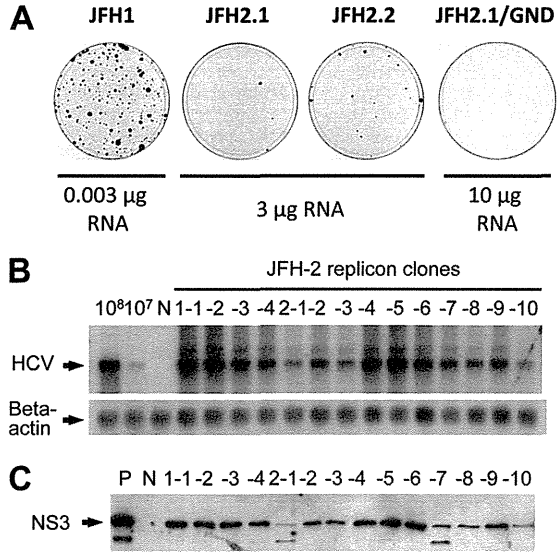

FIG 4 G418-resistant colony formation of JFH-1 and JFH-2 replicons and analysis of JFH-2 replicon cells. (A) Subgenomic RNAs were synthesized *in vitro* by using pSGR-JFH1, pSGR-JFH2.1, pSGR-JFH2.2, and pSGR-JFH2.1/GND as templates. Transcribed subgenomic RNAs were electroporated into HuH-7 cells, and cells were cultured with G418 for 3 weeks before being stained with crystal violet. JFH-1 subgenomic RNA (0.003 μg), 3 μg of JFH-2.1 and JFH-2.2 subgenomic RNA, and 10 μg of JFH-2.1/GND subgenomic RNA were transfected into HuH-7 cells. Experiments were performed in triplicate, and representative staining examples are shown. (B) Northern blot analysis. Total cellular RNA isolated from each of four SGR-JFH2.1 clones (1-1 to 1-4) and 10 SGR-JFH2.2 clones (2-1 to 2-10) was analyzed by using a random-primed DNA probe to detect replicon RNA. Isolated total cellular RNA (4 μg) was separated by denatured agarose gel electrophoresis. After electrophoresis, HCV- and beta-actin-specific RNAs were detected by Northern blot analysis with random-primed DNA probes specific to HCV and beta-actin sequences. Arrows indicate replicon RNA or beta-actin mRNA. (C) Western blot analysis. Cell lysates were prepared from four SGR-JFH2.1 clones (1-1 to 1-4) and 10 SGR-JFH2.2 clones (2-1 to 2-10). The NS3 proteins were detected with rabbit anti-HCV NS3 antibody. Positive-control (P) and negative-control (N) cell lysates were obtained from JFH-1 replicon cells and naive HuH-7 cells.

quences in the JFH-2 viral genome. One sequence contained alanine and isoleucine (AI) at amino acid positions 1204 and 1205, and the other contained methionine and leucine (ML) at the same positions. We referred to these viral genomes containing AI or ML as JFH-2.1 or JFH-2.2, respectively. From the cloning analysis of PCR products, JFH-2.1 populated 19 of 32 clones (59%), and JFH-2.2 populated 13 of 32 clones (41%). To analyze the replication efficiency of the JFH-2 clone, we thus constructed two subgenomic replicon constructs, pSGR-JFH2.1 and pSGR-JFH2.2, as pSGR-JFH1 (11). Synthesized replicon RNAs of JFH-2.1 and JFH-2.2 were independently transfected by electroporation into HuH-7 cells. The transfected cells were then grown for 3 weeks in selection culture that contained 1 mg/ml of G418. Several colonies survived the selection culture, as illustrated by crystal violet staining (Fig. 4A). The JFH2.1/GND replication-incompetent control RNA-transfected cells did not form any colonies, even when 10 μg of RNA was transfected. The colony formation efficiencies of the JFH-2.1 and JFH-2.2 replicons were 0.94 ± 0.54 and 6.43 ± 3.39 CFU/μg RNA, respectively, which were substantially lower than the colony formation efficiency of the JFH-1 subgenomic replicon ($5.32 \times 10^4 \pm 5.02 \times 10^4$ CFU/μg RNA) (11). Four colonies of the JFH-2.1 replicon and 10 colonies of the JFH-2.2 replicon were cloned and expanded for further analysis. Replicon RNA was isolated from each replicon cell clone, and the HCV RNA titer and sequence of the replicon genome were determined (Table 3). The average HCV RNA titer in replicon cell clones was determined by real-time RT-PCR detection as (8.70 ± 4.94) $\times 10^7$ copies/μg of RNA. The size and amount of the replicon RNA in the replicon cells were confirmed by Northern blot analysis (Fig. 4B). We also detected NS3 protein in each clone of replicon cells by Western blot analysis (Fig. 4C). NS3 proteins were mainly found at approximately 70-kDa by polyclonal anti-NS3 antibody; however, an additional signal was also detected at a smaller molecular size in some replicon cells, including the positive-control JFH-1 replicon cells.

Next, we determined the sequences of replicating RNA in each replicon cell clone. Most of the clones, except replicon clone 2.2-8, had at least one nonsynonymous mutation (Table 3). We found nonsynonymous mutations in the NS3, NS5A, and NS5B regions, and three mutations were common among the different replicon genomes. Among the mutations found in the NS3 region, both 1547FL and 1614CW were found in two different replicon cells, and the 1651TN mutation was found in five replicon cells. The 2280QR mutation in NS5A was found in three replicon cells. 2217AS and 2222HQ, which are located in the interferon sensitivity-determining region (ISDR), were each found in a single replicon cell (8). To determine the adaptive effect of these mutations (Fig. 5A), we inserted these mutations (listed in Table 3), except for 1204MK, into pSGR-JFH2.1 and tested the colony formation efficiency of the mutant replicons. The 1204MK mutation was not tested since methionine at amino acid position 1204 was specific for the JFH2.2 sequence. As shown in Fig. 5B, 1547FL, 1614CW, 1651TN, 2222HQ, and 2280QR had weak to moderate adaptive effects for colony formation. Interestingly, the 2217AS mutation in the ISDR strongly enhanced the colony formation to approximately 3×10^4 times that of the parental JFH2.1 replicon (Fig. 5B). We further tested these adaptive mutations in the luciferase reporter replicon format, as described previously (12). SGR-JFH2.1 with the 2217AS construct exhibited significant replication compared to JFH2.1/GND, which is the replication-incompetent negative control. However, other constructs showed no evidence of replication in the transient replication assay (Fig. 5C).

Full-length HCV replication. The 2217AS mutation substantially enhanced RNA replication of the JFH-2.1 subgenomic replicon compared with other mutations. We examined whether a full-length JFH-2 HCV clone with the 2217AS mutation could produce infectious virus. In our previous study, we constructed the J6/JFH1 chimeric construct by replacement of the 5′ untranslated region to the p7 region (EcoRI-BclI) of J6 (1), and we found that J6/JFH1 produces a larger amount of infectious virus in the culture medium (3). We thus used the structural region of the J6CF clone and the NS2 region of the JFH-1 clone from a J6/JFH-1 chimeric virus construct and fused it to the NS3 to 3′X regions of JFH-2.1 with the 2217AS mutation (plasmid pJ6/JFH2/AS) since it was not clear if the structural and NS2 regions of the JFH-2 strain were functionally intact (Fig. 6A). Full-length viral RNA was synthesized from linearized pJ6/JFH2/AS and electroporated into Huh-7.5.1 cells. After two independent transfections, the transfected cells were divided into sub-cell lines to form a total of four sub-cell lines (T1A, T1B, T2A, and T2B). All four sub-cell lines were serially passaged, and HCV core protein, RNA, and infectivity levels in the culture supernatant were monitored (Fig.

TABLE 3 Mutations and RNA titer of the JFH-2 replicon cell clones

Replicon clone	Nucleotide		Amino acid			Replicon titer (no. of copies/μg of RNA)
	Mutation	Position	Mutation	Position	Region	
2.1-1	A→G	2012	E→G	1109	NS3	1.30E+8
	C→A	3638	T→N	1651	NS3	
2.1-2	T→C	3325	F→L	1547	NS3	1.52E+8
	C→A	3638	T→N	1651	NS3	
2.1-3	A→G	5525	Q→R	2280	NS5A	1.09E+8
	A→G	7155	None		NS5B	
2.1-4	C→A	3638	T→N	1651	NS3	1.41E+8
	A→G	7795	None		3′ UTR	
2.2-1	C→G	3528	C→W	1614	NS3	2.33E+7
2.2-2	G→T	5335	A→S	2217	NS5A (ISDR)	3.57E+7
2.2-3	C→G	919	None		*neo*	3.35E+7
	C→A	5352	H→Q	2222	NS5A (ISDR)	
2.2-4	C→A	1223	None		EMCV-IRES[a]	1.05E+8
	C→A	2115	None		NS3	
	G→T	6243	K→N	2519	NS5B	
2.2-5	C→A	3327	F→L	1547	NS3	1.67E+8
2.2-6	T→C	625	None		*neo*	1.09E+8
	C→A	3638	T→N	1651	NS3	
	A→G	5525	Q→R	2280	NS5A	
	T→A	5754	None		NS5A	
	G→A	5803	G→S	2373	NS5A	
2.2-7	C→G	3528	C→W	1614	NS3	6.25E+7
2.2-8	None		None			5.31E+7
2.2-9	C→G	3638	T→N	1651	NS3	6.71E+7
	G→A	5269	A→T	2195	NS5A	
	A→G	5525	Q→R	2280	NS5A	
2.2-10	T→A	2297	M→K	1204	NS3	2.95E+7
	A→G	7815	None		3′ UTR	

[a] EMCV-IRES, encephalomyocarditis virus internal ribosome entry site.

6B and C and Table 4). At the first cell passage, the HCV core protein levels were approximately 300 fmol/liter, and the infectivities were very low. Secreted HCV core protein levels decreased in all of the passaged cells until 25 days after the transfection. However, HCV core protein secretion of passaged T1A cells began to increase from 30 days after transfection. Subsequently, increased core protein secretion was also observed in other passaged cells although at different time points (Fig. 6B and Table 4). The maximum core protein levels in the medium were up to 9,241 fmol/liter in T1B cells at day 75 posttransfection. Infectivity detected in the culture medium was also first increased in T1A, and similar increases were observed with other passaged cells at later time points. Furthermore, specific infectivity (infectivity/HCV RNA or infectivity/HCV core protein) was also higher than in the initial culture medium (Table 4). The passaged cells were immunostained with anti-core monoclonal antibody (Fig. 6D). At 4 weeks after transfection, only a few cells were positive in all four sub-cell lines. However, the number of positive cells increased from 8, 12, 18, or 14 weeks after transfection in T1A, T1B, T2A, or T2B cells, respectively. These results indicate that phenotypic change occurred in the replicating virus after the serial passages of the transfected cells. Before this phenotypic change, the replicating viruses were not able to secrete significant amounts of infectious virus particles due to an unknown defect in infectious virus particle formation or secretion. After the phenotypic change, the robust core protein secretion might have been caused by changes in the efficiency of infectious virus production or secretion. To compare the virus characteristics before and after the phenotypic change, we analyzed T1A culture medium from 5 days, 8 weeks, and 11 weeks posttransfection by density gradient assay (Fig. 6E). The day 5 medium showed a broad density profile both of core protein and HCV RNA, and infectivity was not detected. Interestingly, the peaks of HCV core protein and RNA at around 1.15 mg/ml density became higher at 8 weeks and had a further increase at 11 weeks. Broader minor peaks at the lighter density remained small at week 11. The infectivity peak also became higher at 8 and 11

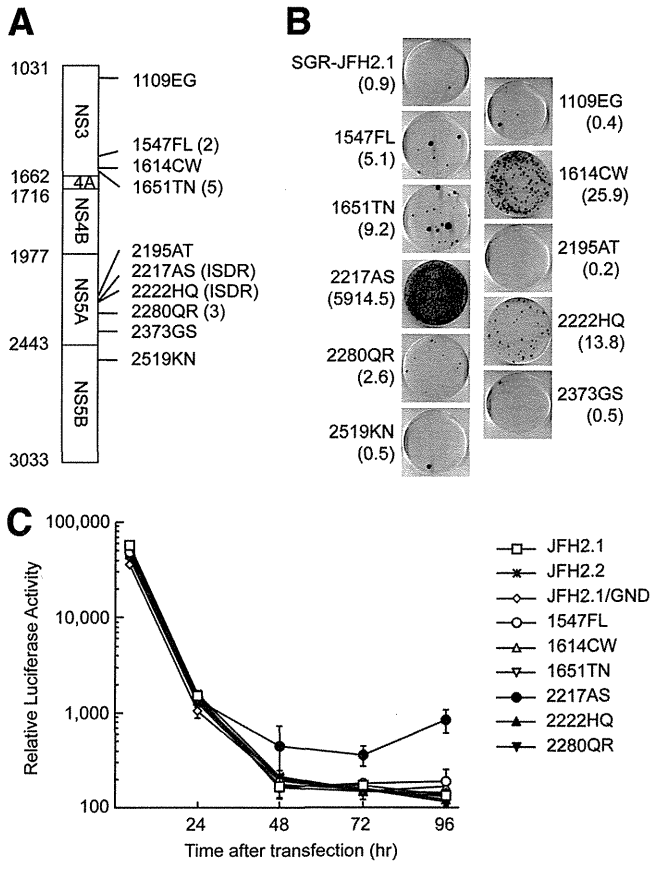

FIG 5 Analysis of the effect on colony formation and transient replication efficiency of mutations detected in replicon cell clones. (A) The box indicates the open reading frame of JFH-2 replicon and amino acid sequences at positions 1031 and 3033 (numbering by full-length JFH-2). The numbers on the left side of the box show the starting position of each protein, with the exception of 3033, which is the end position of NS5B. The numbers with the lines on the right side indicate the mutations introduced in the replicon constructs. 1547FL, 1651TN, and 2280QR mutations were found in 2, 5, and 3 replicon clones, respectively (Table 3). 2217AS and 2222HQ mutations were found in the ISDR (Table 3). (B) Each amino acid mutation found in the replicon genome was introduced into the pSGR-JFH2.1 replicon construct, and colony formation of the JFH2.1 replicon with the wild-type sequence (SGR-JFH2.1) and other mutations was tested. Briefly, transcribed RNA (5 μg) was transfected into HuH-7 cells, and cells were cultured for 3 weeks before being stained with crystal violet. The numbers in the parentheses show the colony formation efficiency (CFU/μg of RNA) of the replicon constructs. (C) Transient replication of JFH-2 subgenomic replicon. HuH-7 cells were transfected with the transcribed RNA from pSGR-JFH2.1/Luc, pSGR-JFH2.2/Luc, pSGR-JFH2.1/Luc/GND (replication-incompetent control), and pSGR-JFH2.1/Luc constructs with adaptive mutations (1547FL, 1614CW, 1651TN, 2217AS, 2222HQ, and 2280QR). Transfected cells were harvested at the indicated time points and at 4 h posttransfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate. Assays were performed in triplicate, and data are presented as means ± standard deviations. The background signal of the luciferase measurement was 129.4 ± 27.4 units.

weeks after transfection. Interestingly, this density profile at 11 weeks posttransfection was quite similar to that of JFH-1 or the J6/JFH1 chimera, as previously described (21, 38). Furthermore, virus-like particles were visualized in the concentrated culture medium by electron microscopic analysis, whereas only unstructured aggregates were found with the mock-transfected control (Fig. 7, left panel; also data not shown). An aliquot of the culture medium was used for immunoelectron microscopy with an E2-specific antibody (AP33), and gold-labeled spherical structures were detected (Fig. 7, middle panel). The overall diameter of the structures (50 to 65 nm) is compatible with the predicted size of HCV.

Characterization of cell culture-adapted J6/JFH2/AS virus. During the serial passages of the transfected cells, the J6/JFH2/AS virus adapted to produce more infectious viruses in the cell culture. We next compared the adapted J6/JFH2/AS virus (T1B cells at day 75 posttransfection) with the J6/JFH1 virus. Huh-7.5.1 cells were inoculated with the viruses at a multiplicity of infection (MOI) of 0.03. The core protein production levels in both the infected cells and the culture medium were increased with similar kinetics after the virus infection, although at lower levels for J6/JFH2/AS virus than J6/JFH1 virus (Fig. 8A). We also tested the neutralization of the infection of these viruses by using mouse anti-CD81 monoclonal antibody and anti-HCV human IgG purified from HCV carrier serum (Fig. 8B). Both antibodies clearly inhibited the infectivity of inoculated virus to Huh-7.5.1 cells. Thus, the J6/JFH2/AS and J6/JFH1 viruses appeared to share similar infection pathways.

Adaptive mutations in the cell culture-adapted J6/JFH2.2/AS virus. We determined the full-length sequence of the HCV genome in the culture medium of T1A and T1B sub-cell lines at 75 days posttransfection by directly sequencing the amplified virus cDNA. We found the following nonsynonymous mutations, in addition to 2217AS, in the viral genomes: 1342ST in NS3 and 2219CR in NS5A of T1A and 148AT in the core protein, 2219CS in NS5A, and 2695TI and 3016LP in NS5B of T1B. These mutations were introduced into the J6/JFH2.2/AS cDNA, and synthetic RNA was transfected into Huh-7.5.1 cells. However, robust virus production was not observed at an early time point after transfection (data not shown). Because the important adaptive mutations might still not be detected in the virus population, we decided to concentrate on the dominant virus population and fix the important mutations in T1A and T1B virus by serial virus passages. We thus repeatedly inoculated naive Huh-7.5.1 cells three times with J6/JFH2/AS virus at a low MOI and harvested the virus when the virus titer plateaued. We sequenced the full-length genome of virus in the culture medium after the third inoculation (T1Ai3 or T1Bi3) and found the following nonsynonymous mutations: 405MK and 417NT in E2, 868MT in NS2, 1642TA in NS3, 1722IV in NS4B, 2204SG in NS5A, and 2695TI in NS5B of T1Ai3; and 148AT in the core protein, 329TS and 356MV in E1, 626VG in E2, 1678IV in NS4A, 1767KR in NS4B, 2219CS in NS5A, and 2695TI and 3016LP in NS5B of T1Bi3 (Fig. 3B). We then introduced these mutations into pJ6/JFH2/AS to construct pJ6/JFH2/AS/mtT1A and pJ6/JFH2/AS/mtT1B. Synthetic RNAs produced from both of the mutation-containing plasmids and control plasmids were transfected into Huh-7.5.1 cells. After the transfection, core proteins were secreted into the culture medium at levels similar to those of JFH-1 RNA-transfected cells but at lower levels than J6/JFH1 RNA-transfected cells (Fig. 9A). HCV RNA levels in the culture medium of J6/JFH2/AS/mtT1A (mtT1A) and J6/JFH2/AS/mtT1B (mtT1B) RNA-transfected cells were less than those in cells transfected with either or JFH-1 J6/JFH1 RNA (Fig. 9B). This discrepancy may be due to the lower detection efficiency of the JFH-1 core protein in the immunoassay, as reported previously (31). Infectivity in the culture medium was also determined. Interestingly, higher infectious titers were detected in the culture medium of the J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B

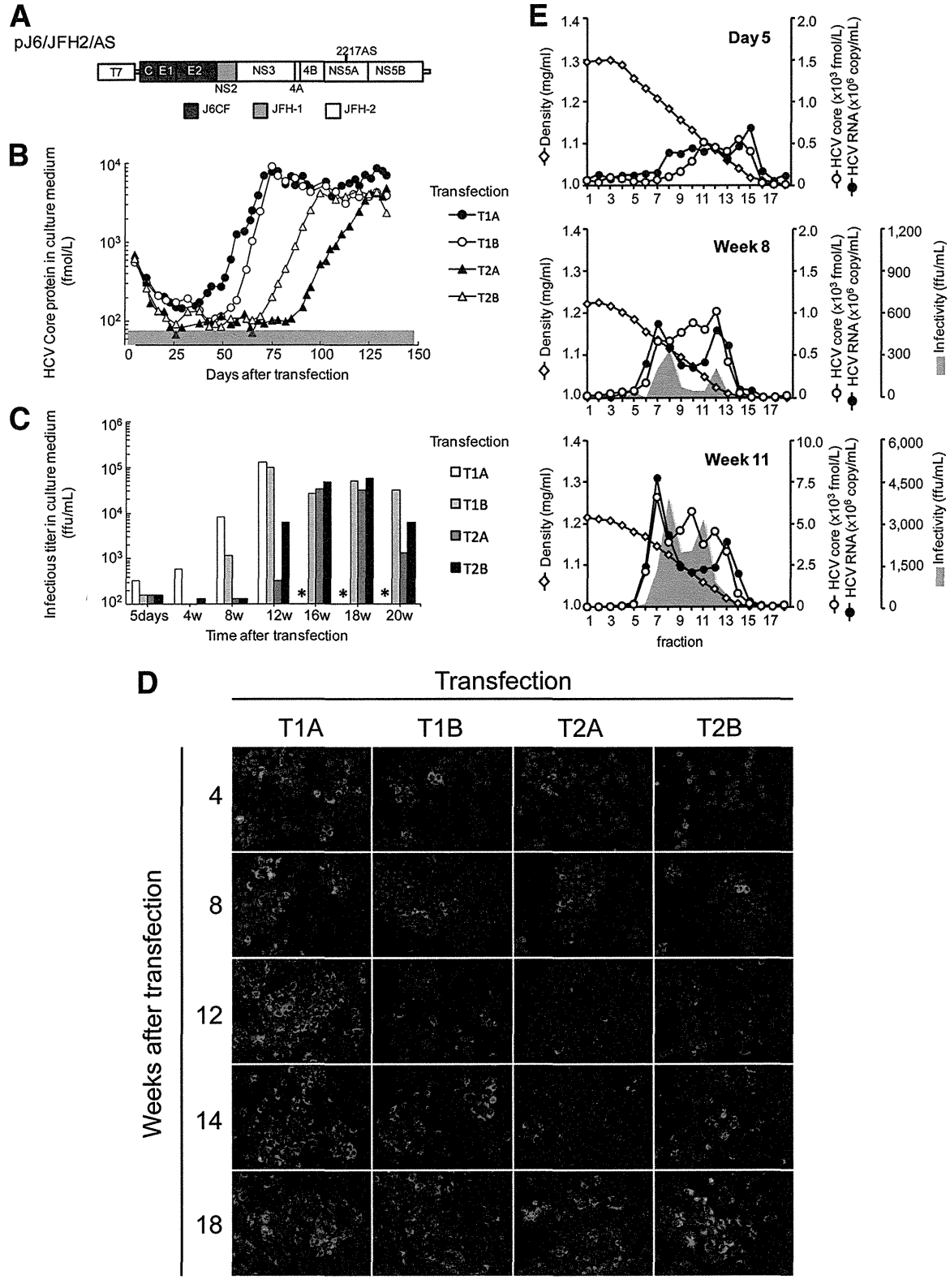

FIG 6 J6/JFH2 chimeric full-length HCV replication. (A) Organization of full-length chimeric JFH-2 construct, pJ6/JFH2/AS. A T7 RNA promoter is located upstream of the 5′ end of the HCV cDNA construct. The 5′ UTR and NS2 region are derived from the JFH-1 strain. Regions of the core protein to E2/p7 are derived from the J6CF strain. The 2217AS adaptive mutation is introduced. (B) Huh-7.5.1 cells were transfected with the transcribed RNA from pJ6/JFH2/AS. Two independently transfected cell lines (transfections 1 and 2 [T1 and T2, respectively]) were divided into two passages, resulting in four independently passaged transfected cell lines (T1A, T1B, T2A, and T2B). At each time point, culture medium was harvested and analyzed for the presence of HCV core protein by Lumipulse Ortho HCV Ag (Ortho-Clinical Diagnostics). The gray area indicates values that are below the detection limit. (C) Infectious titers in the culture supernatant of the passaged transfected cells (T1A, T1B, T2A, and T2B) were determined by focus formation assay. After 16 weeks, the culture media from T1A

TABLE 4 Specific infectivity of culture medium after transfection of J6/JFH2/AS RNA

Cell culture medium (no. of days posttransfection)	Infectivity (FFU/ml)	HCV core protein (fmol/liter)	HCV RNA (no. of copies/ml)	Specific infectivity	
				Infectivity/HCV core protein	Infectivity/HCV RNA (10^4)
T1A (5)	3.20E+1	3.57E+2	2.63E+6	0.09	0.12
T1B (5)	1.60E+1	3.07E+2	2.35E+6	0.05	0.07
T2A (5)	1.60E+1	3.13E+2	2.99E+6	0.05	0.05
T2B (5)	1.60E+1	2.63E+2	3.42E+6	0.06	0.05
T1A (82)	1.28E+4	5.47E+3	2.63E+7	2.34	4.87
T1B (82)	9.83E+3	5.98E+3	2.73E+7	1.64	3.61
T2A (120)	3.17E+3	2.47E+3	8.49E+6	1.28	3.73
T2B (120)	5.83E+3	4.51E+3	2.83E+7	1.29	2.06

RNA-transfected cells than in JFH-1 RNA-transfected cells; however, they were lower than in J6/JFH-1 RNA-transfected cells (Fig. 9C).

Transfected cells were serially passaged, and, importantly, both types of transfected cells (J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B RNA) secreted core protein and HCV RNA at high levels, even at the first passage after transfection, and the levels of HCV core protein and RNA were maintained during the passages (Fig. 10A and B). Infectious titers in the medium of the transfected cells were also measured (Fig. 10C). J6/JFH2/AS/mtT1A secreted a higher infectious titer than J6/JFH2/AS/mtT1B although their HCV core protein levels and RNA levels in the culture medium were similar. To confirm the rapid infectious viral production phenotype of these viruses, we inoculated naive Huh-7.5.1 cells with the culture medium of J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B RNA-transfected cells at 8 and 38 days posttransfection at an MOI of 0.01. All of the inoculated cells secreted core protein and HCV RNA with similar kinetics (Fig. 11A and B). The infectious titer was also determined in the culture medium of the infected and passaged cells (Fig. 11C). mtT1B (day 38 posttransfection) showed lower infectivity at 7 days after inoculation; however, substantial infectivity was detected at 13 and 27 days. The culture medium of mtT1A (day 8 and day 38 posttransfection) was harvested at 20 days after inoculation and analyzed by a sucrose density gradient assay, as described above (Fig. 11D). Major peaks of both HCV core protein and RNA were clearly shown at around 1.15 mg/ml, and the subpeaks of HCV core protein were found in lighter fractions. On the other hand, major peaks of infectivity were found at around 1.0 mg/ml. Compared to the data shown in Fig. 6E, the HCV core and RNA levels and infectivity titer are higher in mtT1A (day 8 and day 38 posttransfection) virus. The similar virus characteristics suggested that J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B viruses do not need further adaptations for autonomous expansion in cultured cells. Thus, we established stable cell culture-adapted virus and constructed recombinant cell culture-adapted infectious HCV clones by reverse genetics.

Human hepatocyte-transplanted uPA/SCID mouse experiment. To determine the *in vivo* infectivity of J6/JFH2/AS virus, we inoculated day 75 culture medium of T1B cells containing 1×10^6 RNA copies of purified J6/JFH2/AS HCV particles and original patient serum also containing 1×10^6 RNA copies into human hepatocyte-transplanted uPA/SCID mice. Inoculation of 1×10^6 RNA copies of cell culture-derived J6/JFH1 virus usually results in robust infection for human hepatocyte-transplanted uPA/SCID mice. Two mice were used for each type of inoculum. Human albumin levels in sera of the inoculated mice were more than 3 mg/ml during the experiment, which supported the high replacement ratio of the human hepatocytes in the mouse liver. Both mice inoculated with patient serum became HCV RNA positive 1 week postinoculation and remained positive during the 4-month observation period (Fig. 12). However, the mice inoculated with J6/JFH2/AS virus in culture medium did not become HCV positive after inoculation (Fig. 12). One mouse inoculated with J6/JFH2/AS virus died 16 days after inoculation, and the cause of death was unknown. HCV RNA was not detected at 7, 14, and 16 days postinoculation. The other mouse inoculated with culture medium was also tested every week for serum HCV RNA and remained negative for 56 days after infection. On day 56, this mouse received a second inoculation with the same culture medium. This mouse was monitored for a total of 63 days, but weekly tests for HCV RNA were continuously negative. Thus, the cell culture-adapted virus in the inoculum may be less viable *in vivo* although the virus acquired robust replication capacity in HuH-7 cells.

Full-length JFH-2 construct. We successfully established J6/JFH2/AS-derived cell culture-adapted viruses. Next, we produced a full-length JFH2/AS virus by using the structural region sequence from JFH-2. pJFH2/AS was constructed according to the viral sequence, and an alanine-to-serine mutation was introduced at amino acid position 2217. JFH2/AS RNA synthesized *in vitro* was electroporated into the Huh-7.5.1 cells, as described above. J6/JFH2/AS RNA was also transfected simultaneously and compared. Two groups of independently transfected cells (transfections 3 and 4 [T3 and T4, respectively]) were analyzed for JFH2/AS and J6/JFH2/AS. Interestingly, JFH2/AS RNA-transfected cells be-

cell lines were not tested (*). (D) The passaged transfected cells were stained with anti-core protein monoclonal antibody (2H9) as a primary antibody at the indicated time points. Green, HCV core protein; blue, 4′,6′-diamidino-2-phenylindole (DAPI) staining. (E) Density gradient analysis of culture supernatant from HCV RNA-transfected Huh-7.5.1 cells. Culture supernatants of transfected cell line T1A collected at 5 days, 8 weeks, and 11 weeks posttransfection were cleared by centrifugation and filtration. Each supernatant was overlaid on the stepwise sucrose density gradient (0%, 10%, 20%, 30%, 40%, 50%, and 60% sucrose) and centrifuged for 16 h at 200,000 × *g* at 4°C. Eighteen fractions were collected from the bottom of the tubes, and the concentration of HCV core protein in each fraction was determined by Lumipulse Ortho HCV Ag. The levels of HCV core protein, HCV RNA, and infectivity were determined in each fraction. Infectivity of the samples from day 5 was negative. Open diamond, buoyant density.

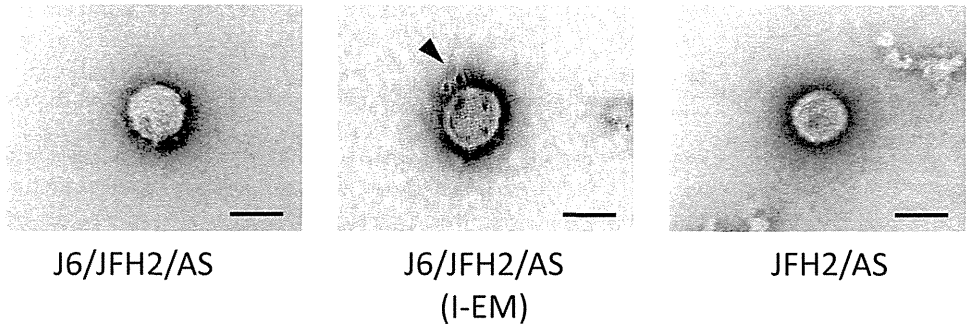

FIG 7 Morphology of JFH-2 virus particles. Negatively stained HCV particles were observed by electron microscopy. J6/JFH-2/AS and JFH2/AS virus particles were purified and observed by electron microscopy by using negative staining. In the middle panel, a J6/JFH-2/AS virus particle was detected by immuno-electron microscopic (I-EM) analysis by using anti-E2 antibody. Arrowhead, gold-labeled antibody. Scale bar, 50 nm.

gan to secrete core proteins earlier than J6/JFH2/AS RNA-transfected cells in this experiment (Fig. 13). Core protein levels were 24,525 and 11,720 fmol/liter in T3 cells at 67 days posttransfection and T4 cells at 63 days posttransfection, respectively. Infectious titers were also determined in the same culture medium at 2.1×10^4 and 4.3×10^4 focus-forming units (FFU)/ml for T3 and T4, respectively. T3 culture medium at day 67 posttransfection was

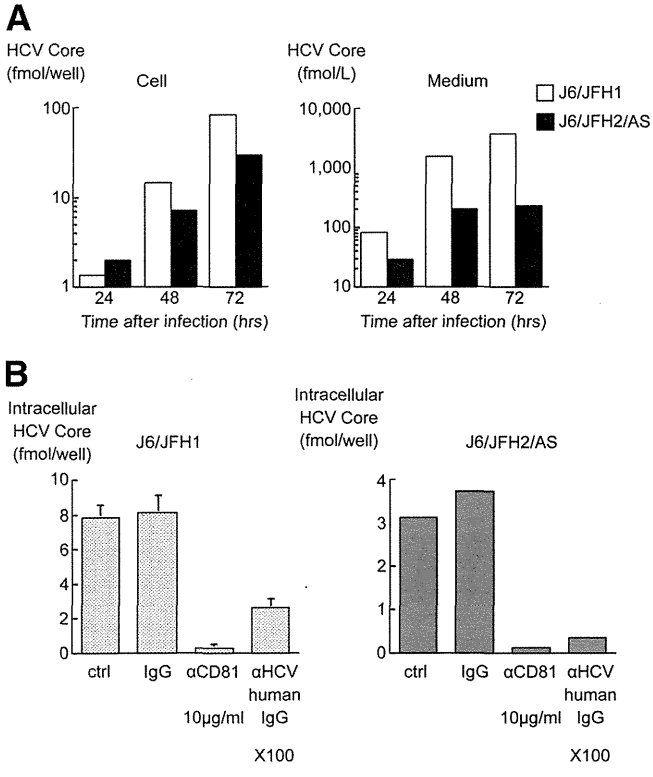

FIG 8 Comparative analysis between J6/JFH1 and J6/JFH2/AS virus. (A) Huh-7.5.1 cells were infected with J6/JFH1 or J6/JFH2/AS virus particles at an MOI of 0.03. HCV core protein production in the inoculated cell lysate and medium was measured at the indicated times. Assays were performed in duplicate, and the average data are represented. (B) Infection with J6/JFH1 and J6/JFH2/AS virus particles was inhibited by adding antibodies to the reaction mixtures. Assays were performed three times independently, and data are presented as means ± standard deviations. Normal human IgG and anti-CD81 monoclonal antibody and anti-HCV human IgG at the indicated concentrations were used. Ctrl, control.

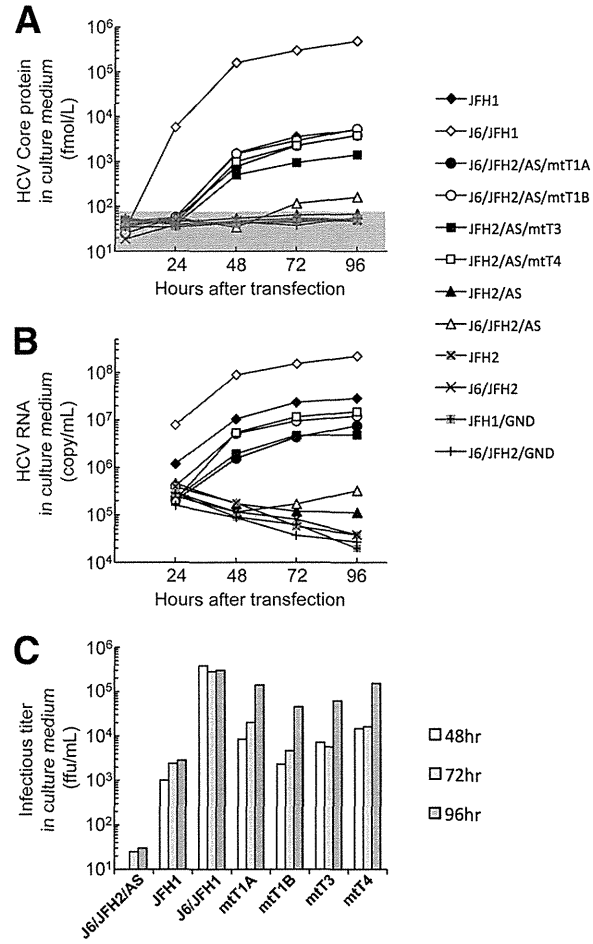

FIG 9 Transient virus production assay of J6/JFH2- and JFH2-related constructs with Huh-7.5.1 cells. Full-length HCV RNA was synthesized from the JFH1, J6/JFH1, JFH2, and J6/JFH2 constructs and their derivatives with mutations and transfected into Huh-7.5.1 cells. (A) HCV core protein levels in the culture medium were determined at 4, 24, 48, 72, and 96 h after transfection. The gray area indicates values that are below the detection limit. (B) HCV RNA levels in the culture medium were also determined at 24, 48, 72, and 96 h after transfection. (C) Infectivity in the culture medium was determined by focus formation assay at 48, 72, and 96 h after transfection. Only positively detected data are shown in the figure. All assays in this figure were performed in duplicate, and the average data are represented.

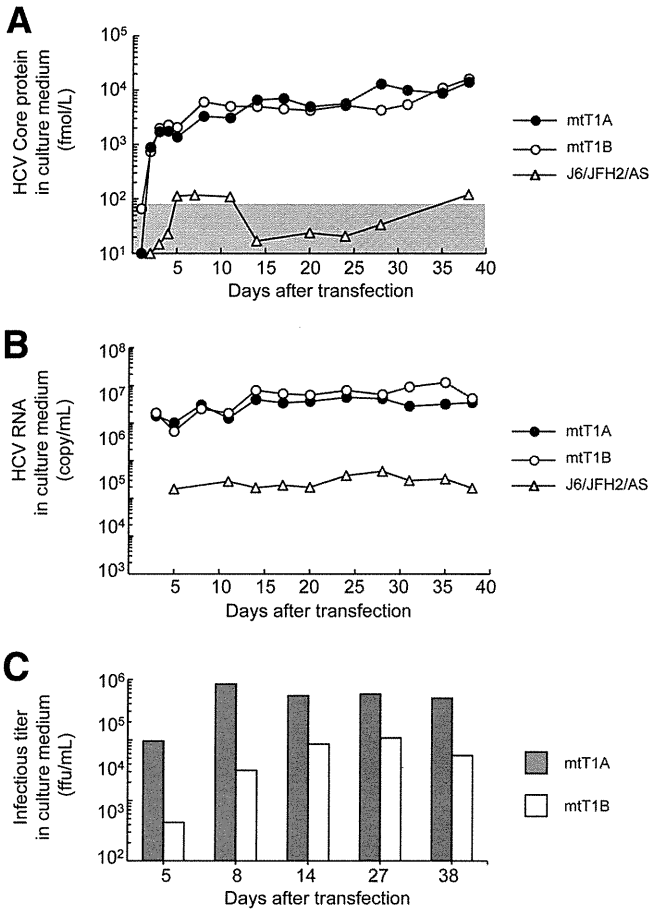

FIG 10 Continuous passage of J6/JFH2/AS cell culture-adapted virus RNA-transfected cells. Full-length HCV RNA was synthesized from the J6/JFH2/AS, J6/JFH2/AS/mtT1A (mtT1A), and J6/JFH2/AS/mtT1B (mtT1B) constructs. RNA-transfected cells were serially passaged until 38 days after transfection, and culture supernatants were harvested at the indicated time points. HCV core protein (A) and HCV RNA (B) levels in the culture media were determined. The data in the gray area were below the detection limit of the assay to detect HCV core protein. (C) Infectivity in the culture medium was determined by focus formation assay at 5, 8, 14, 27, and 38 days after transfection.

also used for electron microscopy analysis. After the density gradient purification, spherical viral particles were detected (Fig. 7, right panel). After the core protein levels plateaued, naive Huh-7.5.1 cells were inoculated with the culture medium, as described above. When the core protein levels plateaued again after the third inoculation of T3 and T4 cells, we sequenced the viral genome in the culture medium (T3i3 and T4i3, respectively) to determine the adaptive mutation. We found the following nonsynonymous mutations: 414IT in E2, 1510EG and 1617RQ in NS3, 2006KQ, 2233AV and 2234NS in NS5A, and 2695TI in NS5B of T3i3; and 387VG in E1, 828VA in NS2, 1225RQ and 1283RG in NS3, 1883VA in NS4B, 2206SA, 2279KN, and 2441CR in NS5A, and 2695TI in NS5B of T4i3 (Fig. 3B). We introduced these mutations into the pJFH2/AS plasmid (pJFH2/AS/mtT3 and pJFH2/AS/mtT4). Synthesized RNA from pJFH2/AS/mtT3 and pJFH2/AS/mtT4 and the related control plasmids was transfected into Huh-7.5.1 cells. HCV core protein levels, HCV RNA levels, and infectivity were monitored in the culture medium of the transfected cells until 96 h after transfection (Fig. 9A to C). JFH2/AS/mtT3 (mtT3) and JFH2/AS/mtT4 (mtT4) secreted similar levels of HCV core protein, RNA, and infectious virus with J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B. Although JFH2/AS/mtT3 secreted slightly higher levels of HCV core protein and RNA than JFH2/AS/mtT4, the secreted infectious virus titers were similar for both viruses. JFH2/AS/mtT3 and JFH2/AS/mtT4 RNA-transfected cells were also serially passaged, and the HCV core proteins were secreted immediately after transfection (Fig. 14A). However, JFH2 and JFH2/AS RNA-transfected cells did not secrete significant amounts of HCV core protein into the culture medium. HCV RNA levels in the culture medium of the RNA-transfected cells were at similar levels for JFH2/AS/mtT3 and JFH2/AS/mtT4 (around 10^7 copy/ml) (Fig. 14B). Infectivity was also detected as higher than 10^4 FFU/ml even at 3 days after the RNA transfection, and this level of infectious titer was maintained during the cell passages (Fig. 14C). We also analyzed JFH2/AS/mtT3 and JFH2/AS/mtT4 culture media by density gradient assay (Fig. 14D). The density profiles with HCV core protein and RNA levels and infectious titers in the fractions were basically similar to those of J6/JFH2/AS-adapted viruses (Fig. 6E and 11D). Taken together, the results described in this section indicate infectious virus was also recovered from the full-length JFH-2 construct with the 2217AS mutation.

Mechanistic analysis of adaptive mutations introduced in the J6/JFH2/AS and JFH2/AS cell culture-adapted viruses. To elucidate the mechanisms of adaptive mutations discovered in J6/JFH2/AS and JFH2/AS virus genomes, we transfected JFH-2 and J6/JFH2 constructs along with possible control constructs into Huh7-25 cells (2) (Fig. 15), which are CD81 defective. The transfection of JFH-1 RNA into Huh7-25 cells results in infectious HCV production, but there was no reinfection into Huh7-25 cells because the cell surface expression of CD81 is essential for HCV infection (10). HCV core protein levels were measured in the culture medium and cell lysate to monitor virus particle secretion and intracellular virus genome replication, respectively (Fig. 15A and B). JFH2/AS, JFH2, J6/JFH2, JFH1/GND, and J6/JFH2/GND RNA-transfected cells did not show increased levels of intracellular core protein expression. However, other RNA-transfected cells showed increased intracellular core protein expression. The cellular core protein level was especially increased at 72 and 96 h after transfection with J6/JFH2/AS RNA, which suggests a higher replication efficiency than J6/JFH; however, core protein secretion was not detected with J6/JFH2/AS, which suggests defective virus particle formation or secretion. Other adaptive mutations in J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B further increased virus genome replication and core protein secretion. In the case of JFH2/AS RNA transfection, cellular core protein expression was not detected, suggesting a lower replication efficiency than that of J6/JFH2/AS. This lower replication efficiency of JFH2/AS may be due to the presence of different sequences in the region of core protein to NS2. However, core protein expression in the cell lysate and culture medium was detected with both JFH2/AS/mtT3 and JFH2/AS/mtT4 RNA transfection. Thus, adaptive mutations in mtT3 and mtT4 are necessary to increase viral genome replication and efficient core protein secretion. JFH-1 and J6/JFH-1 had intracellular core protein expression levels that were similar and high. From the intracellular core protein data, it is clear that J6/JFH2/AS/mtT1A, J6/JFH2/AS/mtT1B, JFH2/AS/mtT3, and JFH2/AS/mtT4 constructs obtained higher replication capacities by

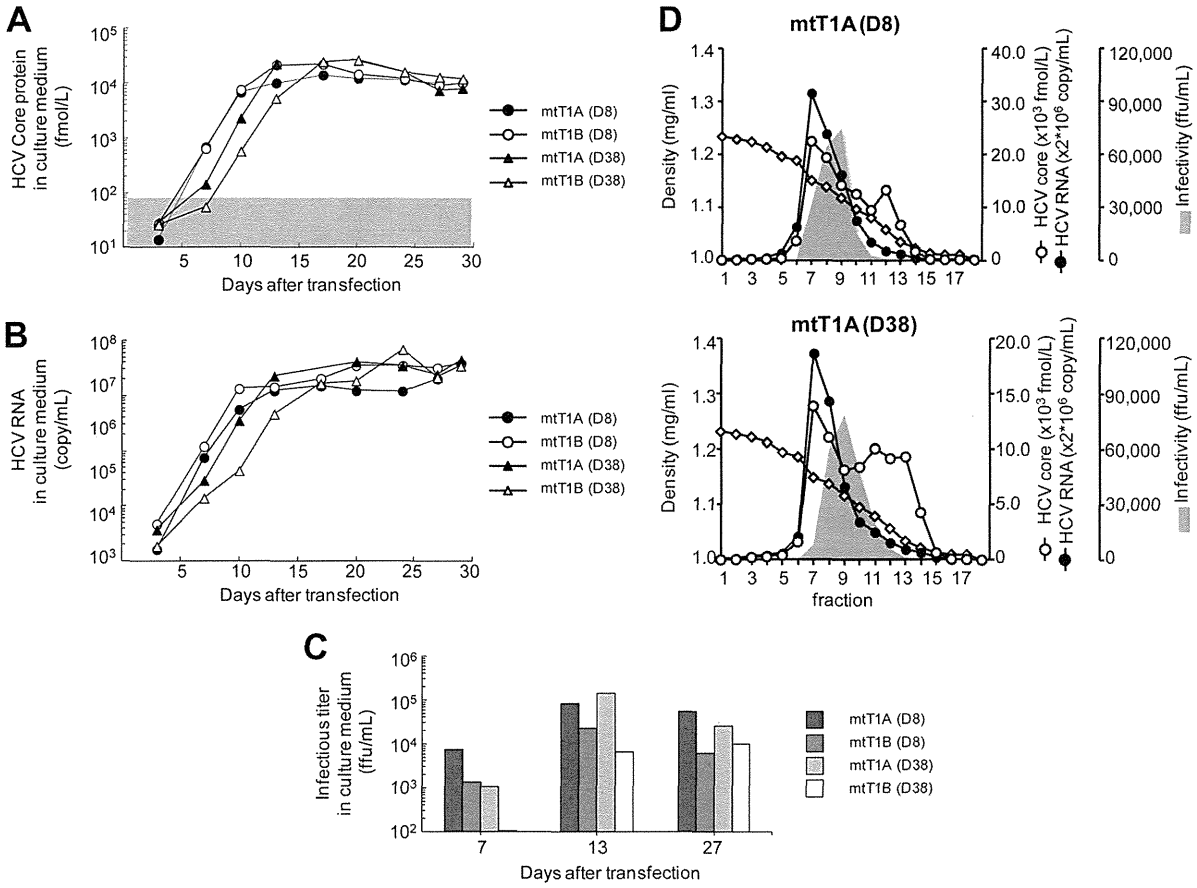

FIG 11 Serial passages of J6/JFH2/AS cell culture-adapted virus infected cells. Naive Huh-7.5.1 cells were inoculated with culture medium of the RNA-transfected cells at an MOI of 0.01. Inoculated cells were serially passaged, and culture supernatants were harvested at the indicated times. HCV core protein (A), HCV RNA (B), and infectivity (C) levels in the culture media were determined. (D) Density gradient analysis of culture supernatant from J6/JFH2/AS/mtT1A cell-culture adapted virus-infected Huh-7.5.1 cells. Culture supernatant of infected Huh-7.5.1 cells with mtT1A (day 8 [D8] D38 posttransfection) was harvested at 20 days after inoculation. Assays were performed as described in the legend of Fig. 6E. Open diamond, buoyant density.

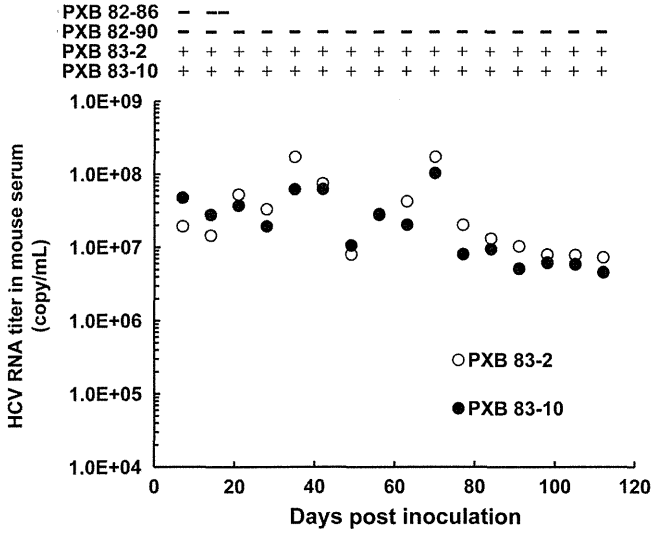

FIG 12 Serum HCV RNA titer in human hepatocyte-transplanted uPA/SCID mice inoculated with JFH-2 serum and cell culture-adapted virus. Mice PXB 82-86 and PXB 82-90 were inoculated with the cell culture-adapted J6/JFH2/AS HCV particles, and mice PXB 83-2 and PXB 83-10 were inoculated with JFH-2 patient serum. Upper and lower panels indicate the results of the RT-PCR and the quantitative detection RT-PCR of HCV RNA in mouse serum, respectively.

adaptive mutations; however, their replication levels are lower than those of JFH-1 and J6/JFH1.

We also analyzed the ratio of extracellular protein to total core protein to analyze the virus secretion efficiency (Fig. 15C). J6/JFH-1 secreted a higher percentage of core protein into culture

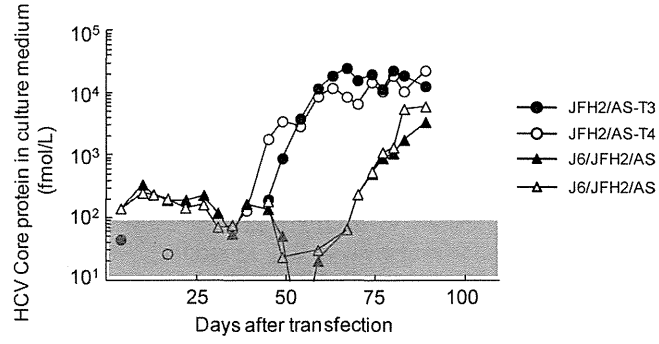

FIG 13 Propagation of a full-length JFH2 virus. Huh-7.5.1 cells were transfected with the transcribed RNA from pJFH2/AS and pJ6/JFH2/AS. Two independently JFH2/AS RNA-transfected cell lines (T3 and T4) were independently passaged. At each time point, culture medium was harvested and analyzed for the presence of HCV core protein.

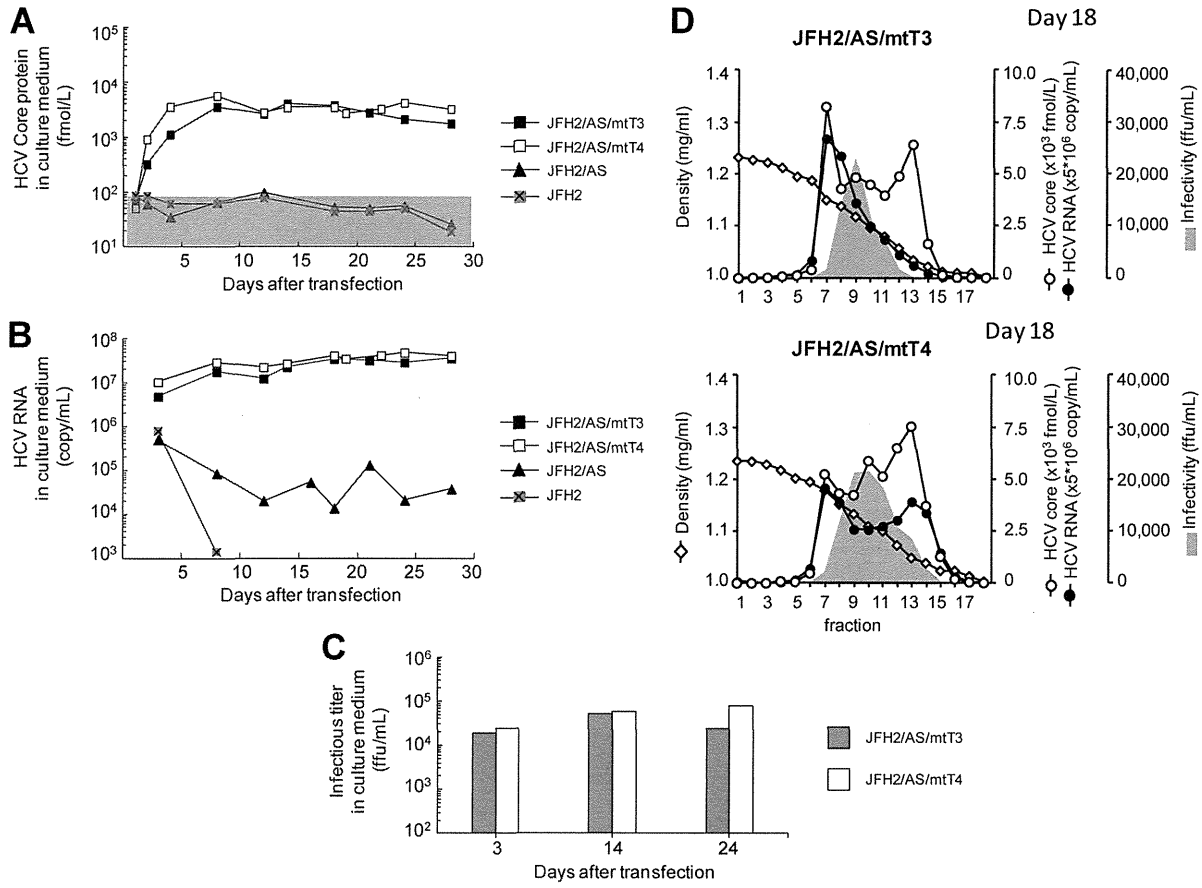

FIG 14 Full-length RNA was synthesized from the JFH2 construct and its derivatives with mutations. RNA-transfected cells were serially passaged, and culture supernatants were harvested at the indicated time points. HCV core protein (A), HCV RNA (B), and infectivity (C) levels in the culture media were determined. The data in the gray area were below the detection limit of the assay to detect HCV core protein. (D) Density gradient analysis of culture supernatant from JFH2/AS cell-culture adapted virus-infected Huh-7.5.1 cells. Culture supernatants of Huh-7.5.1 cells infected with JFH2/mtT3 and JFH2/mtT4 viruses were harvested 18 days after inoculation. Assays were performed as described in the legend of Fig. 6E. Open diamond, buoyant density.

medium than JFH-1. J6/JFH2/AS/mtT1A, J6/JFH2/AS/mtT1B, JFH2/AS/mtT3, and JFH2/AS/mtT4 RNA-transfected cells showed different percentages of secreted core protein. mtT1A and mtT1B constructs showed similar replication levels (Fig. 15B), but mtT1B showed a higher percentage of core protein secretion than mtT1A (Fig. 15C). mtT3 and mtT4 showed similar percentages of core protein secretion, which are higher than the level of JFH1 (Fig. 15C). Because J6/JFH2/AS RNA-transfected cells did not secrete core protein despite intracellular core protein expression (Fig. 15A and B), the adaptive mutant constructs obtained core protein (or virus particle) secretion phenotypes. Thus, during the adaptation process, the viruses obtained both higher replication capacity and core protein secretion capacity by their adaptive mutations.

Other HCV constructs with the 2217AS mutation. The alanine residue at amino acid position 2217 is located in the ISDR of NS5A, and it is conserved among HCV strains including genotype 1 and 2 strains. Because the 2217AS mutation in NS5A is the key mutation for the production of cell culture-adapted HCV, we introduced this mutation into other wild-type HCV constructs, i.e., H77 (genotype 1a), Con1 (genotype 1b), and J6CF (genotype 2a). Synthetic RNAs including the 2217AS mutation were electroporated into Huh-7.5.1 cells, and then the transfected cells were serially passaged. HCV core protein secretion was measured in the culture medium of transfected cells. However, we could not observe the increment of HCV core levels in the culture medium (data not shown). Therefore, we concluded that the 2217AS mutation does not always induce cell culture adaptation in HCV isolates.

DISCUSSION

In previous studies, we have isolated cell culture-infectious HCV, the JFH-1 strain, from a patient with fulminant hepatitis (14, 38). In this report, we isolated another HCV cDNA, named JFH-2, also from a fulminant hepatitis patient. We constructed a subgenomic replicon with the JFH-2 sequence, but its replication efficiency was low. Among the mutations found in the replicon genome, the 2217AS mutation in the ISDR exhibited the strongest adaptive effect. Interestingly, the full-length chimeric or wild-type JFH-2 genome with adaptive mutations could replicate and produce infectious virus particles. Virus infection efficiency was sufficient for autonomous virus propagation in cultured cells.

Several full-length HCV cDNAs have been cloned, and their infectivity has been confirmed *in vivo* with chimpanzee models (18, 39). However, it has been difficult to produce recombinant viral particles and test their infectivity by using cell culture

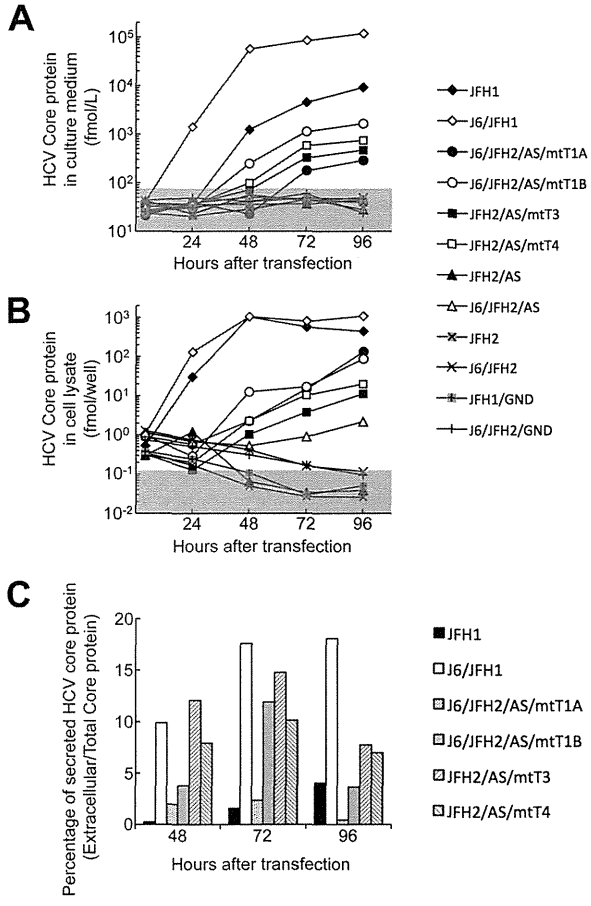

FIG 15 Transient virus production assay of J6/JFH2- and JFH2-related constructs with CD81-defective Huh7-25 cells. Full-length HCV RNA was synthesized from the JFH1, J6/JFH1, JFH2, and J6/JFH2 constructs and their derivatives with mutations and transfected into Huh7-25 cells. (A) HCV core protein levels in culture medium were determined at 4, 24, 48, 72, and 96 h after transfection. The data in the gray area were below detection limit. (B) HCV core protein levels in the cell lysate were determined at 24, 48, 72, and 96 h after transfection. (C) Percentages of secreted HCV core protein from the transfected cells were determined at 48, 72, and 96 h after transfection. Percentages of secreted HCV core protein were calculated only for the indicated viruses. All assays were performed in duplicate, and the data represent average values.

systems (4, 28). Only the JFH-1 strain efficiently replicates in HuH-7 cells and other hepatic and nonhepatic cell lines in subgenomic replicon form (20, 38, 41). Full-length wild-type JFH-1 RNA and chimeric JFH-1 RNA can replicate in HuH-7 cells and produce infectious virus. Since the JFH-1 strain was isolated from a patient with fulminant hepatitis, we assumed that virus strains that cause fulminant hepatitis may replicate efficiently in cultured cells. To identify more HCV clones that can replicate in cultured cells, we isolated the JFH-2 strain from another fulminant hepatitis patient (15). Interestingly, the JFH-2 strain showed a low level of replication in cultured cells in the initial subgenomic replicon experiment. This result may suggest that HCV strains isolated from fulminant hepatitis patients are able to replicate more efficiently than strains from chronic hepatitis patients; however, this hypothesis should be confirmed by testing more HCV strains from patients with fulminant hepatitis. The JFH-2 patient received a course of betamethasone therapy and developed fulminant hepatitis after the withdrawal of betamethasone. It is thus possible that the JFH-2 strain obtained its higher replication capacity in the immune-suppressed host environment. To confirm this hypothesis, we must test the replication efficiency of HCV strains isolated from other immune-suppressed patients, such as patients who are coinfected with HIV, patients who are reinfected after a transplant, and patients who are treated with immunosuppressive agents.

In previous reports, adaptive mutations have been found to enhance viral RNA replication at the expense of virus particle formation efficiency (28). A highly cell culture-adapted Con1 strain can replicate in cultured cells, but it cannot produce infectious virus particles. Interestingly, a highly adapted Con1 strain was not infectious for chimpanzees, while moderately adapted Con1 was infectious. However, the virus recovered from the infected animal was wild-type Con1 virus (5). This result clearly suggests that HCV strains with lower replication efficiencies are favorable for *in vivo* infection. However, we must note that the "replication efficiency" is determined in cultured cells. In the case of JFH-2, we found several adaptive mutations in the subgenomic replicon clones, and the most adaptive mutation, 2217AS, was tested in full-length HCV replication and virus production. After the RNA transfection of J6/JFH2/AS, we could not detect substantial virus secretion for about 30 days. However, after 30 days, significant levels of infectious virus particles were secreted into the culture medium. Naive Huh-7.5.1 cells were inoculated three times with the cell culture-adapted virus. This virus adaptation was also tested with full-length JFH2/AS, and we successfully obtained infectious JFH2/AS virus. Both the J6/JFH2/AS and JFH2/AS viruses acquired the ability for autonomous virus expansion in Huh-7.5.1 cells, and several additional mutations were found in their genomes. Interestingly, the 2695TI mutation in NS5B was commonly found in all of the adapted virus genomes, and isoleucine at amino acid position 2695 is also found in the JFH-1 strain. However, the introduction of only the 2695TI mutation into the J6/JFH2/AS or JFH2/AS virus genome did not restore robust virus production (data not shown). After repeated virus passages, mutations were found throughout the viral genome (in J6/JFH2/AS-T1Ai3 and -T1Bi3 and in JFH2/AS-T3i3 and -T4i3), and we are currently investigating which mutations or combinations of mutations are most important for this adaptation. From the comparisons of cell culture-adapted viruses and their parental virus constructs, adaptive mutations are necessary to increase both viral genome replication and virus particle assembly/secretion efficiency (Fig. 15). The procedure to produce cell culture-adapted HCV was thus established. The adaptive mutations found from the subgenomic replicon assay were introduced into the full-length genome, and the cells transfected with virus RNA were repeatedly passaged until the virus particles were produced.

In vivo infectivity may be inversely related to the replication efficiency in cultured cells, as discussed above. The original JFH-2 patient serum was infectious in human liver-transplanted mice; however, cell culture-adapted J6/JFH2/AS virus was not infectious. The JFH-1 virus was infectious not only for cultured cells but also for chimpanzees and human liver-transplanted mice (10, 38). However, the JFH-1 infection in chimpanzees was only mild and transient without any liver pathology. Thus, the J6/JFH2/AS and JFH2/AS viruses are more cell culture-adapted and attenuated than the JFH-1 virus. It may be worthwhile to test this cell culture-

adapted strain as a live attenuated vaccine candidate to induce protective immunity. However, for ethical reasons, the necessary chimpanzee experiments are not appropriate to perform. Therefore, we should wait for the establishment of immunocompetent small-animal models susceptible to HCV infection to perform this kind of study. Furthermore, future studies should examine the *in vivo* infectivity of the adapted J6/JFH2 and JFH2 viruses isolated in the present study.

The 2217AS mutation is located in the ISDR. In the previous study of the genotype 1b subgenomic replicon, mutations introduced into the ISDR enhanced the colony formation efficiency of the HCV replicons (17, 23). However, mutations in the ISDR impaired the genotype 1b HCV replication in human liver-transplanted mice (9). The exact mechanism of the ISDR is still not clear although the number of mutations in the ISDR is related to the efficacy of interferon therapy (8). Our results in this study also support the concept that the 2217AS mutation in the ISDR enhances replicon replication efficiency although the J6/JFH2/AS virus did not infect human liver-transplanted mice. Further studies are necessary to understand the molecular mechanism of the effects of adaptive mutations in the ISDR.

In the present study, we established a cell culture-adapted HCV strain, JFH-2. The virus could be passaged continuously in naive Huh-7.5.1 cells. This approach may be applicable to the establishment of new infectious HCV clones. Novel antiviral drugs are under development, and some of them will be used in the clinical setting. However, most of them target genotype 1 HCV strains. To eradicate other genotypes of HCV, it is important to establish their replicons and infectious virus culture systems.

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Lymphotropic hepatitis C virus has an interferon-resistant phenotype

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SUMMARY. Hepatitis C virus (HCV) infects and associates with B cells, leading to abnormal B-cell activation and development of lymphoproliferative and autoimmune disorders. This immune perturbation may in turn be associated with the resistance of HCV against the host immune system. The objective of this study was to analyse the effects of HCV infection of B cells on the efficacy of interferon (IFN)-based therapy. The study enrolled 102 patients with chronic hepatitis C who were treated with pegylated IFN plus ribavirin. HCV RNA titres in B cells were compared in patients with rapid viral responder (RVR) *vs* non-RVR, sustained viral responder (SVR) *vs* non-SVR and null viral responder (NVR) *vs* VR. The levels of HCV RNA in B cells were significantly higher in non-RVR, non-SVR and NVR groups. Association between the therapy outcome and the positive B-cell HCV RNA was also investigated in relation to other known viral and host factors. Multivariable analyses showed that the positive B-cell HCV RNA and the minor single-nucleotide polymorphism near the IL28B gene (rs8099917) were independent factors associated with NVR in patients infected with HCV genotype 1. When these two factors were combined, the sensitivity, specificity, positive and negative predictive values for NVR were 92.3%, 98.2%, 92.3% and 98.2%, respectively. Genotype 1 and the presence of one or no mutations in the IFN-sensitivity determining region were associated with higher levels of B-cell HCV RNA. B-cell-tropic HCV appears to have an IFN-resistant phenotype. B-cell HCV RNA positivity is a predictive factor for resistance to IFN-based therapy.

Keywords: B-cell disorders, B-cell tropism, hepatitis C virus, interferon resistance, lymphoproliferative disorders.

INTRODUCTION

Hepatitis C virus (HCV) infects 200 million people worldwide, causing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [1,2]. In addition, HCV infection causes proliferative disorders of B cells, such as mixed cryoglobulinemia [3–5] and B-cell non-Hodgkin's lymphoma [6]. Even when no clinical extrahepatic manifestations are observed, 74% of patients infected with HCV present 'biological' extrahepatic manifestations, such as cryoglobulinemia, high levels of rheumatoid factor, hypocomplementemia and clonal expansion of B cells [7]. Molecular mechanisms of these B-cell abnormalities remain to be clarified. Replication of HCV is observed in the peripheral blood mononuclear cells (PBMCs), especially in B cells of patients with chronic hepatitis C (CH-C) [8–10]. In our previous report, HCV RNA was detected in B cells from 64.0% of CH-C patients and negative-strand HCV RNA in 5.3% of these patients [7]. The presence of lymphoproliferative disorders (LPDs) is associated with HCV RNA detection in B cells. Furthermore, the IFN responses of peripheral B cells from CH-C patients have been reported to be impaired [11]. Taken together, these results indicate that B-cell-tropic HCV exists and may play an important role in the immunological dysfunction of B cells.

As the discovery of HCV, rapid progress has been made in the development of antiviral therapies against HCV infection. The current standard treatment for CH-C, a combination of pegylated interferon (PEG-IFN) and ribavirin for 24 or 48 weeks, has improved clinical responses [12,13]. However, even with this regimen, half of CH-C patients do not achieve sustained clearance of HCV. The likelihood of the response varies greatly, depending on both host and viral factors. Viral factors associated with resistance to interferon (IFN)-based therapy include HCV genotype 1, high viral

Abbreviations: CH-C, chronic hepatitis C; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; LPD, lymphoproliferative disorders; NPV, negative predictive value; NVR, null viral responder; PBMCs, peripheral blood mononuclear cells; PEG-IFN, pegylated interferon; PPV, positive predictive value; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder; VR, viral responder.

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loads and reduced quasispecies diversity in the IFN-sensitivity determining region (ISDR) of NS5A [14], as well as mutations at the 70th and 91st amino acids of the HCV core region [15]. Host factors include age, race, gender, degree of hepatic fibrosis, insulin resistance [16] and interleukin (IL) 28B genotype [17]. Although many of these factors are used for prediction of the response to IFN therapy, it is currently impossible to determine who will attain viral eradication from hepatocytes. Furthermore, examination of all the markers increases medical expenses.

HCV replicates in lymphoid cells at low levels, but they may serve as an extrahepatic reservoir; this is implicated in the recurrence and persistence of HCV infection in immunosuppressed individuals [18]. In addition, low amounts of positive- and negative-strand HCV RNAs are still detected in lymphocytes of sustained viral response (SVR) patients with CH-C after the virus eradication from hepatocytes by IFN-based therapy [19,20]. HCV in lymphoid cells may represent a subpopulation resistant to innate immunity and/or a source of B-cell dysfunction.

The primary purpose of this study was to test the hypothesis that HCV associated with B cells confers resistance to IFN-based therapy and serves as a useful predictive parameter for therapy outcome. The study also evaluated other viral and host factors in conjunction with B-cell HCV RNA as predictive markers for the efficacy of antiviral therapy.

METHODS

Subjects

This study enrolled 102 patients with CH-C who were treated with PEG-IFN plus ribavirin therapy for 24, 48 or 72 weeks (Showa University Hospital, 2005–2010). Diagnosis of HCV infection was based on the detection of anti-HCV antibody and HCV RNA in the serum prior to the initiation of therapy. Clinical characteristics of the HCV-infected patients are shown in Table 1. Missense mutations in the ISDR and in codons 70 and 91 of the core region were analysed in patients infected with HCV genotype 1 by direct sequencing [14,15]. Liver biopsies were performed in 92 patients before the start of therapy; fibrosis was staged from F0 to F4 according to the scheme of Desmet *et al.* [21]. The study protocol was approved by the Ethics Committee of Showa University School of Medicine, Tokyo, Japan. Informed written consent was obtained from each participant and the study followed the ethical guidelines of the 1975 Declaration of Helsinki.

Table 1 Clinical characteristics of HCV-infected patients (n = 102)

Age (years)	54.1 ± 11.4
Male/Female	53/49
ALT (IU/L)	73.8 ± 74.4
Platelets ($\times 10^4/mm^3$)	18.4 ± 6.5
Fibrosis (F0/F1/F2/F3/F4)	1/38/38/9/6
Outcome of IFN therapy (RVR/SVR/NVR)	46 (45%)/60 (59%)/ 15 (15%)
IL28B SNPs (T/T *vs* T/G, G/G)	76/99 (77%) *vs* 23/99 (23%)
Cryoglobulinemia	24/98 (24%)
IgG (mg/dl)	1712 ± 504
IgA (mg/dl)	253 ± 118
IgM (mg/dl)	119 ± 64
Rheumathoid factor (>10 IU/mL)	39/99 (39%)
C3 (<86 mg/dL)	16/99 (16%)
C4 (<10 mg/dL)	4/99 (4%)
CH50 (<20 U/mL)	59/97 (58%)
Presence of B cell clonality	9/71 (12.7%)
HCV genotype (1/2)	77 (75.5%)/25 (24.5%)
Log HCV RNA in serum (log/mL)	5.9 ± 0.82
Positive of HCV RNA in B cells (>10 log/100 ng)	58/102 (57%)

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder

Continuous variables are presented as mean ± standard error.

Isolation of B cells

B cells were isolated using an auto-MACS™ Pro Separator ver.2.0.0 (Miltenyi Biotec K.K., Bergisch Gladbach, Germany). Briefly, PBMCs were obtained from whole blood (30 mL) by centrifugation. Non-B cells were labelled by a cocktail of specific antibodies that were conjugated to biotin, and the mixture was adsorbed with the MACS microbeads to capture the biotin-labelled cells. The cell–microbead mixture was then passed through the auto-MACS magnetic column, and B cells (the flow-through) were collected.

Quantitation of HCV RNA in B cells

Total RNA from each cellular compartment was extracted using the AllPrep® DNA/RNA/Protein Mini kit (Qiagen, Duesseldorf, Germany). HCV RNA levels were determined in 100 ng of each RNA sample by real-time RT-PCR using the primers described previously [22]; this assay has a detection range over 1.0–8.0 log copies. Samples were scored as positive for HCV RNA when titres exceeded 1.0 log copies/100 ng; this threshold excluded contamination of lymphoid cells with serum HCV RNA [7].

IL28B SNP genotyping assay

In 99 patients, the genotype of an IL28B-proximal single-nucleotide polymorphism (SNP) (rs8099917) was determined by real-time PCR. For each patient, genomic DNA (10–100 ng) was purified (Qiagen) and amplified using the

SNP Genotyping Assay specific for rs8099917 (Applied Biosystems, Foster City, CA, USA) according to the TaqMan® GTXpress™ Master Mix Protocol (Applied Biosystems).

Statistical analysis

The mean of continuous variables, with and without normal distribution, was compared by Student's *t* test or by the Wilcoxon test, respectively. Comparison of discontinuous variables was performed by the chi-squared test or Fisher's exact test. A *P* value of <0.05 was considered to be statistically significant. Values with normal distributions were expressed as the mean ± standard error (SE). For variables that were not distributed normally, data were transformed into log values as required. To examine the relation between patient parameters and the outcome of therapy, candidate independent variables were analysed by the Wald test of logistic regression modelling via multivariable analysis. All statistical analyses were performed using JMP ver. 9 software (SAS Institute, Cary, NC, USA).

RESULTS

The levels of HCV RNA in B cells and IFN treatment response

HCV RNA titres in B cells were compared in patients with rapid viral responder (RVR) *vs* non-RVR (Fig. 1a), SVR *vs* non-SVR (Fig. 1b) and null viral responder (NVR) *vs* VR (non-NVR) (Fig. 1c) using univariable analysis. The levels of HCV RNA in B cells were significantly different in all three comparisons ($P = 0.0001$, 0.0012 and 0.0020, respectively). The results suggest that patients whose B cells have less HCV RNA are more sensitive to IFN-based therapy. Mean HCV RNA titres in B cells of NVR patients showed the highest titre among the three groups (RVR: 1.0 ± 0.2, SVR: 1.2 ± 0.2 and NVR: 2.9 ± 0.4 log copies/100 ng RNA). The fraction of patients scoring positive for the presence of HCV RNA in B cells was also significantly different in each comparison: [RVR: 18/46 (39.1%) *vs* non-RVR: 40/56 (71.4%), $P = 0.0010$], [SVR: 26/60 (43.3%) *vs* non-SVR: 32/42 (76.2%), $P = 0.0010$] and [NVR: 14/15 (93.3%) *vs* VR: 44/87 (50.6%), $P = 0.0016$].

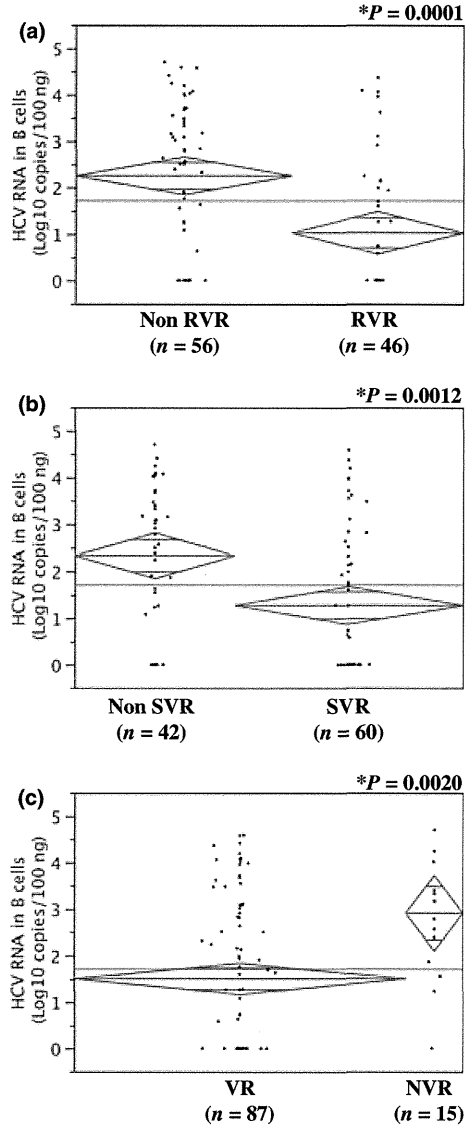

Fig. 1 Comparison of hepatitis C virus (HCV) RNA titres in B cells of patients classified by viral response status: (a) Rapid viral responder (RVR) *vs* non-RVR; (b) sustained viral responder (SVR) *vs* non-SVR; and (c) null viral responder (NVR) *vs* viral responder (VR). A horizontal grey line shows mean of all samples from both groups. Two grey diamonds indicate the averages and 95% confidence interval of each group. Statistical significance was determined by Student's *t* test.

HCV RNA in B cells as a predictive factor for viral response

We next analysed the factors associated with response (RVR, SVR or NVR) to IFN-based therapy. We used the presence of HCV RNA in B cells as a marker of LPD because it is associated with the presence of LPD [7]. Homozygosity for the major allele (T/T) of the IL28B SNP (rs8099917), HCV genotype 2 and low serum HCV RNA levels were significantly associated with RVR by univariable analysis (Table 2a), while the presence of HCV RNA in B cells was associated with non-RVR. When the independence of these factors was assessed by multivariable analysis using a multiple logistic regression model, homozygosity for the major allele (T/T) of the IL28B SNP and lower levels of HCV RNA in sera (<6 log copies/mL) were shown to be independent factors associated with RVR to IFN-based therapy.

In the comparisons of SVR with non-SVR, lower age, higher platelet number, fibrosis stage 1 or 2, the T/T allele of

Table 2 Univariable and multivariable analysis for RVR (a), SVR (b) and NVR (c)

(a) RVR

	Response for therapy		
Univariable analysis	RVR (n = 46)	Non-RVR (n = 56)	P value
Age (years)	52.6 ± 12.0	55.5 ± 10.7	NS
Men	27/46 (59%)	26/56 (46%)	NS
ALT (IU/L)	78.4 ± 68.9	70.0 ± 79.1	NS
Platelets ($\times 10^4/mm^3$)	19.2 ± 6.5	17.8 ± 6.4	NS
Fibrosis stages 1 or 2	36/39 (92%)	41/53 (77%)	NS
IL28B SNPs rs8099917 major allele (T/T)	**39/43 (91%)**	**37/56 (66%)**	**0.0040**
HCV genotype 2	**18/46 (39%)**	**7/56 (13%)**	**0.0019**
Log HCV RNA in serum (log/mL)	**5.45 ± 0.95**	**6.27 ± 0.43**	**<0.0001**
Presence of HCV RNA in B cells (>10 log/100 ng)	**18/46 (39%)**	**40/56 (71%)**	**0.0010**
Multivariable analysis	Odds ratio	95% CI	
IL28B SNP rs8099917 major allele (T/T)	**2.42**	**1.28–5.15**	**0.0116**
HCV genotype 2	1.53	0.84–2.85	NS
HCV RNA in serum <6.0 log/mL	**2.51**	**1.51–4.36**	**0.0006**
Absence of HCV RNA in B cells (<10 log copies/100 ng)	1.38	0.81–2.34	NS

(b) SVR

	Response for therapy		
Univariable analysis	SVR (n = 60)	Non-SVR (n = 42)	P value
Age (years)	**52.1 ± 11.6**	**57.1 ± 10.4**	**0.0247**
Men	32/60 (53%)	21/42 (50%)	NS
ALT (IU/L)	78.1 ± 70.8	67.2 ± 79.7	NS
Platelets ($\times 10^4/mm^3$)	**20.3 ± 7.0**	**15.7 ± 4.6**	**0.0003**
Fibrosis stages 1 or 2	**47/52 (90%)**	**30/40 (75%)**	**0.0477**
IL28B SNPs rs8099917 major allele (T/T)	**52/57 (91%)**	**24/42 (57%)**	**<0.0001**
HCV genotype 2	**21/60 (35%)**	**4/42 (10%)**	**0.0032**
Log HCV RNA in serum (log/mL)	**5.64 ± 0.94**	**6.27 ± 0.35**	**<0.0001**
Presence of HCV RNA in B cells (>10 log/100 ng)	**26/60 (43%)**	**32/42 (76%)**	**0.0010**
Multivariable analysis	Odds ratio	95% CI	
Age <50	1.39	0.77–2.57	NS
Platelets >17 × $10^4/mm^3$	**2.16**	**1.19–4.05**	**0.0139**
Fibrosis stage 1 or 2	1.22	0.56–2.77	NS
IL28B SNP rs8099917 major allele (T/T)	**3.47**	**1.76–7.84**	**0.0009**
HCV genotype 2	1.51	0.70–3.55	NS
HCV RNA in serum <6.0 log/mL	**2.58**	**1.33–5.63**	**0.0085**
Absence of HCV RNA in B cells (<10 log copies/100 ng)	1.66	0.88–3.24	NS

(c) NVR

	Response for therapy		
Univariable analysis	NVR (n = 15)	VR (n = 87)	P value
Age (years)	54.0 ± 2.9	54.2 ± 1.2	NS
Men	10/15 (67%)	43/87 (49%)	NS
ALT (IU/L)	90.5 ± 19.2	71.0 ± 8.0	NS
Platelets ($\times 10^4/mm^3$)	15.5 ± 1.7	19.0 ± 0.7	NS

Table 2 Continued

(c) NVR

Univariable analysis	Response for therapy NVR (n = 15)	VR (n = 87)	P value
Fibrosis stages 1 or 2	4/14 (29%)	11/78 (14%)	NS
IL28B SNPs minor alleles (T/G or G/G)	**12/15 (80%)**	**11/84 (13%)**	**<0.0001**
HCV genotype 1	**15/15 (100%)**	**25/87 (29%)**	**0.0169**
Log HCV RNA in serum (log/mL)	**6.33 ± 0.21**	**5.83 ± 0.09**	**0.0279**
Presence of HCV RNA in B cells (>10 log/100 ng)	**14/15 (93%)**	**44/87 (51%)**	**0.0020**
Multivariable analysis	Odds ratio	95% CI	
IL28B SNP rs8099917 minor allele (T/G or G/G)	**8.51**	**3.52–27.45**	**<0.0001**
HCV RNA in serum >6.0 log/mL	1.54	0.51–4.85	NS
Presence of HCV RNA in B cells (>10 log copies/100 ng)	**4.80**	**1.57–24.85**	**0.0179**

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder
Continuous variables are presented as mean ± standard error.
Bold text indicates statistically significant associations ($P < 0.05$). NS: not significant.

the IL28B SNP, HCV genotype 2 and lower serum levels of HCV RNA were significantly associated with SVR by univariable analysis (Table 2b). Higher platelet counts (>17 × 10^4/mm^3), the T/T allele of the IL28B SNP and lower serum levels of HCV RNA (<6.0 log/mL) were independent factors for SVR by multivariate analysis.

In comparisons of NVR with VR, homozygosity (G/G) or heterozygosity (T/G) for the minor allele of the IL28B SNP, HCV genotype 1, higher serum levels of HCV RNA and the presence of HCV RNA in B cells were significantly associated with NVR by univariable analysis (Table 2c). As none of the NVR patients were infected with HCV genotype 2, multivariable analysis was performed using the 73 samples of HCV genotype 1-infected patients to determine the independent factors associated with NVR. Presence of minor alleles (G/G or T/G) of the IL28B SNP and the presence of HCV RNA in B cells were both found to be independent factors associated with NVR in patients infected with HCV genotype 1.

Stratified analysis of outcome for IFN-based therapy

To assess the utility of HCV RNA in B cells as a predictive factor for the outcome of IFN-based therapy, we repeated the analysis on stratified groups. When the patients were stratified by HCV genotype or by IL28B SNP genotype, the rates of RVR and SVR were higher among cases scoring negative for HCV RNA in B cells (Table 3). In HCV genotype 1-infected patients possessing a minor allele of the IL28B SNP, two of five patients (40%) lacking HCV RNA in B cells achieved RVR and SVR; none of these five patients had a NVR. In contrast, none of 14 patients with positive HCV

Table 3 Stratified analysis of outcome for the IFN-based therapy (n = 100)

	IL28B SNP rs8099917	HCV RNA in B cells	Total	RVR	SVR	NVR
HCV genotype 1 (n = 77)	T/T (n = 58)	Positive	38	15/37 (41%)	23/37 (62%)	2/37 (0.5%)
		Negative	20	11/20 (55%)	14/20 (70%)	1/20 (0.5%)
	T/G, G/G (n = 19)	Positive	14	0/14 (0%)	0/14 (0%)	12/14 (86%)
		Negative	5	2/5 (40%)	2/5 (40%)	0/5 (0%)
HCV genotype 2 (n = 23)	T/T (n = 19)	Positive	4	2/4 (50%)	2/4 (50%)	0/4 (0%)
		Negative	15	12/15 (80%)	15/15 (100%)	0/15 (0%)
	T/G, G/G (n = 4)	Positive	2	0/2 (0%)	1/2 (50%)	0/2 (0%)
		Negative	2	2/2 (100%)	2/2 (100%)	0/2 (0%)

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder

RNA in B cells achieved either RVR or SVR; 12 of these 14 patients (86%) had a NVR. These results suggest that the absence of HCV RNA in B cells is a useful predictor of the response to IFN-based therapy. The ability to predict the response is defined based on sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) (Table 4). In genotype 1-infected patients, sensitivity, specificity, PPV and NPV for NVR according to genetic variation near IL28B were 100%, 87.7%, 65.0% and 100%, respectively. When both IL28B SNP variants and the presence of HCV RNA in B cells were combined, specificity and PPV were higher (specificity 98.2% and PPV 92.3%). In HCV genotype 2-infected patients possessing a major allele of the IL28B SNP, two of four patients (50%) with HCV RNA-positive B cells failed to achieve SVR. In contrast, all 15 patients with HCV RNA-negative B cells achieved SVR. Even in patients with minor alleles of the IL28B SNPs, both of two patients with HCV RNA-negative B cells achieved SVR. In genotype 2-infected patients, sensitivity, specificity, PPV and NPV for SVR according to the presence of HCV RNA in B cells were 90.0%, 100%, 100% and 60.0%, respectively.

These combined results indicate that the minor alleles of IL28B SNPs plus the presence of HCV RNA in B cells are useful predictors for NVR in genotype 1-infected patients, and the presence of HCV RNA in B cells is a predictor for non-SVR in genotype 2-infected patients.

Lymphotropic HCV has an IFN-resistant phenotype

We further characterized the viral phenotype of IFN resistance for B cell-tropic HCV and the effects of IL28B genotype on HCV RNA titre in B cells. Figure 2a shows that HCV RNA titres in B cells were significantly higher in genotype 1-infected s than in genotype 2-infected patients. The positive rates of HCV RNA in B cells were 68.8% (53/77) and 24.0% (6/25), respectively (P = 0.0001). These results suggest that genotype 1 HCV can infect and/or associate with B cells more efficiently than genotype 2. However, HCV RNA titres in B cells did not differ between patients bearing the major allele (T/T) of the IL28B SNP and those with the minor alleles (G/G or T/G) (data not shown), indicating that the IL28B genotype does not affect HCV infection of (or

Table 4 Predictive factors for outcomes of the IFN-based therapy in patients infected with HCV genotypes 1 and 2

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Genotype 1: Predictive factor for NVR				
IL28B T/G,G/G	100	87.7	65.0	100
IL28B T/G, G/G and HCV RNA in B cells (+)	92.3	98.2	92.3	98.2
Genotype 2: Predictive factor for SVR				
IL28B T/T	85.7	33.3	90.0	25.0
HCV RNA in B cells (−)	90.0	100	100	60.0

HCV, hepatitis C virus; NVR, null viral responder; PPV, positive predictive value; SVR, sustained viral responder.

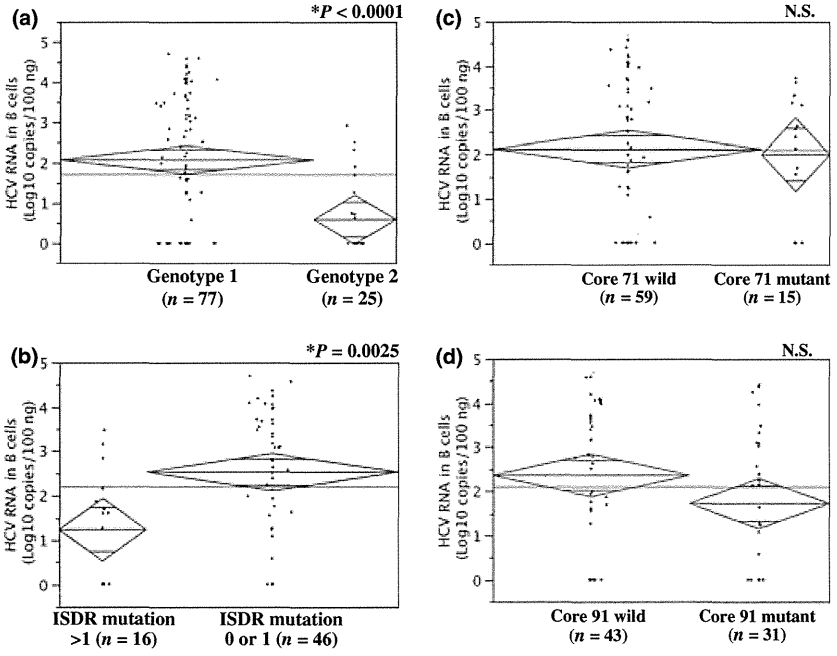

Fig. 2 Comparison of hepatitis C virus (HCV) RNA titres in B cells of (a) patients infected with HCV genotypes 1 and 2; (b) patients infected with HCV with 0-1 interferon sensitivity determining region amino acid mutations *vs* those with >1 mutations; (c) patients infected with HCV with codon 70 amino acid mutation of the core region *vs* those with wild type; and (d) patients infected with HCV with codon 91 amino acid mutation of the core region *vs* those with wild type. A horizontal grey line shows mean of all samples. Two grey diamonds indicate the averages and 95% confidence interval of each group. Statistical significance was determined by Student's t test. N.S.: not significant.

association with) B cells. We next analysed the effects of viral sequence and quasispecies diversity, which are known to affect the outcome of the IFN-based therapy, on HCV RNA titres in B cells. The number of nucleotide mutations in the ISDR of the NS5A region and missense mutations at codons 70 and 91 of the core region were determined in samples from patients infected with HCV genotype 1. Figure 2b shows that the HCV RNA levels in B cells were higher in patients infected with HCV with 0 or 1 mutation in the ISDR (ISDR 0-1) than in those harbouring HCV with more than one ISDR mutations (ISDR > 1). Additionally, the fraction of patients with HCV RNA in B cells was higher among those infected with ISDR 0-1 HCV [ISDR 0-1: 38/46 (82.6%) *vs* >1: 9/16 (56.3%), $P = 0.0463$]. However, substitutions at core positions 70 or 91 did not associate with HCV RNA titre in B cells (Fig. 2c,d). These results suggest that the number of mutations in ISDR, but not core mutations, affect the persistence of HCV in B cells.

DISCUSSION

While the human hepatocyte is the primary target for HCV infection, HCV also has lymphotropism, especially toward B cells [7,23]. Previous reports demonstrated the *in vitro* and *in vivo* association of HCV with B cells [7,24,25]. In our earlier report, we detected the replication of HCV in B cells in about 5% of HCV-infected patients and the presence of HCV RNA in about 64% of these patients [7]. Stamataki *et al.* [25] also demonstrated that HCV JFH1 bound (but did not replicate in) B cells in the cell culture system and that the virion binding B cells became more stable than free virions. These results suggest that the HCV isolates can be classified into three subgroups. The first subgroup consists of HCV isolates that neither infect nor adhere to B cells. A second subgroup (which encompasses most HCV isolates) can associate with B-cell surface receptors, but do not replicate efficiently in these cells. Such binding may induce activation and signalling, contributing to prolonged B-cell survival. The third subgroup (detected in only 5% of patients) consists of HCV isolates capable of infecting B cells and replicating efficiently in these cells.

The present study showed that HCV isolates that have infected and/or associated with B cells had an IFN-resistant phenotype. HCV RNA titres in B cells were significantly higher in patients with poor responses to IFN-based therapy (Fig. 1). Furthermore, the presence of HCV RNA in B cells was one of the factors determining the outcome of IFN-based therapy (Table 2). The precise mechanism of these clinical effects remains unknown; B-cell-associated viruses may be more stable than free virions [25]. Although current antiviral therapies can eliminate free HCV virions from sera, HCV is thought to survive in lymphocytes [19,20]. Peripheral blood memory B cells infected with HCV may be recruited to the liver of patients with CH-C [26]. Lymphotropic HCV may survive even after the IFN-based therapy and re-infect hepatocytes through the infiltration of HCV-infected B cells to the liver.

Another important issue is that HCV infection of and/or association with B cells may also contribute to IFN resistance by inducing dysfunction in B cells. The patients with HCV-positive B cells had at least one abnormality of LPD markers, indicating that HCV infection of and/or association with B cells reflected the presence of B cell-disorders [7]. In fact, HCV has been reported to bind naïve B cells through CD81, leading to the abnormal activation of B cells in the absence of ligation with the B cell receptor [24].

Many predictive markers (both viral and host factors) are associated with IFN-based treatment outcomes. Prediction of NVR and/or non-SVR status might allow hepatologists to pay closer attentions to poorly responding patients while minimizing unnecessary therapy. Recently, the genotypes of IL28B SNPs have been reported to be a valuable predictor for the outcome of IFN-based therapy [17,27,28]. In the present study, multivariable analyses showed that the genotypes of IL28B SNPs and the presence of HCV RNA in B cells were independent predictive markers for NVR in genotype 1-infected patients (Table 2c). (No genotype 2-infected NVR patients were detected in this study.) The combination of IL28B minor alleles and the presence of HCV RNA in B cells are especially valuable for predicting the NVR *vs* VR (viral responder) distinction in patients infected with HCV genotype 1 and for predicting the SVR *vs* non-SVR distinction in patients infected with HCV genotype 2. In patients infected with HCV genotype 1, the PPV for NVR in individuals with the IL28B minor allele was significantly elevated from 65.3% to 92.3% by the addition of HCV RNA in B cells as a predictive marker (Table 4). Similarly, increases in sensitivity, specificity, PPV and NPV for SVR were all higher in HCV genotype 2-infected patients when the presence of HCV RNA in B cells was used as a marker. The detection of HCV RNA in B cells may serve as a useful parameter for predicting the outcome of IFN-based therapy in patients infected with HCV genotype 2 although further studies with an increased population size are warranted.

Multiple host and viral factors are proposed as predictors of outcomes for IFN-based therapy. Host genotype (IL28B SNP alleles) and mutations in the virus (including the ISDR and the HCV core region) have been reported as significant pretreatment predictors of response to PEG-IFN and ribavirin combination therapy [29,30]. The present study shows that patients with B-cell-associated HCV RNA typically are infected with genotype 1, and their HCV has fewer mutations in the ISDR. Lerat *et al.* [8] have shown that PBMCs from patients infected with genotype 1 exhibit a higher detection rate of positive- and negative-strand HCV RNA, and their results are consistent with our observations: HCV ISDR mutations, but not substitutions of the core region, affect HCV RNA titres in B cells. HCV ISDR mutations may affect HCV infection of B cells through the effects on IFN signalling [31]. Neither viral titres nor the detection of HCV

RNA in B cells was associated with the genotypes of IL28B SNPs, which are thought to be the strongest genetic factor predicting the outcome of IFN-based therapy. Detail mechanisms underlying the association of the genotype of IL28B SNPs with the outcome of the IFN-based therapy, remain unknown. Expression levels of interferon λ mRNA and/or *in situ* cytokine levels in liver may differ between patients with the major or minor alleles of IL28B SNPs. It is possible that the circumstance of the innate immunity against HCV in liver is different from that in B cells. It is speculated that the presence of HCV RNA in B cells is linked to the interferon-resistance phenotype of the virus itself and/or the host immune-disorders triggered by the abnormal activation of B cells in patients. In conclusion, HCV that infects or associates with B cells appears to present an IFN-resistant phenotype, and the presence of HCV RNA in B cells is a useful predictive marker for resistance to IFN-based therapy.

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