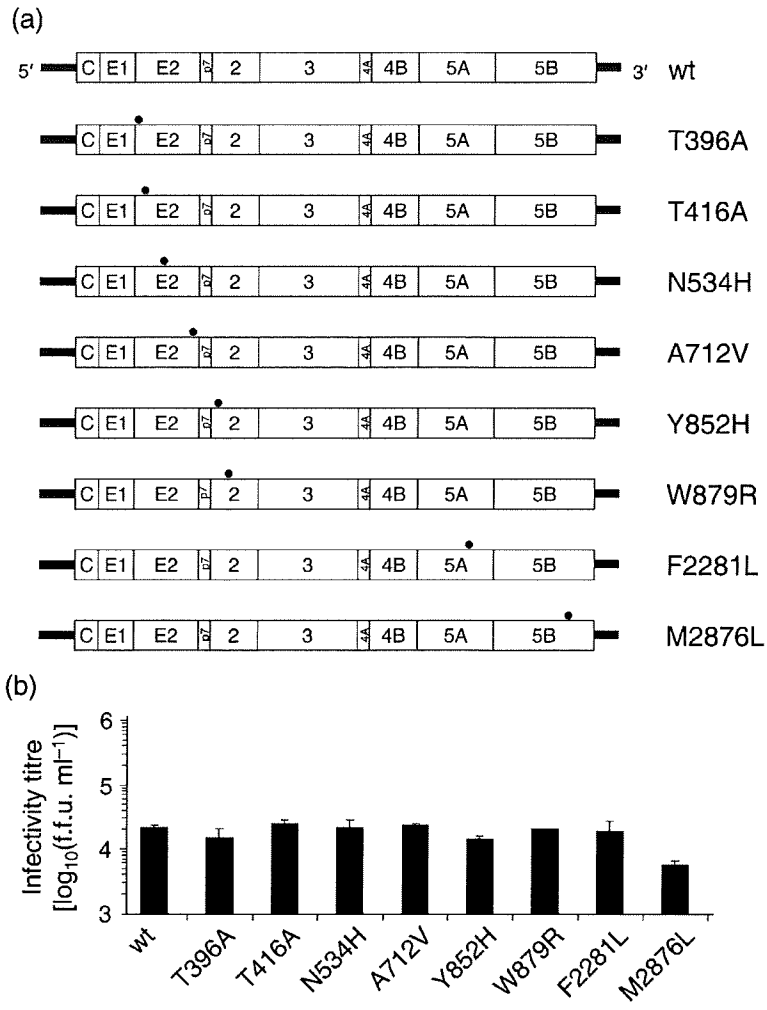

Fig. 2. Effects of individual mutations on the production of infectious HCV. (a) Schematic representation of the wild-type (wt) and mutant chimeric HCV J6/JFH1 genomes. HCV J6/JFH1 mutants with a single point mutation are shown. The adaptive mutations T396A, T416A, N534H, A712V, Y852H, W879R, F2281L and M2876L are indicated by ●. (b) The *in vitro*-transcribed mutant J6/JFH1 RNAs were electroporated into Huh-7.5 cells to generate recombinant mutant viruses. The infectivity titres of the culture supernatants were measured by titration assay. Then, naïve Huh-7.5 cells were infected with each virus at an m.o.i. of 0.01 and cultured for 12 days. The culture supernatant was collected every day from 1 to 12 days post-infection. The ability of each mutant virus to release infectious virus particles was examined by titration assay. Infectivity titres reached maximal levels at 10 days post-infection and the maximal infectivity titres were plotted. Error bars represent SD for triplicate measurements.

ml^{-1}) was calculated as shown in Table 2. The recombinant mutant viruses, R-27, R-38 and R-47, had higher specific-infectivity titres (1 : 46, 1 : 35 and 1 : 54, respectively) than the wild-type virus P-1 (1 : 197), suggesting that the particles released from cells infected with the R-27, R-38 and R-47 viruses are more infectious than those released from cells infected with the wild-type J6/JFH1 virus.

Efficient expression of HCV proteins in Huh-7.5 cells infected with the adaptive mutants

To investigate further the mechanism of adaptive mutations, we performed immunofluorescence staining of the infected cells. Huh-7.5 cells (6×10^4 cells per 24-well plate) were infected with the P-1, R-27, R-38 and R-47 viruses (1.2×10^4 f.f.u.) at an m.o.i. of 0.2. Cells were fixed 5 days post-infection and stained for immunofluorescence. Approximately 90 % of the cells were HCV-positive in the P-1-, R-27-, R-38- and R-47-infected cells (Fig. 4a). We next examined protein synthesis by immunoblotting for the HCV core and NS3 proteins. Immunoblot analysis of the cell lysates demonstrated that the levels of the core and NS3 proteins in cells infected with the R-27, R-38 and R-47 viruses were 2.0- to 2.5-fold higher than those in cells infected with the P-1 virus (Fig. 4b, c), suggesting that these mutant viruses have a replicative advantage.

Growth curves of infectious HCV after transfection of RNAs or infection with HCV

To determine whether the replicative advantage is at the level of entry or replication/translation of the genome, we examined one-step growth curves by transfecting equivalent amounts of RNAs of the wild-type and the mutant viruses into Huh-7.5 cells by means of electroporation (Fig. 5a, b). The intracellular and extracellular core protein levels were quantified by core protein-specific ELISA at the indicated times. The one-step growth curves showed that the intracellular and extracellular core protein levels increased with very similar kinetics in the cells transfected with the wild-type and adapted RNAs (Fig. 5a, b).

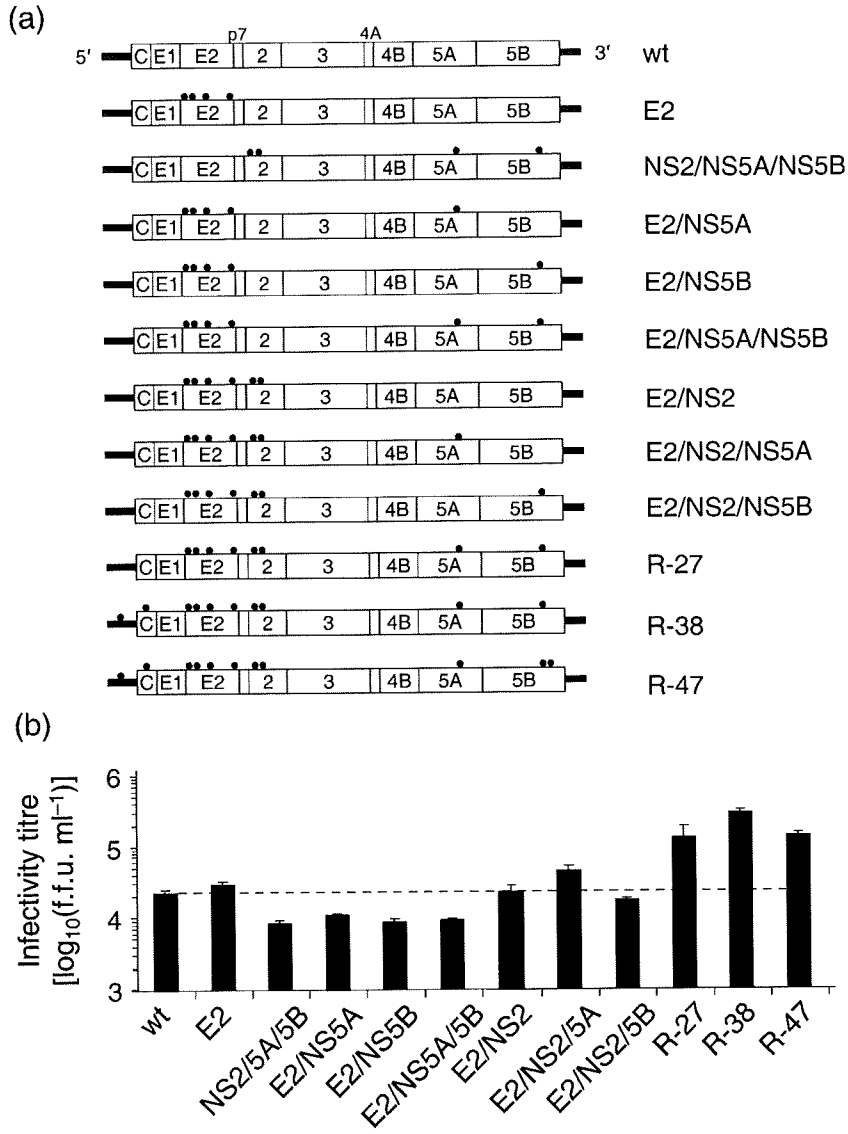

Fig. 3. Effects of combination of adaptive mutations on the production of infectious HCV. (a) Schematic representation of the wild-type (wt) and mutant chimeric HCV J6/JFH1 genomes. The HCV J6/JFH1 genomes with a combination of adaptive mutations at nt 146 (U to A) in the 5′-UTR and amino acid changes at K78E, T396A, T416A, N534H, A712V, Y852H, W879R, F2281L and M2876L are indicated by ●. (b) Recombinant mutant viruses with a combination of mutations were generated. Naïve Huh-7.5 cells were infected with each virus at an m.o.i. of 0.01 and cultured for 12 days. The ability of each mutant to release infectious virus particles was examined by titration assay. Infectivity titres reached maximal levels at 10 or 11 days post-infection and the maximal infectivity titres were plotted. Error bars represent SD for triplicate measurements.

We next examined the growth curves of the core protein levels by infecting cells with the recombinant viruses. The intracellular and extracellular core protein levels in cells infected with the P-1, R-27, R-38 and R-47 viruses were quantified. Huh-7.5 cells (1.2×10^5 cells per 12-well plate) were infected with these viruses at an m.o.i. of 0.2. The intracellular core protein levels in cells infected with the R-27, R-38 and R-47 viruses were 3- to 5-fold higher at day 1 post-infection than those in the P-1-infected cells. The intracellular core protein levels in the cells infected with the mutant viruses were 7- to 11-fold higher at day 3 post-infection than those in the P-1-infected cells (Fig. 5c). The extracellular core protein levels in the P-1-infected cells were comparable to the levels in cells infected with mutant viruses at day 1 post-infection. However, the extracellular core protein levels in cells infected with the R-27, R-38 and R-47 viruses increased more rapidly and reached 4.4- to 5.8-fold higher at day 3 post-infection than those in cells infected with the P-1 virus (Fig. 5d). Taken together, these data suggest that the adaptive mutants have advantages at the entry level, rather than the virus replication/translation level.

Table 2. Specific-infectivity titres of the recombinant adaptive mutant viruses

Virus	HCV RNA copies [$\log_{10}$(GE ml^{-1})]	Infectivity titre [$\log_{10}$(f.f.u. ml^{-1})]	Specific infectivity (f.f.u.:GE)
P-1	6.6±0.1	4.3±0.1	1:197
R-27	6.8±0.1	51±0.2	1:46
R-38	6.9±0	15.4±0.1	1:35
R-47	6.9±0.1	5.1±0.1	1:54

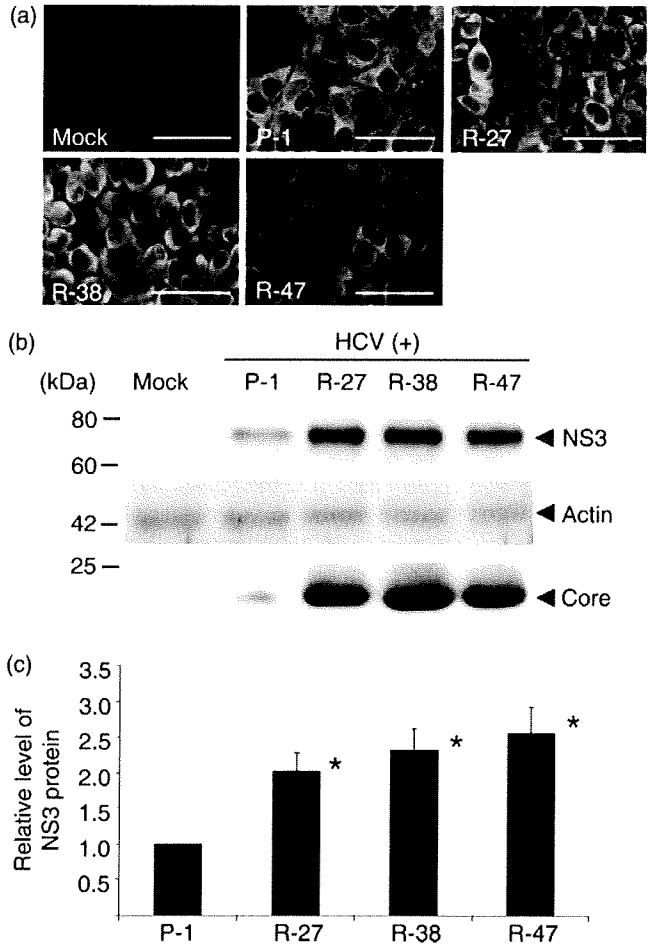

Fig. 4. Efficient expression of HCV proteins in Huh-7.5 cells infected with the adaptive mutants. Huh-7.5 cells (6×10^4 cells per 24-well plate) were infected with 200 µl P-1, R-27, R-38 or R-47 virus (6×10^4 f.f.u. ml^{-1}) at an m.o.i. of 0.2. (a) Cells were fixed 5 days post-infection and stained for immunofluorescence with anti-HCV-positive sera. Bars, 10 µm. (b) Immunoblot analysis of core and NS3 proteins in Huh-7.5 cells infected with R-27, R-38 and R-47 viruses. Data are representative of three independent experiments. (c) Quantification of the data shown in (b). Intensities of the gel bands were quantified by using the Scion Image for Windows program. The level of actin served as a loading control. Error bars represent SD for triplicate measurements. The difference between P-1 and the adaptive mutant (R-27, R-38 or R-47) was significant (*$P<0.05$ by Student's *t*-test).

Blocking of virus attachment and entry with anti-CD81 antibody

To determine whether the adapted mutant viruses have advantages at the entry level, we examined CD81-dependent entry into Huh-7.5 cells. Naïve Huh-7.5 cells were incubated with CD81-specific or non-specific antibody prior to inoculation. We scored infection by immunofluorescence at 24 h post-infection. As shown in Fig. 6(a), the anti-CD81 antibody inhibited the entry of the mutant viruses R-27, R-38 and R-47, as well as the wild-type virus, in a dose-dependent manner, suggesting that interaction between CD81 and HCV E2 glycoprotein is crucial for virus entry for all of these viruses. However, infections by the mutant viruses R-27, R-38 and R-47 were less dependent on CD81 than the wild-type virus. This result suggests that the mutations in the E2 glycoprotein confer an advantage to the mutant viruses at the entry level. We further analysed the mutant viruses to determine which mutation(s) is important for the advantage at the entry level. We infected Huh-7.5 cells with mutant viruses with a single point mutation in the E2 glycoprotein, such as T396A, T416A, N534H or A712V, or with all of the four mutations in E2. Blocking of virus entry with the anti-CD81 antibody was examined as shown in Fig. 6(b). Infection by the mutant virus N534H, as well as the mutant viruses E2, R-27, R-38 and R-47, was less dependent on CD81 than infection by the wild-type virus, whereas the other mutant viruses T396A, T416A and A712V showed a similar pattern to the wild type. These results indicate that the N534H mutation in the E2 region confers an advantage to the adaptive mutant viruses at the entry level.

DISCUSSION

In this study, we established an efficient HCV-production system by serial passaging of Huh-7.5 cells infected with the chimeric HCV J6/JFH1. Sequence analyses revealed that the adapted viruses possessed more than eight non-synonymous mutations in the genomes. Reverse-genetics analysis revealed that the recombinant viruses R-27, R-38 and R-47 exhibited higher expression of the HCV proteins than the wild-type virus. Moreover, we demonstrated that the N534H mutation in the E2 glycoprotein confers an advantage to the mutant viruses at the entry level.

The adaptive mutant viruses possessed four mutations (T396A, T416A, N534H and A712V) in E2. Two of these mutations (T416A and N534H) are in the regions that are involved in E2–CD81 binding and are, therefore, the possible target for neutralizing antibodies inhibiting E2–CD81 interactions (Helle & Dubuisson, 2008). The blocking of virus attachment and entry with CD81-specific antibody in this study revealed that the infections by the E2 R-27, R-38, R-47 and N534H mutants were less dependent on the CD81 molecule than that by the wild type J6/JFH1, suggesting that the N534H mutation gives the mutant viruses a selective advantage at the entry level. The N534H mutation is located in the sixth of 11 *N*-glycosylation sites, and is predicted to remove this *N*-glycosylation. The removal of *N*-glycosylation sites has been shown to have variable effects on CD81 binding and infectivity (Owsianka *et al.*, 2006; Roccasecca *et al.*, 2003). The glycans at positions 417, 532 and 645 (E2N1, E2N6 and E2N11) were shown to reduce the sensitivity of HCV pseudoparticles to antibody neutralization and to reduce the access of CD81 to its binding site on E2 (Goffard *et al.*, 2005). JFH-1 virus with the N534K mutation spread faster than the wild-type

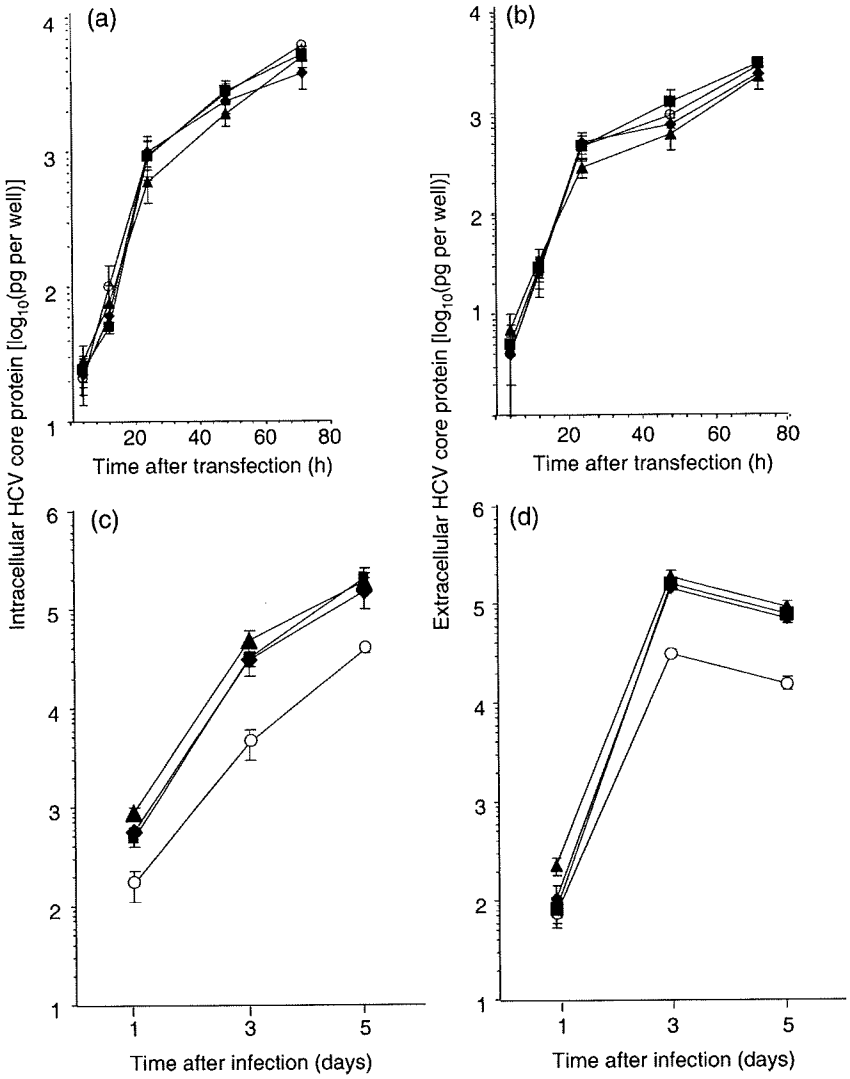

Fig. 5. Effects of adaptive mutations on the production of intracellular and extracellular core protein after transfection of *in vitro*-translated HCV RNAs or after infection of recombinant HCV. (a, b) After electroporation of 10 μg *in vitro*-translated HCV RNAs P-1 (○), R-27 (▲), R-38 (◆) and R-47 (■) into Huh-7.5 cells (5×10^6), the cells were divided into five sets, replated into a six-well plate and cultured. The cells and culture supernatants were harvested at the time points given. Intracellular (a) and extracellular (b) core protein levels were quantified by core protein-specific ELISA. (c, d) After Huh-7.5 cells (1.2×10^5 cells per 12-well plate) were infected with the P-1 (○), R-27 (▲), R-38 (◆) and R-47 (■) viruses at an m.o.i. of 0.2, the cells and culture supernatants were harvested at the time points given. Intracellular (c) and extracellular (d) core protein levels were quantified by core protein-specific ELISA.

JFH-1 virus after two successive amplifications in naïve cells, although the numbers of infectious viruses in the supernatant of transfected cells were initially low (Delgrange *et al.*, 2007). Our results in the growth curves of the viruses in the transfected cells and infected cells were consistent with their report. The CD81 inhibition assay in this study demonstrated clearly that the N534H mutation of the J6/JFH-1 virus confers a selective advantage for J6/JFH-1 at the entry level. To our knowledge, the present study is the first to prove that the mutation at site N534 gives infectious HCV a selective advantage at the entry level. These results raise two possibilities. One is that the N534H mutation in the E2 glycoprotein removes *N*-glycosylation and this mutant E2 glycoprotein possesses a higher affinity for the CD81 molecule, resulting in efficient entry to the cells. Another possibility is that the E2 glycoprotein with the N534H mutation gains higher affinity for other HCV receptors. Further investigation will be required to elucidate the mechanism of this adaptive mutation.

Our results showed that a combination of the mutations in E2, together with four additional mutations in NS2, NS5A and NS5B, resulted in higher infectivity of HCV, suggesting that the additional four mutations possess an advantage at different steps.

NS2 is a membrane-associated cysteine protease (Grakoui *et al.*, 1993; Hijikata *et al.*, 1993b; Lorenz *et al.*, 2006). The N terminus of NS2 consists of one or more transmembrane domains, whilst the C-terminal domain of NS2, together with the N-terminal one-third of NS3, forms the NS2–3 protease, an enzyme that catalyses a single cleavage at the NS2/NS3 boundary. The crystal structure of the C-terminal domain of NS2 has recently been determined and reveals a dimeric protease containing two composite active sites (Lorenz *et al.*, 2006). Jones *et al.* (2007) showed that NS2 and p7 are essential for HCV infectivity. The Y852 and W879 residues are located in the hydrophobic region of NS2. Although the exact topology of NS2 is disputed, the Y852H and W879R mutations would be predicted to lie

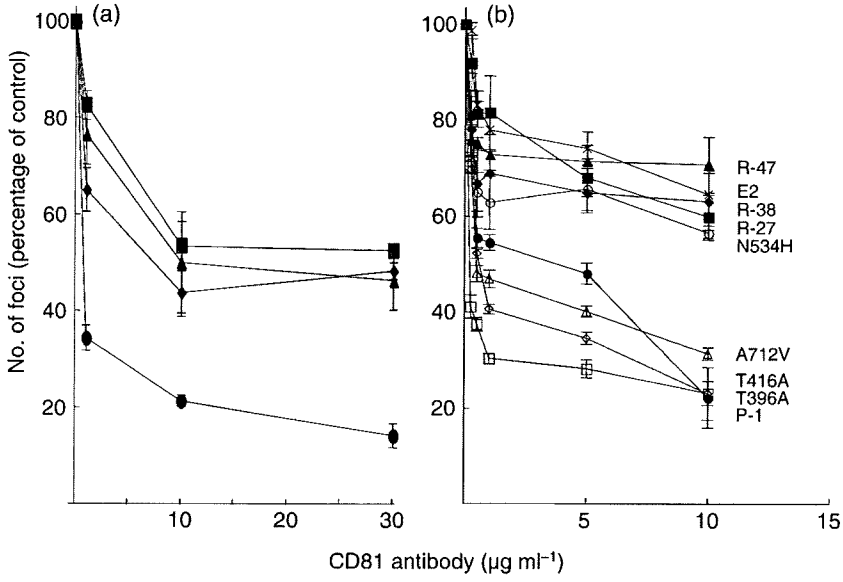

Fig. 6. Blocking of virus attachment and entry with anti-CD81 antibody. (a) Huh-7.5 cells (2×10^5 cells per six-well plate) were pretreated with 0, 1, 10 or 30 μg CD81 antibody (clone JS-81) ml^{-1} for 1 h and then infected with the wild-type (●, P-1) or recombinant mutant (■, R-27; ◆, R-38; ▲, R-47) viruses at an m.o.i. of 0.5. The cells were cultured for 24 h. The infection was monitored by HCV immunofluorescence and the numbers of HCV-positive foci were counted. Each result is expressed as a fraction of the number of foci observed in wells that received the control antibody instead of anti-CD81. Error bars represent SD for triplicate measurements. (b) Huh-7.5 cells (2×10^5 cells per six-well plate) were pretreated with 0, 0.25, 0.5, 1, 5 or 10 μg CD81 antibody ml^{-1} for 1 h and then infected with the wild-type (●, P-1) or recombinant (■, R-27; ◆, R-38; ▲, R-47; ×, E2; □, T396A; ◇, T416A; ○, N534H; △, A712V) viruses at an m.o.i. of 0.01. Blocking of virus entry with anti-CD81 antibody was examined. The infection was monitored by HCV immunofluorescence and the number of HCV-positive foci was counted.

within the second and third transmembrane domains, respectively (Yamaga & Ou, 2002). Murray *et al.* (2007) demonstrated that the A880P mutation increased infectious virus production significantly in the context of the J6/JFH1 genome, suggesting that the mutations in the transmembrane domain of NS2 play an important role in HCV infectivity. It is possible that the Y852H and W879R mutations in the transmembrane domain affect the topology and localization of NS2, and thereby HCV infectivity. Interestingly, NS2 has been found to interact with all other HCV NS proteins in *in vitro* pull-down assays, as well as cell-based colocalization and co-immunoprecipitation experiments (Dimitrova *et al.*, 2003; Hijikata *et al.*, 1993b), suggesting a role for NS2 as part of the replication complex.

Sequence analyses of HCV replicon cells revealed that highly adaptive mutations lie within the NS4B, NS5A and NS5B coding regions, with the majority clustering in NS5A. However, the mechanism underlying the replication enhancement is not known (Bartenschlager & Sparacio, 2007). The mutant viruses possessed an F2281L mutation that was located in domain II of NS5A. NS5A is an RNA-binding phosphoprotein composed of three domains that are separated by trypsin-sensitive low-complexity sequences (LCS I and LCS II) and an N-terminal amphipathic α-helix that anchors the protein stably to intracellular membranes (Brass *et al.*, 2002; Penin *et al.*, 2004; Tellinghuisen *et al.*, 2004). According to the X-ray crystal structure of domain I, it forms a dimer with a claw-like shape that can accommodate a single-stranded RNA molecule (Tellinghuisen *et al.*, 2005). Domain III of NS5A plays an important role in virus assembly and the production of infectious particles (Appel *et al.*, 2008; Masaki *et al.*, 2008; Tellinghuisen *et al.*, 2008). However, the role played by domain II of NS5A in the HCV replication cycle is unknown. Further examination will be required to clarify the effects of the F2281L mutation on the infectivity of the virus. Kaul *et al.* (2007) reported the V2941M mutation in NS5B in the context of the JFH1 genome. Lohmann *et al.* (2001) reported the R2884G mutation in the context of Con1-based replicon cells. Amino acid substitutions within NS5B may favour HCV replication and virus production in ways that remain to be determined.

Miyanari *et al.* (2007) proposed that HCV NS proteins and replication complexes are recruited to lipid droplet-associated membranes by the HCV core protein and that this recruitment is critical for producing infectious viruses. Cholesterol and sphingolipid associated with HCV particles are important for virion maturation and infectivity (Aizaki *et al.*, 2008). We speculate that the additional four mutations in NS2, NS5A and NS5B may confer an advantage in the maturation of virus particles or modification of virus envelopes with cholesterol and sphingolipid. Further investigation will be necessary to elucidate the mechanism of the adaptive mutations in NS2, NS5A and NS5B.

In conclusion, we have developed an efficient HCV-production system by passaging HCV J6/JFH1-infected Huh-7.5 cells. We have demonstrated that an efficient HCV-production system could be obtained by introducing adaptive mutations into the J6/JFH1 genome. The J6/JFH1-derived mutant viruses presented here would be a good tool for producing HCV particles with enhanced infectivity and for studying the molecular mechanism of HCV entry.

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Double-Filtration Plasmapheresis plus IFN for HCV-1b Patients with Non-Sustained Virological Response to Previous Combination Therapy: Early Viral Dynamics

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Key Words
Chronic hepatitis C · Double-filtration plasmapheresis · Early viral dynamics · Genotype 1b · High viral load · Interferon β · Non-sustained virological responder · Peginterferon plus ribavirin combination therapy

Abstract
Double-filtration plasmapheresis (DFPP) was approved in Japan in April 2008 for the retreatment of chronic hepatitis C patients with genotype 1b and high viral loads, whose hepatitis C virus was not eradicated by earlier IFN therapy or by pegylated IFN plus ribavirin (PEG-IFN/RBV) combination therapy. In this study, we assessed the early viral dynamics of 9 patients with non-sustained virological response to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction of ≥1 log in the viral load in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment. By contrast, DFPP plus consecutive intravenous IFN-β for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks. The viral load declined by ≥2 log in 50% (3/6) at 4 weeks after the start of treatment. DFPP plus consecutive intravenous IFN-β for 4 weeks is a promising treatment for non-sustained virolgical response patients.

Introduction

Hepatitis C virus (HCV) infection is the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) in industrialized countries. HCV infection is manageable, however, and its complications can be prevented by antiviral therapy [1, 2]. Currently, the most effective treatment for chronic HCV infection is based on pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy [3]. Nonetheless, sustained

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virological response (SVR) rates for those infected with the most resistant genotypes (HCV-1a and HCV-1b) still hover around 50% [3, 4].

To surmount this SVR rate with combination therapy, several trials have been undertaken, two of which are: (1) retreatment with combination therapy and (2) double-filtration plasmapheresis (DFPP). By the protocol-defined primary analysis of the former, the SVR rate has been 16% at most, even for a 72-week induction group [5].

The use of DFPP [approved in Japan in April 2008 for the retreatment of chronic hepatitis C (CHC) patients with genotype 1b and high viral loads] together with IFN administration has produced a substantial reduction in the viral load during the early stages of treatment and has effected a high SVR [6], suggesting that this treatment is a new modality for CHC patients in difficult-to-treat states. In this study, we used DFPP plus IFN to enhance the efficacy of the treatment of CHC patients whose HCV was not eradicated by earlier PEG-IFN/RBV combination therapy, and we assessed early viral dynamics associated with SVR.

Patients and Methods

Patients

Nine patients (aged 43–66 years) whose HCV had not been eradicated by earlier PEG-IFNα-2b plus RBV combination therapy carried out between 2008 and 2009 were enrolled in this study. The patients were divided into 2 groups: partial responders (PR; relapse after the end of therapy) and non-responders (NR; no disappearance of HCV RNA during therapy). All the patients were confirmed to be HCV RNA positive with high transaminase levels persisting for 6 months or longer, and with HCV RNA genotype 1b at levels exceeding 10^5 log IU/ml in blood (as determined before the start of therapy by real-time PCR). Also, the patients were negative for hepatitis B surface antigen. Patients with platelet counts of $\leq 10 \times 10^4/\mu l$, leukocyte counts of $\leq$3,000/μl, or hemoglobin levels of ≤12 g/dl were excluded from the study.

Each patient gave written informed consent and agreed to receive concomitant DFPP, and the study was approved by the review board of the Kobe Asahi Hospital.

DFPP and Blood Collection

Blood collected from the peripheral vein for DFPP by a Plasmaflo™ OP-18W filter (Asahi Kasei Medical, Tokyo, Japan) was separated into plasma and cell components. The virus was then removed from the plasma by a second filter (Cascadeflo™ EC-50W; Asahi Kasei Medical) of an average pore size of 30 nm. For each session, the final volume of treated plasma was 50 ml/kg; the number of sessions was 5 over 2 weeks, and the time of DFPP, based on the reduced plasma fibrinogen levels during DFPP, was decided by the physicians and as required by the patients.

Types of IFN for 4 Weeks with DFPP

During DFPP, the patients were treated with different kinds of IFN: patient 1 with PEG-IFNα-2b plus RBV for 4 weeks; patients 2 and 3 with IFN-β 3 MU twice daily for 2 weeks and PEG-IFNα-2a plus RBV for 2 weeks; patients 4 and 9 with IFN-β 3 MU twice daily for 2 weeks and IFN-β 6 MU daily for 2 weeks; patient 5 with IFN-β 3 MU twice daily for 10 days and IFN-β 6 MU daily for 18 days, and patients 6, 7 and 8 with IFN-β 3 MU twice daily for 4 weeks. The dose of PEG-IFNα-2b was 1.5 μg/kg and 180 μg of α-2a per week. The RBV dose was 800 mg/day with α-2b and 600–800 mg/day with α-2a. After DFPP plus IFN treatment for 4 weeks, all patients were scheduled to receive PEG-IFN/RBV combination therapy (patient 1: PEG-IFNα-2b 1.5 μg/kg per week plus RBV 800 mg/day; patients 2–9: PEG-IFNα-2a 180 μg per week plus RBV 600–800 mg/day).

Amino Acid Substitutions in the Core Region (aa 30 and aa 91) and Number of IFN Sensitivity-Determining Region Mutations

We measured pre-treatment factors such as prediction of clinical outcome of therapy, amino acid sequence variation in the NS5A region (referred to as IFN sensitivity-determining regions) and in the core protein regions (aa 70 and aa 91) of HCV with a given genotype, and the viral load.

HCV RNA Measurement

The quantity of HCV RNA was measured by real-time PCR (detection limit 1.2 log IU/ml), by HCV core antigen (detection limit 20 fmol/l), and by RT-PCR (Amplicor HCV monitor v 2.0; Roche; detection limit 50 IU/ml).

Virus Removal at Second Filter Inlet and Outlet

Plasma was collected twice from the inlet and outlet of the second filter during 1 session of DFPP: once when the treated plasma volume reached half of the target quantity, and once when DFPP was completed. The change in the quantity of HCV RNA was evaluated through the plasma samples collected.

Viral Reduction and Viral Response Rate

The quantity of HCV RNA was converted to a log value at the beginning of the treatment (A) and at each of the virus measurement points (B). Δlog was then calculated: $\Delta\log = \log A - \log B = \log (A/B)$.

Evaluation of DFPP Safety

The subjective and objective adverse events of DFPP were observed, and five clinical factors were measured (platelet and lymphocyte counts, and hemoglobin, albumin and fibrinogen levels) before the first session of DFPP, before successive sessions on the second, third, fourth, fifth and sixth days, and 2 weeks after the last session.

Statistical Analysis

Statistical analysis consisted of analysis of variance for patient background factors, and the paired t test for quantities of HCV RNA at the second filter inlet during DFPP. The t test was used for viral load reductions and Fisher's exact test for viral response rates among the groups. The t test was 2-tailed, and differences of $p < 0.05$ were considered significant.

Table 1. Early viral dynamics with DFPP plus IFN treatment

Case	Age/ sex	Type of IFN for 4 weeks with DFPP	Viral dynamics after DFPP+IFN						Viral dynamics of previous treatment (PEG-IFN/RBV)			Viral mutation		
			before treatment	log drop			unit		before treatment	log drop 4 wks	unit	out-come	aa 70	aa 91	ISDR
				24 h	1 wk	2 wks	4 wks								
1	66/M	PEG-IFNα-2b/RBV 4 wks	6,510	0.5	0.6	0.6	1.1	fmol/l	452	0.7	KIU/ml	NR	wild	wild	0
2	65/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	7.5	0.4	1.3	2.6	1.0	log IU/ml	2,800	ND	KIU/ml	PR	wild	wild	0
3	52/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	5.8	0.4	1.0	1.6	+0.2	log IU/ml	6.3	0.2	log IU/ml	NR	wild	wild	1
4	47/F	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	6.8	0.6	0.3	0.4	0.4	log IU/ml	2,900	0.3	KIU/ml	NR	mutant	mutant	1
5	52/F	IFN-β (3 MU 2/day) 10 days → IFN-β (6 MU 1/day) 18 days	5.5	1.4	1.5	1.2	1.9	log IU/ml	782	0.6	fmol/l	NR	wild	mutant	1
6	61/F	IFN-β (3 MU 2/day) 4 wks	6.5	1.2	3.4	5.0	4.8	log IU/ml	8,450	2.6	fmol/l	NR	wild	wild	0
7	66/F	IFN-β (3 MU 2/day) 4 wks	5.3	0.0	0.8	1.2	1.3	log IU/ml	11,500	0.8	fmol/l	NR	mutant	wild	1
8	43/F	IFN-β (3 MU 2/day) 4 wks	3,460	0.5	0.2	1.3	2.2	fmol/l	745	0.1	fmol/l	NR	wild	mutant	1
9	43/M	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	7.2	0.6	1.4	2.5	2.9	log IU/ml	426	0.1	KIU/ml	NR	wild	wild	0

PEG-IFN/RBV: PEG-IFNα-2a (180 μg per week) plus RBV (600–800 mg/day) or PEG-IFNα-2b (1.5 μg/kg per week) plus RBV (800 mg/day). IFN-β: 3 MU twice daily or 6 MU daily.
ND = Not done; aa = amino acid; ISDR = interferon sensitivity-determining region.

Results

Of the 9 patients, 1 was PR and 8 were NR. Virus mutation in the core region was as follows: wild type (7 patients) and mutant type (2 patients) at aa 70; wild type (6 patients) and mutant type (3 patients) at aa 91. IFN sensitivity-determining regions demonstrated mutation 1 (5 patients) and mutation 0 (4 patients), while mutation 2 was not seen in any patient. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction in the viral load of ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The early viral dynamics after DFPP plus consecutive intravenous IFN-β treatment for 4 weeks showed a reduction in the viral load of ≥1 log in 33% (2 of 6 patients), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The reduction of the viral load by ≥2 log was observed in 50% (3 of 6 patients) at 4 weeks after the start of treatment (table 1).

Discussion

New drugs to replace IFN as well as drugs that can be used in combination with IFN are being actively developed. Also, attempts are being made to find ways to physically remove HCV particles from the blood. Granulocyte apheresis, plasma exchange and hemofiltration have been applied to HCV-infected patients for the treatment of cryoglobulinemia and vasculitis, modalities which have been shown to reduce HCV RNA in the blood during treatment [6–11]. The mechanisms of the clinical results of plasmapheresis have been described, whereby HCV in the blood is related to the effects of IFN therapy that could be enhanced by removing the virus from blood [12–14]. Low-density lipoprotein-cholesterol apheresis and plasma exchange in hypercholesteremic patients with HCV infection reduces the quantity of HCV RNA in the blood of some patients [15]. Hemodialysis, hemofiltration and peritoneal dialysis in chronic dialysis patients infected with HCV significantly lower HCV RNA levels in the blood [16]. Combined granulocyte apheresis with IFN therapy for CHC [17–19] and the prerequisite for early reduction of the virus in the treatment of CHC [20, 21] are essential. Thus, the potential effectiveness of IFN therapy combined with early physical removal of the virus is of particular interest.

Asahina et al. [22] studied HCV dynamics in both serum and peripheral blood mononuclear cells in 44 patients, with HCV genotype 1b and high viral loads, randomly assigned to 4 treatment groups: (1) combination therapy with 6 MU daily of IFNα-2b plus 800 mg of RBV; (2) monotherapy with 6 MU daily of IFNα-2b; (3) monotherapy with twice-daily intravenous administration of 3 MU of IFN-β, and (4) monotherapy with daily intravenous administration of 6 MU of IFN-β. HCV RNA levels measured serially by highly sensitive real-time PCR and HCV dynamics in both serum and peripheral blood mononuclear cells have demonstrated a 'biphasic' pattern. The exponential decay slopes of the second phase have been significantly higher in the combination or the twice-daily dose regimen groups than in group 2 or 4 (0.10 ± 0.08 vs. 0.02 ± 0.09 or 0.16 ± 0.09 vs. 0.02 ± 0.04 day^{-1}; $p < 0.05$ and $p < 0.0005$, respectively) [22]. Kim et al. [23] observed that a daily dose of IFN-β 6 MU for 4 weeks effects a 2 log decrease in the HCV RNA load in 7 patients with genotype 1b and high viral loads.

In this study, early viral dynamics were assessed in the 9 patients non-SVR to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks reduced the viral load by ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9), and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. DFPP plus consecutive intravenous IFN-β treatment for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively.

The prerequisite for early virological response (EVR; indicating negative HCV RNA at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those who do not reach EVR fail to respond to further therapy. Treatment discontinued in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is highly desirable [24].

To be able to predict SVR with PEG-IFN/RBV treatment, reduction of the HCV RNA viral load by week 4 is considered essential. A 2 log reduction in the HCV RNA viral load by week 4 is a prerequisite to achieving SVR with PEG-IFN/RBV treatment [25]. In our study of DFPP plus consecutive intravenous IFN-β treatment for 4 weeks, a reduction in the viral load of ≥2 log was achieved in 50% (3 of 6 patients) at 4 weeks after the start of treatment.

From the above considerations, DFPP plus consecutive intravenous IFN-β treatment for 4 weeks is a promising regimen for non-SVR patients with genotype 1b and high viral loads, previously treated with PEG-IFN/RBV therapy. Further study is needed to elucidate the SVR rate in a larger number of patients given DFPP plus IFN treatment, especially with consecutive intravenous IFN-β.

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Disclosure Statement

No conflict of interest exists.

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Original Article

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Outcome and Early Viral Dynamics with Viral Mutation in PEG-IFN/RBV Therapy for Chronic Hepatitis in Patients with High Viral Loads of Serum HCV RNA Genotype 1b

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Key Words
Chronic hepatitis · Early viral dynamics · IFN/RBV resistance-determining region · HCV RNA genotype 1b · High viral load · PEG-IFN/RBV combination therapy · Virological response, prediction

Abstract
We investigated whether sustained virological response (SVR) and non-SVR by chronic hepatitis C patients to pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy are distinguishable by viral factors such as the IFN/RBV resistance-determining region (IRRDR) and by on-treatment factors through new indices such as the rebound index (RI). The first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h) were calculated. The subject patients were divided into 3 groups based on RI-1st and RI-2nd: an RI-A group (RI-1st ≤1.0), an RI-B group (RI-1st >1.0 and RI-2nd <0.7) and an RI-C group (RI-1st >1.0 and RI-2nd ≥0.7). The SVR rate was 71.4% (10/14) in the RI-A group, 46.2% (6/13) in the RI-B group and 20.0% (3/15) in the RI-C group ($p = 0.005$ between the RI-A group and the RI-C group). In IRRDR ≥6 and IRRDR ≤5 the SVR rate was 81.3% (13/16) and 23.1% (6/26) ($p = 0.0002$), respectively. By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group (≥6 mutations in the IRRDR) and 7.7% (1/13) in the RI-C group (≤5 IRRDR mutations) ($p = 0.0003$).

Introduction

Recently, global consensus has obtained that a combination of IFN or pegylated IFN plus ribavirin (PEG-IFN/RBV) is the treatment of choice for chronic hepatitis C (CHC). Notwithstanding this treatment regimen, sustained virological response (SVR) rates of those infected with the most resistant genotypes [hepatitis C virus (HCV)-1a and -1b] still hover at ~50% [1, 2]. It is therefore worthwhile to identify the predictive factors that allow the selection of patients who would achieve eradication

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of HCV RNA either before or during therapy, especially since IFN/RBV combination therapy is costly and has several side effects [3].

Predictors of the effectiveness of IFN-based therapy can be classified into pretreatment and on-treatment factors. Pretreatment factors comprise: (1) host factors such as age, gender, obesity, alcohol consumption, hepatic iron overload, fibrosis, immune responses and co-infection with other viruses, and (2) viral factors that mainly include viral genotypes and loads, particular amino acid sequence variations in the NS5A region [4, 5] and in the core protein region of HCV [6] within a given genotype. Moreover, the mean number of mutations in variable region 3 (V3) plus its upstream flanking region of NS5A [amino acid 2334–2379, referred to as IFN/RBV resistance-determining region (IRRDR)] is significantly higher in HCV isolates obtained from patients who later achieve SVR by PEG-IFN/RBV than in those from non-SVR patients. On-treatment factors are mainly related to viral kinetics within the first few weeks of treatment [7].

In the current study, with the aim of investigating whether SVR and non-SVR can be distinguished by viral factors such as IRRDR and by on-treatment factors through new indices such as the rebound index (RI), we calculated the first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h), as proposed by Nomura et al. [8].

Patients and Methods

The 42 patients included in this study, who all demonstrated high viral loads (>100 KIU/ml) of serum HCV RNA of genotype 1b, had been diagnosed with CHC on the basis of abnormal serum alanine aminotransferase persisting for at least 6 months, and of positive HCV RNA assessed by RT-PCR. None of the patients was positive for hepatitis B surface antigen or other liver diseases (autoimmune hepatitis, alcoholic liver disease). All the patients received a regimen of PEG-IFNα-2b (peginterferon alpha-2b; PegIntron; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/kg/week, subcutaneously) in combination with RBV (ribavirin; Rebetol; Schering-Plough) 600–1,000 mg/day for 48 weeks. RBV was administered at a dose of 600 mg/day (3 capsules) to patients weighing <60 kg, 800 mg/day (4 capsules) to those weighing <80 kg and 1,000 mg/day (5 capsules) to those weighing ≥80 kg.

The efficacy of the combination therapy was evaluated by HCV RNA negativity determined by qualitative RT-PCR analysis at the end of therapy (end of therapy response) and 6 months after the completion of therapy (SVR). The amount of HCV RNA was also measured quantitatively by RT-PCR (Amplicor HCV monitor v. 2.0; Roche) before therapy. The lower detection limit of the assay was 5 KIU/ml. Samples collected during and after therapy were also determined by qualitative RT-PCR (Amplicor; Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml.

SVR was defined as undetectable serum HCV RNA at 24 weeks after the cessation of treatment, and non-SVR as detectable HCV RNA at 24 weeks after the discontinuation of treatment. Informed consent was obtained from all patients enrolled in the study after thoroughly explaining the aims, risks and benefits of the therapy.

The amount of HCV core antigen was assessed by the IRM assay (Ortho Clinical Diagnostics, Tokyo, Japan), which provides a good correlation between the amount of HCV core antigen and the amount of HCV RNA, as shown in our previous study [9]. The HCV core antigen was measured on days 0, 1 (24 h), 7 (1 week) and 14 (2 weeks) according to the detection limit of 20 fmol/l established by the manufacturer.

RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h [8].

The patients were divided into 3 groups based on RI-1st and RI-2nd: group A (RI-1st ≤1.0), group B (RI-1st >1.0 and RI-2nd <0.7) and group C (RI-1st >1.0 and RI-2nd ≥0.7).

NS5A sequence analysis (IRRDR) was performed as described [4]. Briefly, the sequences of the amplified fragments were determined by direct sequencing without subcloning with the use of a Big Dye Deoxy Terminator cycle sequencing kit and an ABI 337 DNA sequencer (Applied Biosystems, Japan). The aa sequences were deduced and aligned with Genetyx Win software v. 7.0 (Genetyx Corp., Tokyo, Japan). Numbering of aa throughout the manuscript is according to the polyprotein of HCV genotype 1b prototype HCV-J.

Statistical Analysis

Differences between the groups were assessed by the χ^2 test, Fisher's exact test or Student's t test, the Mann-Whitney test and the Kruskal-Wallis test. $p < 0.05$ was considered statistically significant.

Results

Of the 42 patients treated with combination therapy, 19 (45.2%) achieved SVR and 23 (54.8%) were still HCV RNA positive (non-SVR) 6 months after therapy. No significant differences were observed in patient characteristics between SVR and non-SVR, except in platelet counts and the degree of fibrosis (table 1), or among the RI-A, -B and -C groups (table 2).

The SVR rate was 71.4% (10/14), 46.2% (6/13) and 20.0% (3/15) in the RI-A, -B and -C groups, respectively, with a significant difference between the RI-A and -C groups ($p = 0.005$), but not significant between the RI-A and -B groups and the RI-B and -C groups (fig. 1). In the 14 patients of the RI-A group, HCV RNA turned negative

Table 1. Host-dependent, virus-related profile by response (SVR and non-SVR)

	SVR	Non-SVR	p value
Gender (M/F), n	11/8	13/10	NS
Age, years	56.7 ± 8.8	59.3 ± 10.5	NS
HCV RNA level, KIU/ml	1,685 ± 1,477	1,660 ± 1,363	NS
HCV core antigen, fmol/l	7,044 ± 6,763	9,343 ± 12,563	NS
Body weight, kg	59.9 ± 11.5	59.8 ± 13.6	NS
Treatment history (retreatment/naïve)	6/13	13/10	NS
Platelet count ($\times 10^4/mm^3$)	18.7 ± 4.4	14.8 ± 5.4	NS
F0, 1/F2, 3	12/2	5/10	0.004

Table 2. Host-dependent, virus-related profile by response (RI-A, -B and -C groups)

	RI-A	RI-B	RI-C	p value
Gender (M/F), n	7/7	9/4	8/7	NS
Age, years	60.0 ± 5.9	58.5 ± 9.4	56.1 ± 12.8	NS
HCV RNA level, KIU/ml	1,401 ± 1,014	2,053 ± 1,286	1,593 ± 1,772	NS
HCV core antigen, fmol/l	6,084 ± 5,106	7,674 ± 5,038	11,000 ± 15,837	NS
Body weight, kg	62.1 ± 16.6	59.5 ± 10.4	58.2 ± 10.1	NS
Treatment history (retreatment/naïve)	3/11	7/6	9/6	NS
Platelet count ($\times 10^4/mm^3$)	15.3 ± 3.5	18.3 ± 5.9	16.3 ± 6.0	NS
F0, 1/F2, 3	7/3	5/4	5/5	0.004

Table 3. SVR rate between IRRDR ≤5 and IRRDR ≤6 in RI-A, -B and -C groups

	RI-A		RI-B		RI-C	
	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6
SVR	3	7	2	4	1	2
Non-SVR	3	1	5	2	12	0
SVR rate, %	50.0	87.5	28.6	66.7	7.7	100
p value	NS		NS		0.0024	
			0.0003			

by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and was positive in 1 patient throughout the treatment. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, on and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients (fig. 2).

The SVR rate was 81.3% (13/16) in the group with ≥6 mutations in IRRDR, and 23.1% (6/26) in those with ≤5 (fig. 3), with a significant difference between the 2 groups ($p = 0.0002$).

By combining RI and IRRDR, the SVR rate was 87.5% (7/8) in the RI-A group (IRRDR ≥6) and 7.7% (1/13) in the RI-C group (IRRDR ≤5) (table 3), with a significant difference between the 2 groups ($p = 0.0003$).

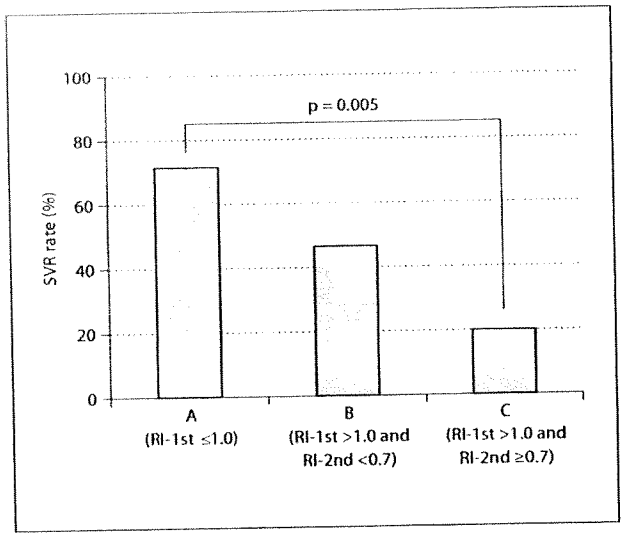

Fig. 1. SVR rate in RI-A, -B and -C groups. The overall SVR rate was 71.4, 46.2 and 20.0%, respectively. Significant difference in SVR rate is indicated.

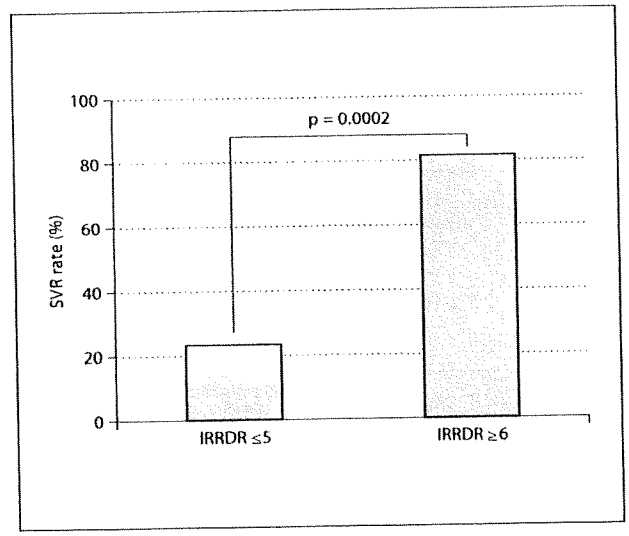

Fig. 3. SVR rate and IRRDR number. The SVR rate was 23.1% in IRRDR ≤5 and 81.3% in IRRDR ≥6, which was significantly different.

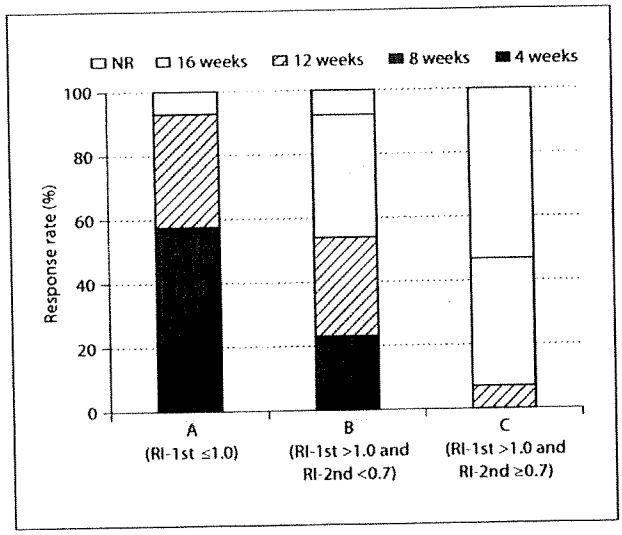

Fig. 2. Relation between response time and virus dynamics. In the 14 patients of the RI-A group, HCV RNA turned negative by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and remained positive throughout the treatment in 1 patient. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, at and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients.

Discussion

The importance of early virological response (EVR; signifying HCV RNA negative at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those not reaching EVR do not respond to further therapy. Discontinuation of treatment in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is worth investigating [10].

Treatment with IFN induces a decline in HCV RNA levels that can be mathematically measured in 2 phases. The decline in the first phase, usually measured at 24 or 48 h, probably reflects direct inhibition of intracellular production and release of HCV [11], with IFN efficacy ranging from about 70% (approx. 0.7 log units) for standard IFN (given 3 times a week) to more than 90% (1 log unit) for high daily doses of standard IFN or PEG-IFN (given once a week) [12, 13]. The decline in the second phase, beginning after 24–48 h, is slower and more variable than that in the first phase, and is thought to reflect continued inhibition of replication and the gradual elimination of virus-infected cells [11]. The decay in the first phase has little correlation with the IFN dose, but is more rapid with PEG-IFN than with standard IFN preparations [10].

Lowering HCV RNA during the first phase is essential for efficient elimination of HCV during the second phase. Decreases in HCV RNA titers within the first 24–48 h after the start of IFN would, therefore, be a dependable estimate of antiviral efficacy [12, 13].

Early viral kinetics, determined up to week 2, are believed to express the therapeutic effect of PEG-IFN. The concentration of PEG-IFNα-2b in serum peaks after 24 h, then declines gradually [14, 15]. The viral load is thus reduced by 24 h but increases in week 1 [16, 17]; with a large dose of PEG-IFN at each administration, it decreases markedly at 24 h but then increases in week 1 regardless of the dose. In the responder group, however, the viral load continues to decline each week thereafter [17].

In this study, we used new indices proposed by Nomura et al. [8]: RI-1st and RI-2nd calculated from early viral kinetics. RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and the RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h. In the SVR group, a number of patients demonstrated no increase in the viral load at week 1. Patients with a high RI-2nd were regarded as poor responders or non-responders to PEG-IFN. The RI-2nd of those other than non-responders was below 0.7; therefore, 0.7 was adopted as the reference value for RI-2nd, and the patients were divided into 3 groups based on RI-1st and RI-2nd: the RI-A group (RI-1st $\leq$1.0), the RI-B group (RI-1st >1.0 and RI-2nd <0.7) and the RI-C group (RI-1st >1.0 and RI-2nd $\geq$0.7). The SVR rate of the RI-A, RI-B and RI-C groups was 71.4% (10/14), 46.2% (6/13) and 20% (2/10), respectively ($p = 0.005$ between the RI-A group and the RI-C group). RIs are also associated with the early clearance of HCV RNA that is related to SVR.

In the RI-A group 21.4% (3/14), 35.7% (5/14) and 35.7% (5/14) became HCV RNA negative by weeks 4, 8 and 12, respectively. In the RI-B group 7.7% (1/13), 15.4% (2/13), 30.8% (4/13) and 38.5% (5/13) became HCV RNA negative by weeks 4, 8, 12, and at and after week 16, respectively. In the RI-C group 6.7% (1/15) and 40.0% (6/15) became HCV RNA negative by week 12, and at and after week 16, respectively. It is believed that the simplified RI-1st and RI-2nd are evidential indices for determining the therapeutic efficacy of PEG-IFN/RBV treatment.

We have previously reported that the high degree of sequence variation in IRRDR (IRRDR $\geq$6) significantly correlates with SVR, whereas the low degree of sequence variation in this region (IRRDR $\leq$5) correlates with non-SVR [4]. A significant correlation between the rapid reduction of HCV core antigen titers and the degree of sequence variation in IRRDR has been observed. This, in particular, suggests a possible influence of IRRDR $\geq$6 on HCV replication kinetics during IFN-based therapy, especially that the direct effect of IFN begins a few hours after the first dose.

In this study, the SVR rate was 81.2% (13/16) with IRRDR $\geq$6 and 23.1% (6/26) with IRRDR $\leq$5 ($p = 0.0002$), strongly suggesting that IRRDR $\geq$6 would be a useful marker for the prediction of SVR.

By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group (RI-1st $\leq$1.0) with IRRDR $\geq$6, signifying that about 90% of these patients turned SVR and were, thus, believed to be very good responders. An SVR rate of 7.7% (1/13) was obtained in the RI-C group with IRRDR $\leq$5 ($p = 0.0003$).

In conclusion, we propose that IRRDR combined with RIs is the most sensitive predictive factor for SVR and non-SVR. With the aid of RIs and IRRDR, a more effective PEG-IFN/RBV treatment could be within reach. A more detailed investigation with a larger number of subjects is needed to confirm the current results in patients given PEG-IFN/RBV combination therapy.

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Disclosure Statement

No conflict of interest exists.

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Analysis of Neutralizing Antibodies against Hepatitis C Virus in Patients Who Were Treated with Pegylated-Interferon *plus* Ribavirin

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RUNNING TITLE: ANTI-HCV NEUTRALIZING ANTIBODIES IN SERA OF HCV-INFECTED PATIENTS

Key words: hepatitis C virus, neutralizing antibody, pegylated-interferon *plus* ribavirin, treatment outcome

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ABSTRACT

The role of neutralizing antibodies (NAb) in determining responses to antiviral therapy has not been defined well. By using hepatitis C virus (HCV) cell culture system with the J6/JFH1 strain of HCV genotype 2a, we analyzed NAb responses in patients with chronic hepatitis C who received pegylated-interferon *plus* ribavirin (PEG-IFN/RBV) antiviral therapy. A total of 65 patients chronically infected with HCV genotype 1b were enrolled in this study. Of all the 65 patients, 34 (52%) patients achieved early virological response (EVR), with the remaining 31 patients (48%) being Non-EVR. Twenty-seven patients (42%) achieved sustained virological response (SVR), with the remaining 38 patients (58%) being Non-SVR. Thus, NAb titers were significantly higher in sera of patients who achieved EVR and SVR than those of Non-EVR and Non-SVR, respectively. Rather unexpectedly, NAb titers did not significantly decrease when measured even one year after disappearance of HCV RNA. On the other hand, when change ratios of NAb titers before and after disappearance of HCV RNA were compared between patients with different treatment outcomes, we noticed that the change ratio of NAb_{50} titers of patients who achieved an EVR was significantly lower than that of Non-SVR. In conclusion, our present results suggest that NAb titers were significantly associated with clinical responses to PEG-IFN/RBV therapy.

INTRODUCTION

Hepatitis C virus (HCV), an enveloped, positive-stranded RNA virus, is a member of the Genus *Hepacivirus*, the Family *Flaviviridae*. The life cycle of the virus, including viral attachment and entry to the cells, genome replication, protein synthesis and virion assembly, has recently been studied using an HCV cell culture system (12). An estimated 170 million individuals are infected with HCV worldwide. The current standard therapy is based on a combination of pegylated-interferon *plus* ribavirin (PEG-IFN/RBV) and, with this treatment regimen, viral eradication can be achieved in around 50% of the patients infected with HCV-1b.

Both viral and host factors play important roles in the control of viral infection. Whereas viral factors help to adjust the cellular environment to support viral replication, host factors generally function to combat the viral invasion either by actively blocking the virus replication through innate and/or acquired immune responses or by having the infected cells die out by themselves through apoptosis so that the virus can no longer replicate in the infected cells.

Acquired immune responses of the host involve cell-mediated immunity and humoral immunity. The importance of cellular immunity in combating HCV infection has been well documented (4, 14). On the other hand, humoral immune responses in protection against and/or recovery from HCV infection may be of less importance. Nevertheless, it has been reported that the neutralizing antibody (NAb) responses play an important role in the prevention of infection and in limiting viremia (10, 13, 16). Indeed, patients chronically infected with HCV were reported to possess relatively high titers of cross-reactive NAb (1). It is reported that patients with chronic hepatitis C infection also have high NAb titers to envelope protein of HCV-like particles (HCV-LPs) (2). Humoral and cellular immune responses are also important in determining response to antiviral therapy with IFN/RBV (7). We previously reported that the degree of antibody responses to the NS5A protein of HCV was correlated with early virological response after the initiation of PEG-IFN/RBV therapy (8). However, the role for NAb in determining responses to PEG-IFN/RBV antiviral therapy has not been well documented.

In the present study, we have established an experimental system to measure NAb titers using hepatitis C virus cell culture (HCVcc) model, and measured NAb titers in patients with chronic hepatitis C who were treated with PEG-IFN/RBV. Our data revealed that good treatment outcome was associated with higher NAb titers in patients chronically infected with HCV-1b.

MATERIALS AND METHODS

Cells

Huh-7.5 cells (3), a kind gift from Dr. C. M. Rice (Rockefeller University, New York, NY, USA), were propagated in Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% fetal bovine serum (FBS; Biowest), 0.1 mM nonessential amino acids (Invitrogen), 100 IU penicillin per ml and 100 μg streptomycin per ml (Invitrogen). Cells were grown at 37°C in a CO_2 incubator.

Virus

The J6/JFH1 strain of HCV (11) was a kindly gift from Dr. C. M. Rice. Virus stocks were produced in Huh-7.5 cells, and the viral titers were determined by focus forming units (FFU) assay in Huh-7.5 cells, as described previously (5). The viral stocks were kept at -80°C until ready for use.

Patients and serum samples