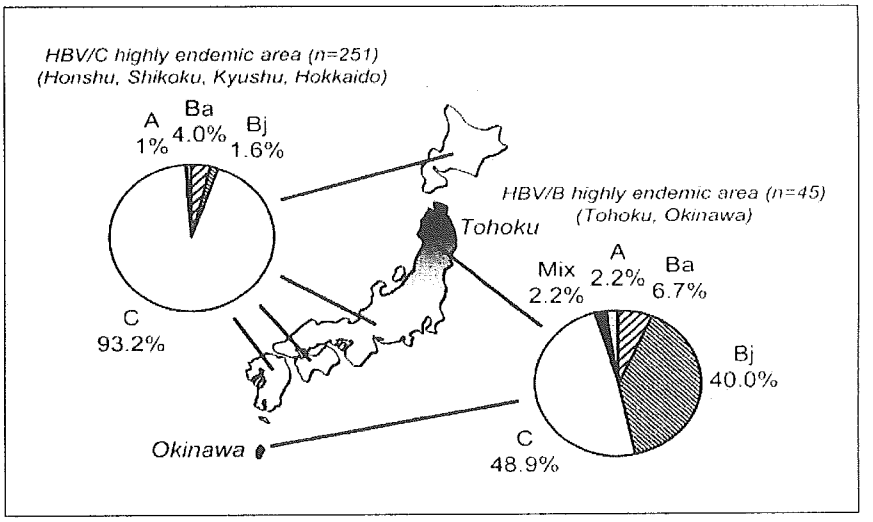

Fig. 1. The geographic distribution of HBV genotypes in Japan. In the Tohoku district, the northern area of mainland Japan, and Okinawa, the most southern islands, 48.9% of HCC patients were HBV/C, 6.7% were HBV/Ba, and 40.0% were HBV/Bj. In contrast, in other parts of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 93.2% were HBV/C, 4.0% were HBV/Ba and 1.6% were HBV/Bj.

Table 1. Characteristics of 296 HBsAg-positive Japanese patients with HCC collected from all over Japan

Male:female	223:73
Age, years	55.1 ± 10.8[a]
Total bilirubin, mg/dl	1.5 ± 1.9
AST, IU/l	78.5 ± 103.9
ALT, IU/l	63.0 ± 69.8
ALP, IU/l	321.1 ± 225.4
γ-GTP, IU/l	108.4 ± 174.4
HBeAg, % positive	35.0
Anti-HBe, % positive	64.8
HBV genotype	
HBV/A	3 (1.0%)
HBV/Ba	13 (4.4%)
HBV/Bj	22 (7.4%)
HBV/C	256 (86.5%)
Mix	2 (0.7%)

[a] Mean ± SD.

Table 2. Clinical findings of the HCC patients with HBV genotypes of Ba, Bj or C

	HBV genotype		
	Ba	Bj	C
Age, years	55.4 ± 12.9	66.6 ± 10.6	54.0 ± 10.7
		p < 0.01	p < 0.01
Total bilirubin, mg/dl	1.0 ± 0.4	1.2 ± 0.7	1.5 ± 2.0
AST, IU/l	173.9 ± 352.6	51.6 ± 42.1	82.6 ± 113.4
ALT, IU/l	102.4 ± 162.9	33.9 ± 16.8	66.5 ± 74.9
ALP, IU/l	147.7 ± 126.6	209.8 ± 95.4	343.9 ± 238.0
		p < 0.05	
γ-GTP, IU/l	78.6 ± 55.9	63.1 ± 45.9	110.5 ± 186.7
			p < 0.05

GTP level of the HBV/C patients was significantly higher than those with HBV/Ba and HBV/Bj, respectively ($p < 0.05$).

Geographic Distribution of HBV Genotypes

The geographic distribution of HBV genotypes was area-specific in Japan (fig. 1). This specific distribution of HCC patients was in accord with that of all the patients including asymptomatic carriers, CH and LC patients, as described previously [7]. Namely, in the Tohoku district, the northern area of the Japanese mainland, and Okinawa, the most southern islands, 22 (48.9%) of HCC patients were HBV/C, 3 (6.7%) were HBV/Ba, and 18 (40.0%) were HBV/Bj. In contrast, in other areas of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 234 (93.2%) were HBV/C, 10 (4.0%) were HBV/Ba, and 4 (1.6%) were HBV/Bj ($p < 0.01$).

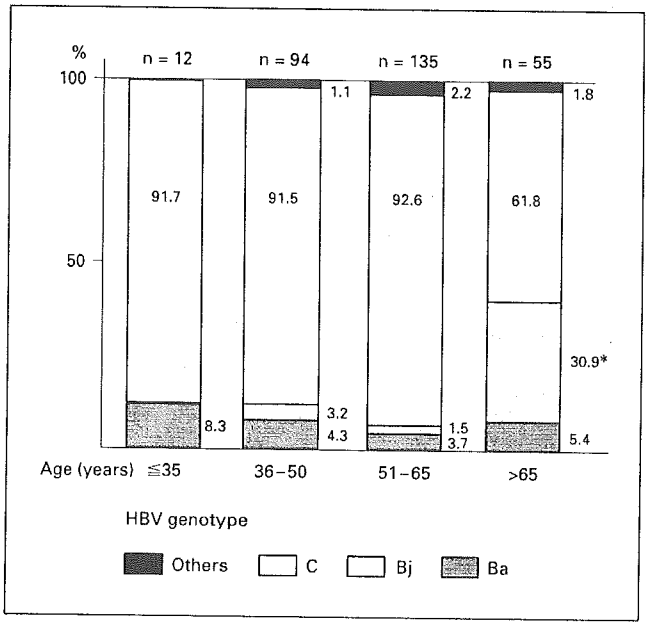

Fig. 2. The distribution of HBV genotypes in each age group. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of patients had HBV/C while 30.9% had HBV/Bj (* $p < 0.01$, group aged older than 65 years vs. other age groups). More patients with HBV/Ba were in the younger aged group, although the number of patients with HBV/Ba was small in all the groups.

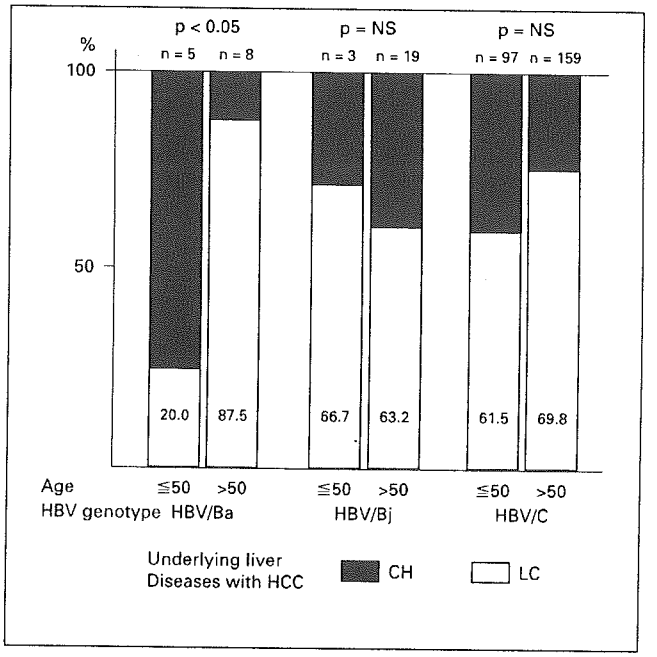

Fig. 4. The underlying liver diseases, chronic hepatitis (CH) or liver cirrhosis (LC), in HCC patients. In patients with HBV/Ba, only 25.0% of the group aged 50 years or younger had LC, while 85.7% of the group aged older than 50 years had LC ($p < 0.01$). However, in patients with HBV/Bj or HBV/C, the ratios of the underlying liver diseases were approximately identical even when compared by age.

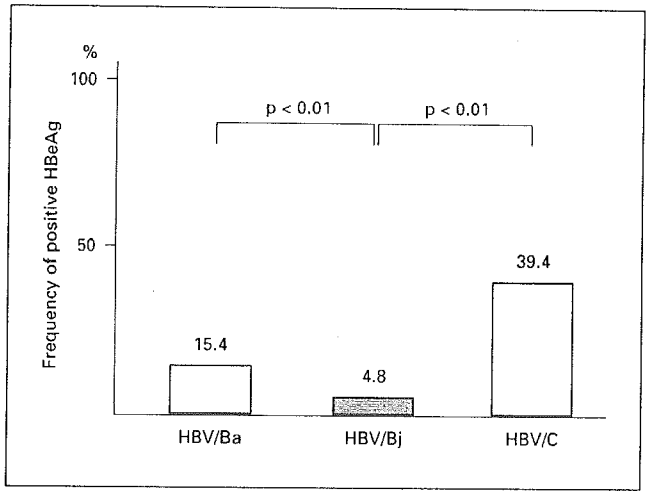

Fig. 3. The frequency of patients with positive HBcAg in each HBV genotype. The frequency of positive HBcAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$).

Mean Age and Frequency of Positive HBeAg among Patients with Each Genotype

The mean age of HBV/Bj patients (66.6 ± 10.6 years) was significantly higher than those with HBV/Ba (55.4 ± 12.9 years, $p < 0.01$) and HBV/C (54.0 ± 10.7 years, $p < 0.01$; table 2). The distribution of HBV genotypes in each age group is shown in figure 2. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of the patients had HBV/C while 30.9% had HBV/Bj ($p < 0.01$, group aged older than 65 years vs. other age groups). HBV/Ba tended to be found in the younger age group although the number of patients with HBV/Ba was small in all groups.

The frequency of positive HBeAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$; fig. 3).

Underlying Liver Diseases

All HCC patients had underlying chronic liver diseases, such as CH or LC. We compared the underlying liver diseases among those aged 50 years or younger and those aged older than 50 years by HBV genotype (fig. 4). In 13 patients with HBV/Ba, only 1 (20.0%) of the 5 patients aged 50 years or younger had LC, while 7 (87.5%) of the 8 patients aged older than 50 years had LC ($p < 0.05$). However, in patients with HBV/Bj or HBV/C, the ratios of underlying liver diseases were approximately identical even when compared by age.

Discussion

The clinical and virologic features of patients with chronic HBV infection are specific according to their HBV genotypes [4, 15]. However, to date, there has been no report on the relationship between the HBV genotypes of Ba, Bj and C, and the clinical characteristics of HCC patients. We therefore analyzed the relationship between the clinical characteristics of Japanese HCC patients identified throughout Japan, and their HBV genotypes, including the HBV subtypes of Ba and Bj. In this study, we demonstrated that HBV/Ba (4.4%), HBV/Bj (7.4%) and HBV/C (86.5%) were found in Japanese HCC patients, and that there were distinct clinical differences among the three HBV genotypes, in geographic distribution, age distribution, and the frequency of positive HBeAg.

Of the Japanese patients with chronic HBV infection, including asymptomatic carriers, CH, LC and HCC, 1.7% were HBV/A, 12.2% HBV/B, 84.7% HBV/C, 0.4% HBV/D, and the others 1.0%, as reported previously [7]. In this study, we collected 296 serum samples from patients with HCC throughout Japan. In addition, we recently developed a new method for detecting HBV/Ba and HBV/Bj with restriction fragment length polymorphism [11]. Thus, we showed that 1.0% was HBV/A, 4.4% HBV/Ba, 7.4% HBV/Bj, 86.5% HBV/C, and mixed genotype 0.7% in Japanese HCC patients. This prevalence in HCC patients is almost identical to that in all patients with chronic HBV infection [7]. In addition, the geographic distribution of HBV/B and HBV/C in HCC patients is also identical to that in all patients. However, when we analyzed the HBV subtypes of HBV/Ba and HBV/Bj in patients with HBV/B, a high proportion of patients with HBV/Bj is found in the highly endemic HBV/B area, the Tohoku district and Okinawa, while the prevalence of HBV/Ba is approximately identical between the highly endemic HBV/C area, the other areas of Japan, and the highly endemic HBV/B area. Thus, HBV/Bj is specifically distributed in the Tohoku district and Okinawa.

As reported previously, HBV/Ba is ubiquitous in all Asian countries including Japan, although HBV/Bj is specific to Japan and is not found in other countries [11]. In Okinawa, it is reported that a high proportion of patients with chronic HBV infection have HBV/B and a good prognosis compared with patients with HBV/C [16, 17]. In contrast, in Taiwan, close to Japan, a higher proportion of patients aged 50 years or younger with HBV/B have HCC and CH [15]. The underlying liver diseases in those who developed HCC were compared among each HBV genotype group. In the HBV/Ba group, up to 75% of the patients aged 50 years or younger had CH as the underlying liver disease, compared with patients aged over 50 years. On the other hand, in the group with HBV/Bj or HBV/C, more than 60% of the patients had LC regardless of their age. The mean age of the patients with HBV/Ba in Japan is more than 10 years younger than those with HBV/Bj. So, more younger patients with HBV/Ba tend to have CH than the other patients. However, the molecular mechanism is unclear why patients with HBV/Ba develop HCC at a younger age and often have CH.

It is unclear why Japanese patients with HBV/B have a good prognosis while Taiwanese patients with HBV/B often have more advanced liver diseases, such as HCC. The frequency of patients positive for HBeAg in the HBV/Ba and HBV/C groups was higher than in the HBV/Bj group. So, the viral activity of HBV may be higher in patients with HBV/Ba or HBV/C than those with HBV/Bj. Thus, these differences in subtypes of HBV/Ba and Bj could be one of the reasons why the discrepancy in prognosis exists between Japanese and Taiwanese patients with HCC.

The differences in DNA sequences between HBV/Ba and HBV/Bj can be characterized in the core gene [10]. It has been reported that HBV/Ba, not HBV/Bj, recombines with HBV/C in the core gene. The product of the core gene is reported to be a cytotoxic T-cell epitope [18], suggesting that patients with HBV/Ba and HBV/C may be exposed to severe immune responses for destroying hepatocytes compared with those with HBV/Bj. In addition, patients with HBV/Ba more often have core promoter mutations at nucleotide 1762/1764 than those with HBV/Bj [11], which is associated with more advanced liver diseases [6, 19]. Taken together, these facts may indicate a poor prognosis in patients with HBV/Ba compared to those with HBV/Bj.

In the patients with HBV/C, the mean ALP and γ-GTP levels were higher than those with the other genotypes. In this study, there may exist some bias of regarding the tumor size of HCC between patients with HBV/C and the other patients. It is considered that more patients with a rather large size of HCC were found in the patients with HBV/C, resulting in elevation in ALT and γ-GTP levels.

To investigate the hepatocarcinogenesis and risk factors of HCC, it is important to study the differences in host, environmental and viral factors. The various genetic alterations, such as mutations of cancer-associated genes or loss of some chromosomes, are found in the HCC cells [20]. However, the genetic polymorphism varies among populations [21]. The differences in host genomes are still unknown between Japanese and other Asian populations. The association of environmental factors, such as air, water and food contaminated with some chemical agents, and HCC is still unclear, although aflatoxin affects the mutation of p53 in HCC [22]. However, with respect to the viral factors, a survey of the distribution of HBV genotypes or subtypes will be important clues for solving these problems.

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Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

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Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

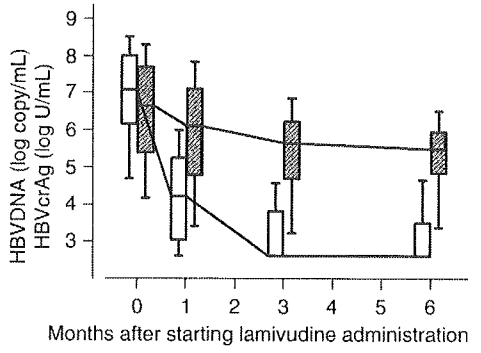

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

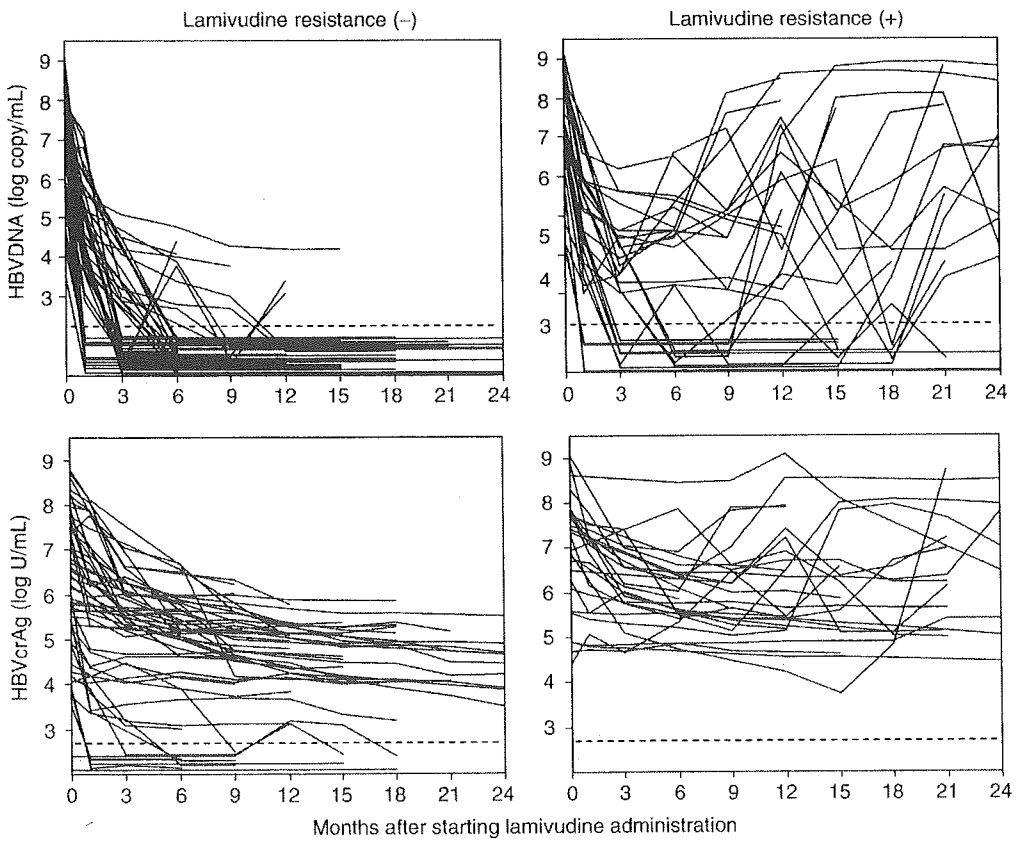

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

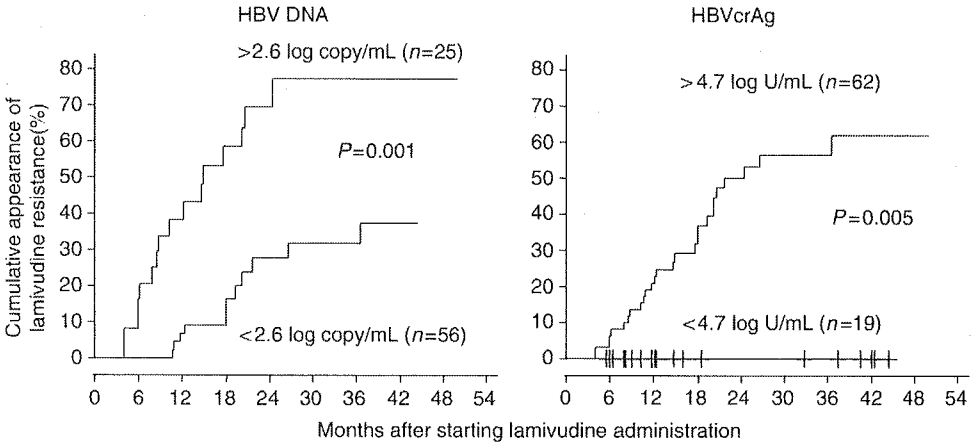

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

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Cell-based models of sustained, interferon-sensitive hepatitis C virus genotype 1 replication

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Abstract

We have previously reported hepatitis C virus (HCV) replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV cDNA plasmid (pT7-flHCV-Rz) and infected with vaccinia-T7 polymerase. We hypothesized that the use of replication-defective adenoviral vectors expressing T7 (Ad-T7pol) or cell lines stably transfected with T7 (Huh-T7) would alleviate cell toxicity and allow for more sustained HCV replication.

CV-1, Huh7, and Huh-T7 cells were transfected with pT7-flHCV-Rz and treated with Ad-T7pol (CV-1 and Huh7 only). Protein and RNA were harvested from cells on days 1, 2, 3, 5, 7, and 9 post-infection. No cytotoxicity was observed at 9 days post-infection in any cell type. HCV positive- and negative-strand RNA expression were strongest during days 1–3 post-infection; however, HCV RNA remained detectable throughout the 9-day observation period. Furthermore, transfection with a replication-incompetent plasmid suggested that efficient HCV replication is dependent upon NS5B gene expression. Finally, after 1–2 days of IFN treatment, HCV positive-strand levels decreased significantly compared to HCV-infected but untreated samples ($p < 0.05$).

In conclusion, these refined binary systems offer more durable and authentic models for identification of host cellular processes critical to HCV replication and will permit longer-term analysis of virus–host interactions critical to HCV pathogenesis and the treatment of genotype 1 infections.

Keywords: Hepatitis C virus; HCV; Replication; Genotype 1; Adenovirus vector; Huh-T7

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, including hepatitis, cirrhosis, and hepatocellular carcinoma (Alter et al., 1999). The combination of interferon (IFN) and ribavirin (RBV) is the standard treatment for chronic HCV infection; however, their effectiveness remains limited (McHutchison and Poynard, 1999). The lack of a full-length HCV tissue culture model has limited not only the ability to screen novel antiviral agents but also the ability to precisely characterize the antiviral effect of IFN, particularly against genotype 1 infections.

We recently reported successful cell-based HCV replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV cDNA plasmid and infected with a recombinant vaccinia vector encoding T7 polymerase (Chung et al., 2001). However, HCV replication driven by vaccinia-based vectors is restricted to short-term studies due to the cytotoxic

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effects of vaccinia. Moreover, vaccinia encodes two proteins, E3L (Chang et al., 1992; Watson et al., 1991) and K3L (Carroll et al., 1993; Gale et al., 1996), that act as potent inhibitors of the IFN-induced double-stranded RNA-activated protein kinase (PKR). Due to these limitations, we sought to further refine our HCV replication model using alternative, less disruptive modes of T7 polymerase delivery.

We hypothesized that the use of replication-defective adenoviral vectors expressing T7 or cell lines stably expressing T7 would alleviate cell toxicity and allow for more sustained HCV replication.

Recombinant replication-defective adenoviral vectors have comparable infectivity to vaccinia vectors. These adenoviral vectors cannot replicate inside infected cells, because they lack the E1A and E1B proteins necessary for viral vector replication. Moreover, these vectors lack the E3 gene that inhibits immune responses by interacting with cytoplasmic MHC class-I molecules (Wold and Gooding, 1989). Thus, adenoviral vectors maintain infectivity and protein delivery with minimal cytotoxicity. By transfecting the HCV cDNA construct into Huh7 cell lines stably expressing T7 polymerase (Huh-T7) (Schultz et al., 1996), the need for viral delivery systems was removed altogether. Using these alternative delivery methods, we have established a refined HCV replication model that produces more sustained viral RNA replication, leads to less perturbation of host genes, and represents a more authentic system for studying virus–host interactions relevant to HCV pathogenesis. These refined models were also utilized to characterize the antiviral kinetics of IFN on HCV replication.

2. Materials and methods

2.1. Cell lines

CV-1 cells (American Type Culture Collection, Manassas, VA) and Huh7 (Dr. Robert Lanford, Southwest Foundation for Biomedical Research) and Huh-T7 (Dr. Stanley Lemon, University of Texas) (Schultz et al., 1996) were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

2.2. Plasmids and transfection-infection

The binary replication system has been described previously and is capable of successful positive-strand and negative-strand HCV RNA synthesis, efficient HCV protein production, and quasispecies generation (Chung et al., 2001; Contreras et al., 2002). Briefly, a plasmid containing the infectious full-length genotype 1 cDNA sequence corresponding to the H77 prototype strain (Yanagi et al., 1997) was adapted at its 5′ and 3′ termini with the T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz (hereafter referred to as H77). As a negative control, a mutant plasmid in which the GDD active site of the NS5B RNA-dependent RNA polymerase (RdRp) was mutated to AAG (hereafter referred to as $H77_{GDD \rightarrow AAG}$) was generated by site directed mutagenesis (Quick Change; Stratagene; La Jolla, CA). This substitution is associated with replication-incompetence in replicon models (Blight et al., 2000). H77 and $H77_{GDD \rightarrow AAG}$ were used to transfect CV-1, Huh7, or Huh-T7 cells at 70% confluency on 6-well plates with Lipofectamine (Invitrogen, Carlsbad, CA). Plasmids were transfected at concentrations of 1 μg/well for CV1 cells and 3 μg/well for Huh7 and Huh-T7 cells. Transfection efficiency was assessed by co-transfection with 0.1 μg/well of phRL-TK (Int^-) (Promega, Madison, WI) and luciferase activity quantified using the Dual-Luciferase reporter assay system (Promega). For CV-1 and Huh7 cells, T7 polymerase was delivered using a recombinant vaccinia virus vector (vTF7-3) (Fuerst et al., 1986) or a recombinant adenovirus vector (Ad-T7pol) 24 h after H77 transfection. In control experiments, a replication-defective adenovirus vector lacking the T7 polymerase gene (Ad-Psi5) was used. Adenoviral vectors were provided by the Harvard Gene Therapy Initiative's Viral Vector core (Boston, MA).

2.3. X-gal staining of pOS8-transfected cells

To compare the transfection and infection efficiency of the vaccinia and adenovirus vectors, the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase gene, was co-transfected into cells. After 48 h, cultured cells were washed with PBS, fixed with 0.25% glutaraldehyde for 1 h at 4 °C, and stained with 0.1% 5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously (Hiasa et al., 1998; Miyake et al., 1996).

2.4. Interferon experiments

Interferon alpha 2b was obtained from Schering Plough (Kenilworth, NJ). For CV-1 and Huh7 cells, 100–1000 IU/mL of IFN was added 5 h after infection with adenovirus vector. For Huh-T7 cells, 100–1000 IU/mL of IFN was added 5 h after transfection with H77. Medium with or without IFN was changed at day 1 post-infection and every 2 days thereafter.

2.5. Cellular RNA extraction and qualitative strand-specific rTth RT-PCR

Cells were washed three times with phosphate-buffered saline. RNA was extracted using TRIzol (Invitrogen; Carlsbad, CA), and treated two times for 4 h with DNase I using the DNA-free kit (Ambion; Austin, TX) following the manufacturer's protocol. RNA was quantified by UV spectrum analysis, and adjusted to 0.3 μg/μL. HCV RNA was detected utilizing a previously described qualitative strand-specific *rTth* reverse transcription PCR (RT-PCR) assay (Castet et al., 2002; Lanford et al., 1995). For detection of negative-strand HCV RNA, 1 μg of RNA in 10 μL of dietyl pyrocarbonate-treated water was layered with mineral oil and heated at 95 °C for 1 min, and lowered to 70 °C. A 20 μL mixture containing 10 pM of HCV-II sense primer (5′-CAC TCC CCT GTG AGG AAC T-3′, nucleotides [nt] 38–56 of the 5′UTR) (Laskus et al., 1997), 1× RT buffer (Applied Biosystems; Foster City, CA), 1 mM $MnCl_2$, 200 μM (each) deoxynucleoside triphosphate, and 5 U of *rTth* enzyme

(Applied Biosystems) was then added. The temperature was dropped to 60 °C for 2 min for annealing and then raised to 70 °C for 20 min for the cDNA reaction. To inactivate the RT activity of r*Tth*, chelating buffer (Applied Biosystems) was added. Forty microliters of the prewarmed PCR mixture containing 10 pM of HCV-I antisense primer (5′-TGG ATG CAC GGT CTA CGA GAC CTC-3′, nt 342–320 of the 5′UTR) (Laskus et al., 1997) and 3.75 mM $MgCl_2$ was added. Twenty-five cycles of PCR (94 °C 15 s, 58 °C 30 s, 72 °C 30 s) were performed.

For GAPDH measurements, RT was carried out using an oligo $d(T)_{16}$ primer under standard conditions (Hiasa et al., 2003). The cDNA product was subjected to 25 cycles of PCR (95 °C 1 min, 60 °C 2 min, 73 °C 2 min), using 50 pM of the GAPDH sense and antisense primers (forward primer 5′-GAA GGT GAA GGT CGG AGT-3′, reverse primer 5′-GAA GAT GGT GAT GGG ATT TC-3′), 0.1 mM of each dNTP, 2.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase. Reaction products were separated on 1.5 % agarose gels.

To ensure efficient removal of plasmid DNA after DNase I treatment, a qualitative PCR was performed. The plasmid DNA was completely digested as no PCR products were observed using this approach.

2.6. RNase protection assay

Antigenomic HCV RNA was detected as described previously (Chung et al., 2001). Briefly, utilizing the sense-oriented [α-^{32}P] UTP-labeled probe (corresponding to 98 nucleotides of the 3′ terminal HCV genome), antigenomic RNA was generated by in vitro transcription using T7 polymerase from the vector pHCV-3′T (Chung and Kaplan, 1999). Transcripts were generated using the RPA III kit according to the manufacturer's instructions (Ambion).

2.7. Real-time quantification of HCV positive- and negative-strand RNA

Positive- and negative-strand HCV RNAs were quantified by real-time PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany) and SYBR green I dye as described previously (Blackard et al., 2005). One microgram of RNA was used for cDNA synthesis in a mixture containing 5 U of r*Tth* and 10 pM of the appropriate RT primer (HCV-I for positive-strand HCV RNA or HCV-II for negative-strand HCV RNA). cDNA was purified with the High Pure PCR product purification kit (Roche Diagnostics).

Positive- and negative-strand HCV PCR amplifications were performed with 2 μL of purified cDNA in a reaction mixture containing 1 μL of LightCycler Fast Start DNA Master SYBR Green I, 4 mM of $MgCl_2$, and 5 pM of antisense primer KY78 (5′-CTC GCA AGC ACC CTA TCA GGC AGT-3′, nt 311–288 of the 5′UTR) and 5 pM of sense KY80 (5′-GCA GAA AGC GTC TAG CCA TGG CGT-3′, nt 68–91 of the 5′UTR). The PCR consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of the following thermal conditions: 15 s at 95 °C, 5 s at 70 °C, and 15 s at 72 °C. All samples were analyzed in triplicate. The sensitivity of the PCR for HCV was previously determined to be approximately 230 copies HCV/μL.

For quantification of GAPDH mRNA, RT was performed with the same amount of RNA used for HCV positive- and negative-strand analysis, using the oligo $d(T)_{16}$ primer under standard conditions. For real-time PCR amplification of GAPDH, a commercial GAPDH primer set (Roche Search LC, Mannheim, Germany) was used under the recommended conditions. For real-time PCR amplification of LacZ, sense (5′-GCC TGC GAT GTC GGT TTC CGC GAG G-3′) and antisense primers (5′-GCC AGC GCG GAT CAT CGG TCA GAC G-3′) were utilized under the following conditions: 10 s at 95 °C, 10 s at 68 °C, 16 s at 72 °C (Dobson et al., 1990). The sensitivity of detection was approximately 210 copies/μL.

DNA was quantified measuring SYBR green I dye incorporation into PCR products at 530 nm following manufacturer's instructions. An HCV standard curve was generated using a PCR product corresponding to nucleotides 38–342 of the 5′UTR. At the end of each run, a DNA melting curve was performed to control for sample homogeneity and quality. In a subset of samples, electroporation and sequencing were performed to confirm the correct identity of the amplified PCR product. Data were expressed as the copy number of HCV positive-strand (or negative-strand) RNA per molecule of GAPDH. This analysis was done in quadruplicate for each sample and presented as the mean and standard deviation. Each value was analyzed statistically using the SPSS 10.0 software (SPSS, Chicago, IL). Differences in mean values were compared using the Mann–Whitney *U*-test.

2.8. Western blotting analysis

Cells were washed twice with PBS, and lysed with 100 μL of Nonidet P-40 buffer (0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1% SDS). Protein lysate concentrations were measured using the DC protein assay Kit (Bio-Rad, Hercules, CA). Forty microliters of protein lysate were utilized. Separated products were then blotted onto Immobilon-P membranes, and each membrane was incubated with the relevant antibody. The ECL Kit (Amersham Pharmacia, Buckinghamshire, UK) was used for detection. Monoclonal antibody to HCV core protein (515s) (Kashiwakuma et al., 1996) was provided by Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; monoclonal antibody to β-galactosidase was purchased from Promega. Appropriate species-specific conjugated secondary antibodies were obtained commercially (Amersham Pharmacia).

2.9. ELISA for HCV core antigen

Cell culture lysates were adjusted to 0.2 mg/mL. HCV core antigen concentrations were quantified using the HCV core protein ELISA kit (Ortho-Clinical Diagnostics, Raritan, NJ) following the manufacturer's instructions (Bouvier-Alias et al., 2002). Core ELISA data were expressed as fmol of HCV core antigen per μg of total protein. The lower level of detection for this assay was less than 1.5 pg/mL.

3. Results

3.1. Replication-defective adenoviral vectors successfully replicate HCV RNA without cytotoxicity

To compare the transfection and infection efficiency of the vaccinia and adenovirus vectors, the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase gene, was transfected into cells that were then infected with either vaccinia-T7 (vTF7-3) or Ad-T7pol at a multiplicity of infection (MOI) of 10. The parental replication-incompetent vector Ad-Psi5 was used as an adenoviral vector control. At an MOI of 10, each viral vector efficiently expressed β-galactosidase in approximately 50% of cells 24 h after infection (Fig. 1A). Using trypan-blue staining, cell injury was observed in cells transfected with vaccinia-T7 but not in cells transfected with the Ad-T7pol or Ad-Psi5 vectors (data not shown).

Ribonuclease protection assay for negative-strand HCV RNA (Fig. 1B) and Western blotting for HCV core protein (Fig. 1C) were performed 24 h after infection. Expression of negative-strand HCV RNA was lower after Ad-T7pol infection compared to vTF7-3 infection, yet was clearly detectable. Similarly, HCV

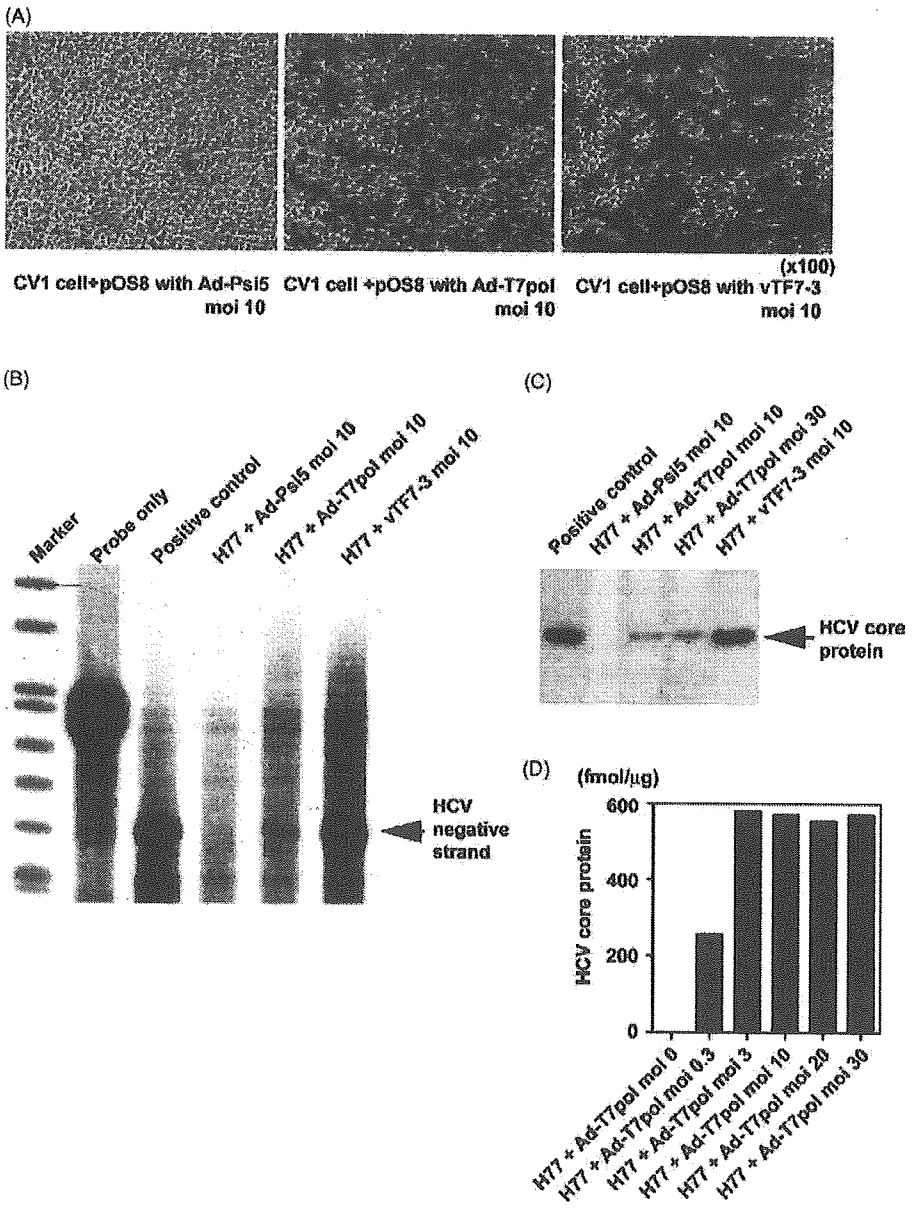

Fig. 1. Comparison of the transfection and infection efficiency of HCV replication system using vaccinia-T7 and adeno-T7 vectors: (A) after transfection with the pOS8 plasmid, cells were infected with virus vectors using control adenovirus (Ad-Psi5), recombinant adeno-T7 polymerase (Ad-T7pol), or vaccinia-T7 polymerase (vTF7-3) at an MOI of 10. (B) RPA for negative-strand HCV RNA was performed with H77 plasmid as a positive control. (C) Western blotting for HCV core protein was performed on CV-1 cell lysates 24 h after infection. (D) Quantitative HCV core ELISA results indicated that an MOI of 10 was optimal for adenoviral-driven HCV protein production.

core protein production was less robust after Ad-T7pol infection; nonetheless, it was clearly detectable. Negative-strand HCV RNA and HCV core protein were not detected when the control Ad-Psi5 vector was used. Quantitative HCV core ELISA results suggested that an MOI of 10 was optimal for adenoviral-driven HCV protein production (Fig. 1D); therefore, an MOI of 10 was selected for all subsequent experiments.

In contrast to increased HCV RNA synthesis and protein production in transfected/infected cells, LacZ mRNA levels decreased rapidly after day 1 and were not detectable after day 7 (data not shown).

3.2. Adenoviral-T7-driven HCV replication is dependent on an intact HCV polymerase gene

The $H77_{GDD \rightarrow AAG}$ mutant (Fig. 2A) was used to assess whether the HCV RNA polymerase gene (NS5B) was necessary for viral replication. By qualitative RT-PCR of the 5′UTR, HCV negative-strand synthesis was detectable only in the presence of both H77 and Ad-T7pol (Fig. 2B) in CV-1 cells. The absence of detectable negative-strand HCV RNA upon transfection of the mutant plasmid ($H77_{GDD \rightarrow AAG}$) indicates that replication was dependent on an intact polymerase sequence. H77 + Ad-T7pol expressed significantly higher core protein levels compared to $H77_{GDD \rightarrow AAG}$ + Ad-T7pol (Fig. 2C), further suggesting an intact polymerase sequence is necessary for robust HCV protein production. Ribonuclease protection assay demonstrated the presence of HCV negative-strand in CV-1, Huh7, and Huh-T7 cell lines on day 2 (Fig. 2D). However, negative-strand HCV RNA was not detected when the $H77_{GDD \rightarrow AAG}$ mutant was transfected, indicating that an intact NS5B sequence was necessary for negative-strand HCV RNA synthesis.

3.3. Kinetic analysis of HCV RNA synthesis and core protein production

The data described above suggest that the Ad-T7pol replication system results in efficient HCV RNA and protein

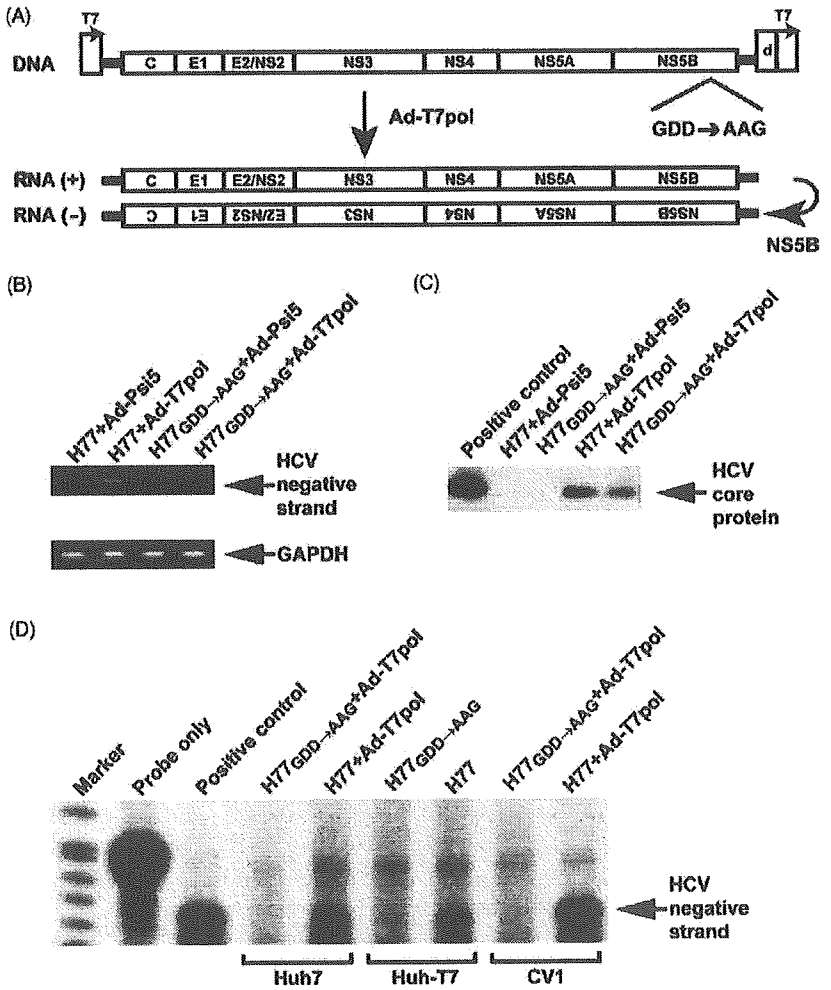

Fig. 2. (A) A control plasmid was prepared by mutating the active site motif from GDD to AAG in the NS5B RNA-dependent RNA polymerase sequence ($H77_{GDD \rightarrow AAG}$). (B) A qualitative strand-specific RT-PCR for negative-strand HCV RNA was performed as previously described (Lanford et al., 1995). (C) Western blotting analysis demonstrated that transfection/infection with H77 + Ad-T7-pol also resulted in HCV core protein production. (D) Ribonuclease protection assay demonstrated detectable negative-strand HCV RNA in CV-1, Huh7, and Huh-T7 cell lines on day 2.

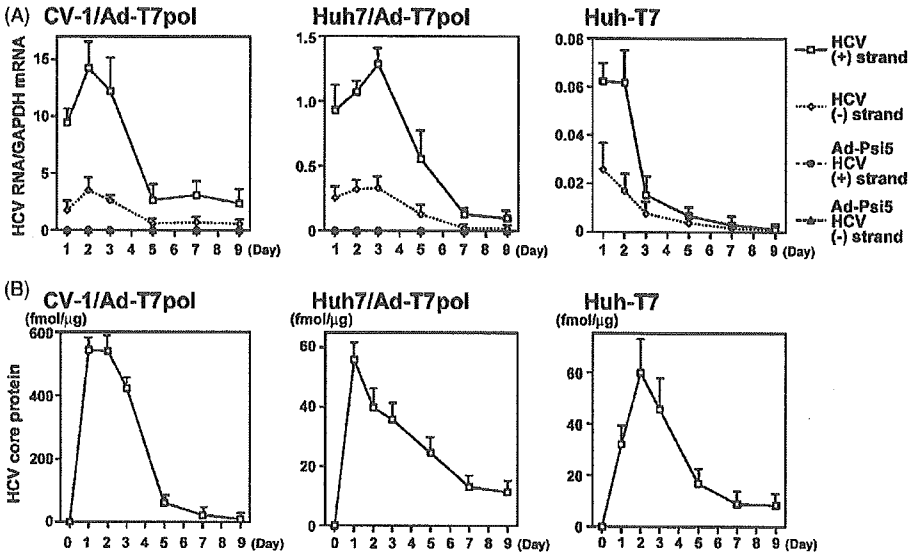

Fig. 3. (A) Real-time PCR of HCV positive- and negative-strand RNA was performed as described. Data are expressed as the strand-specific HCV copy number per molecule of GAPDH. Error bars indicate the mean ± standard error (S.E.) of four replicates. (B) A quantitative core ELISA measured HCV protein production in CV-1, Huh7, and Huh-T7 cell lines. Data are expressed as fmol (mean ± S.E.) of HCV core per μg of total protein for three replicates.

expression without inducing cell toxicity. Thus, the time course of strand-specific HCV RNA synthesis (Fig. 3A) and HCV protein production (Fig. 3B) was examined further in CV-1 and Huh7 cells (transfected with H77 and infected with Ad-T7pol), as well as Huh-T7 cells (transfected with H77). Both positive- and negative-strand HCV RNA were detectable for the entire 9-day experiment in each cell line. Positive-strand HCV RNA levels increased significantly after infection and continued to be expressed at high levels for 3 days and diminished thereafter. Negative-strand HCV RNA synthesis paralleled that of positive-strand throughout the time course; however, the quantity of negative-strand HCV RNA was approximately 10% of positive-strand levels. This is consistent with positive-/negative-strand ratios reported from infected human liver samples (Komurian-Pradel et al., 2004; Laskus et al., 1998). As expected, HCV RNA was not detected in CV-1 or Huh7 cells infected with the Ad-psi5 control vector.

Using an identical experimental approach, HCV core protein production was expressed strongly during days 1–3 in all cell lines examined, and diminished with similar kinetics as HCV RNA (Fig. 3B). Similar to HCV RNA, HCV core protein was detectable for the entire 9-day experiment in each cell line.

To circumvent potential perturbations in the cellular environment due to transfection/infection with viral vectors, experiments in a Huh7 cell line stably transfected with T7 polymerase (Huh-T7) were performed. After transfection of H77 into these cells, positive- and negative-strand HCV RNA were detected (Fig. 3A), as well as HCV core protein (Fig. 3B), throughout the entire 9-day experiment. Interestingly, HCV RNA levels were lower in Huh-T7 cells than in CV-1 and Huh7 cells, although core levels were not appreciably different between Huh7 and Huh-T7 cells.

3.4. IFN efficiently inhibits HCV expression

Utilizing these refined models of HCV replication, the inhibitory effects of IFN on HCV expression were examined. To determine the potential effects of IFN on cellular gene translation, the plasmid OS8 was transfected, and LacZ mRNA levels were measured in the presence of several doses of IFN. LacZ mRNA expression was slightly reduced; however, no significant toxicity in cells exposed to IFN was observed using trypan-blue staining (data not shown).

In CV-1 cells (Fig. 4A), HCV positive-strand RNA levels were significantly decreased in the presence of 1000 IU/mL IFN at day 2 (14.21 ± 3.95 versus 8.55 ± 0.61, $p < 0.05$). A trend toward reduced HCV RNA was also observed on day 3 (12.23 ± 5.43 versus 4.13 ± 0.74, $p < 0.10$). In Huh7 cells, a significant decrease of HCV positive-strand was observed on days 2 (1.07 ± 0.07 versus 0.63 ± 0.14, $p < 0.05$) and 3 (1.29 ± 0.13 versus 0.44 ± 0.15, $p < 0.05$). In Huh-T7 cells, a significant decrease was also observed on day 2 (0.06 ± 0.03 versus 0.03 ± 0.007, $p < 0.05$). For HCV negative-strand RNA (Fig. 4B), only day 3 IFN-treated CV-1 cells had significantly decreased levels compared to untreated cells (2.60 ± 0.41 versus 0.97 ± 0.31, $p < 0.05$).

HCV core protein expression was approximately 10-fold higher in CV-1 cells compared to either Huh7 or Huh-T7 cells (Fig. 4C). IFN treatment of CV-1 cells did not appear to have a large effect on HCV core protein levels; however, HCV core levels were decreased in IFN-treated CV-1 cells compared to untreated cells at days 3 (423.51 ± 25.73 fmol/μg versus 190.92 ± 35.25 fmol/μg, $p < 0.05$) and 5 (60.24 ± 12.89 fmol/μg versus 34.15 ± 0.76 fmol/μg, $p < 0.05$). For Huh7 and Huh-T7 cells, HCV core expression was significantly reduced when treated with IFN compared to untreated cells at all time points ($p < 0.05$).

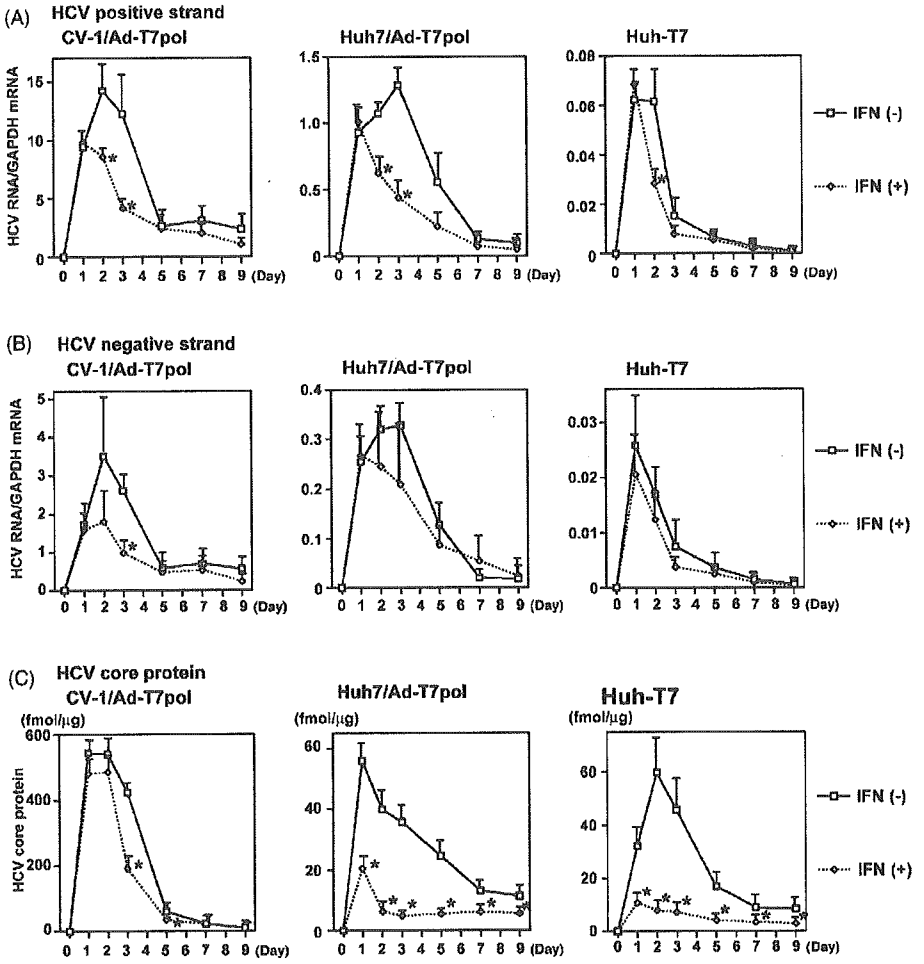

Fig. 4. (A) Real-time PCR of positive-strand HCV RNA was performed in the presence of 1000 IU/mL IFN. Error bar indicates mean ± S.E. for four replicates ($^{*}p < 0.05$; $^{**}p < 0.10$). (B) Real-time PCR of negative-strand HCV RNA was also performed in the presence of 1000 IU/mL IFN. (C) Quantitative core ELISA also demonstrated decreased HCV protein production in IFN-treated cells.

4. Discussion

Because of the cytotoxic nature of vaccinia virus, long-term evaluation of HCV RNA synthesis and protein production, as well as characterization of the inhibitory effects of antiviral agents, such as IFN and RBV, was not possible using our previous replication model. By using adenovirus-derived T7 vectors, vaccinia-induced cytotoxicity was removed, allowing sustained detection of HCV replication and protein production for 9 days in multiple cell types. The refined binary HCV replication system efficiently synthesized HCV negative-strand RNA, an important indicator of ongoing, active viral replication, in an NS5B-dependent manner, as no negative-strand HCV RNA was detected upon transfection of an NS5B mutant. Using Huh7 cell lines stably expressing T7 polymerase (Huh-T7), dependence of the replication models on any viral vectors was removed. Quantities of HCV RNA synthesis and protein production in Huh-T7 cells were not as robust as in CV-1 or Huh7 cells transfected with Ad-T7. However, sustained HCV replication in Huh-T7 cells, with no obvious signs of cytotoxicity, suggests that this viral vector-independent replication model will be useful for future studies of virus–host interactions and the development of antiviral agents with activity against HCV genotype 1.

These binary systems offer several advantages over currently available HCV replication systems. First, these replication models do not require continuous antibiotic selection as do current replicon systems (Blight et al., 2000; Frese et al., 2001; Guo et al., 2001; Lohmann et al., 1999). Second, the requirement of highly adaptive viral mutations for efficient replicon activities that are not necessarily viable in vivo (Bukh et al., 2002) may limit the interpretability of certain findings obtained from replicon systems. Because the refined replication models do not require continuous selection and do not possess highly 'adaptive' viral mutations, they are more authentic for characterization of antiviral agents, virus–host interactions, and viral fitness. Third, these replication systems can be used to study HCV replication in a variety of hepatocyte- and non-hepatocyte-derived cell types; in contrast, replicon systems only replicate efficiently in Huh7 cells. Most importantly, these replication systems use a full-length infectious genotype 1a cDNA construct that yields an

Table 1
Several similarities and differences between the vaccinia and adenovirus systems exist

	Vaccinia	Ad-T7
T7 polymerase delivery	Vaccinia virus	Adenovirus
Cytotoxicity	Yes	No
HCV replication	(+) and (−) strand synthesis; high levels	(+) and (−) strand synthesis; low but effective levels
Duration of replication	24 h	9 days
HCV protein production	Yes	Yes
Quasispecies generation	Yes	Yes
IFN inhibits replication	Yes	Yes

authentic dual-function template in vivo that is both translated and transcribed. Moreover, transfected cells in our replication systems are able to express all HCV structure and non-structural proteins (Lin et al., 2005). Thus, they are more likely to carry out authentic HCV RNA replication than replicon systems based on sub-genomic constructs.

Several significant differences exist between the vaccinia and adeno-T7 replication systems (Table 1). Both are capable of positive- and negative-strand HCV RNA synthesis, protein production, and quasispecies generation without the need for cell culture adaptive mutations (Chung et al., 2001; Contreras et al., 2002; Blackard and Hiasa et al., unpublished data). HCV replication is inhibited significantly by IFN in both systems. However, the vaccinia-based system replicates at much higher levels than the adeno-T7-based system; yet, HCV RNA synthesis occurs for at least 9 days in the former due to the lack of vector-induced cytotoxicity. Nonetheless, the decrease of HCV RNA synthesis and protein production after 3 days suggests an inhibitory effect exerted by key host cells proteins, such as protein kinase R (PKR), since adenoviruses do not inhibit PKR function as does vaccinia virus (Chang et al., 1992; Watson et al., 1991). Further examination of host antiviral pathways that limit robust long-term viral replication in culture is necessary.

We used these refined replication systems to explore the kinetics of HCV RNA synthesis and protein production in the presence of IFN. When 1000 IU/mL of IFN was added to the culture medium of HCV-expressing cells, there was no difference in HCV positive- or negative-strand quantity compared to untreated cells at day 1. Despite this lack of short-term antiviral activity, HCV RNA was significantly decreased in IFN-treated cells at days 2 and 3, suggesting that the full effects of IFN may require at least 24 h.

This cell-based HCV replication system has already been used to examine the interaction between HCV protein expression and host type I IFN signaling components in the Jak-STAT kinase pathway (Lin et al., 2005). Recently, in vitro systems that support infectious HCV production have been reported. However, these systems are based on HCV genotype 2a (Wakita et al., 2005; Zhong et al., 2005) and do not support replication in cells other than the highly permissive Huh-7 cell line and its derivatives. Importantly, the replication systems described here are based on genotype 1a isolate and replicate in several hepatocyte- and non-hepatocyte-derived cell lines. Thus, these refined replication models provide the opportunity to explore HCV molecular biology and the interactions between antiviral agents and specific HCV and/or host proteins that are relevant to genotype 1 infection.

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