research Program of the NIAID, NIH.

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FEBS Letters 581 (2007) 1983–1987

Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Received 15 February 2007; revised 31 March 2007; accepted 5 April 2007

Available online 20 April 2007

Edited by Hans-Dieter Klenk

Abstract We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.
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Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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doi:10.1016/j.febslet.2007.04.021

human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *XbaI* (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAG-CGTCTAGC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGG-CTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested

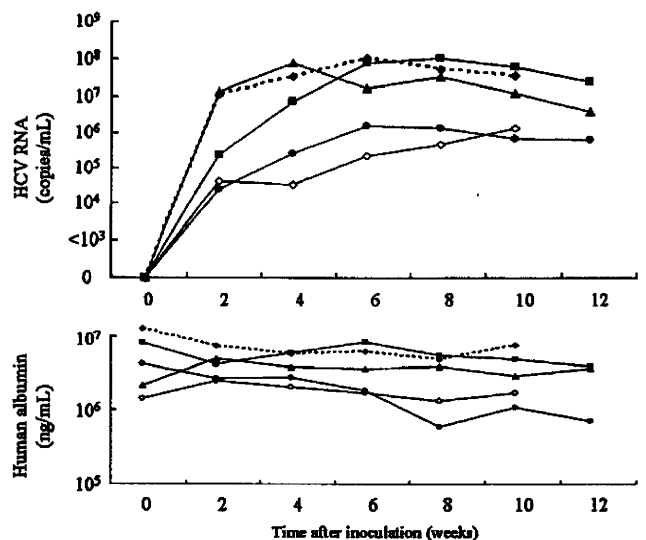

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).

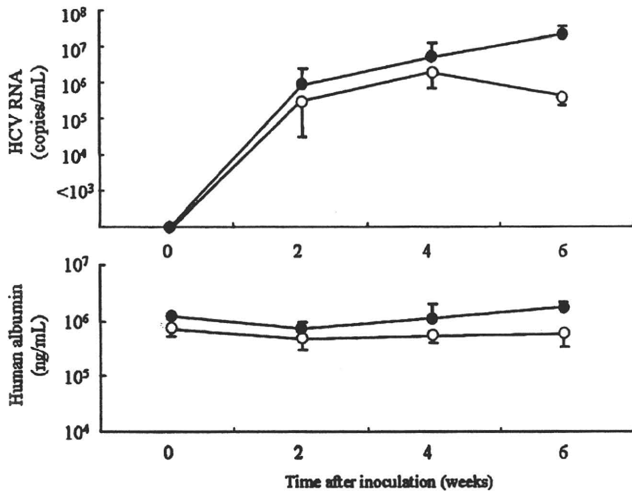

Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean ± S.D.

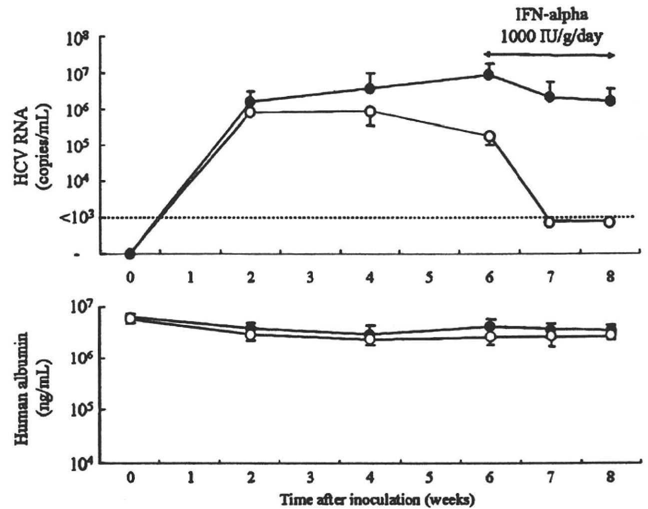

Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μl) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean ± S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment reduced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

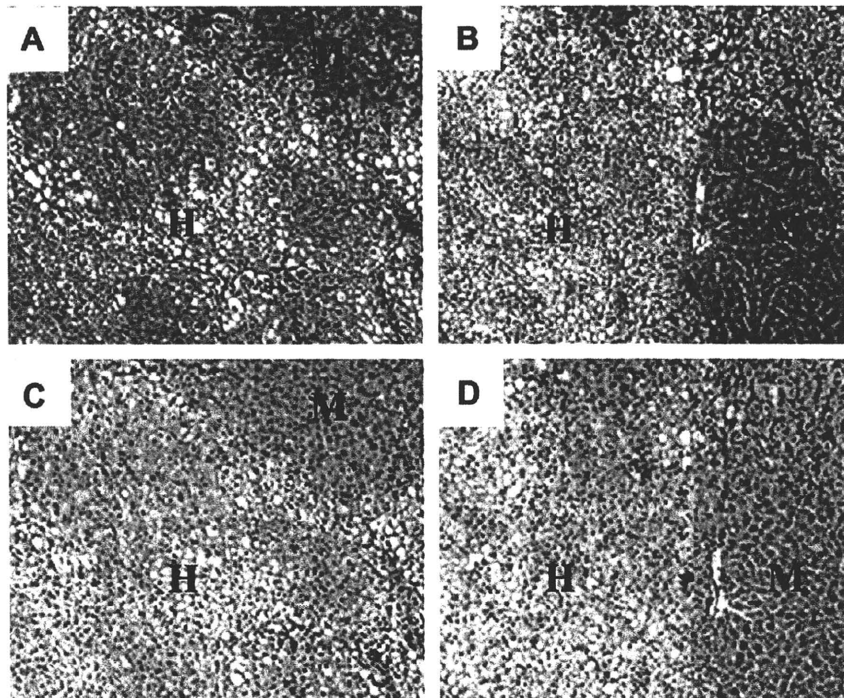

Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, ×100.)