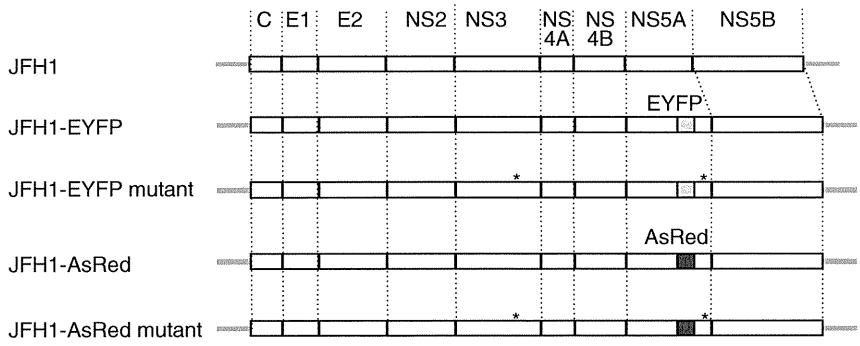


Figure 1 Schematic of the JFH1-based hepatitis C virus constructs. EYFP or AsRed was inserted in-frame into the *Cpo*I site of NS5A (JFH1-EYFP or JFH1-AsRed). The adaptive mutations in NS3 and NS5A (T4209A and C7653T) are indicated by asterisks (JFH1-EYFP mutant or JFH1-AsRed mutant).

reporter sequences, does not affect viral genomic replication,[12] while manipulation of the domain resulted in the loss of viral particle secretion possibly through abrogation of NS5A-core protein–protein association that is crucial for the viral particle assembly.[16] These problems have made it to difficult to analyze the entire viral life cycle in living cells. Recently, Han *et al.* reported a mutant HCV-JFH1 clone that expressed green fluorescent protein (GFP)-tagged NS5A and which could propagate and secrete infectious virus particles.[17]

In this study, we took advantage of the mutant fluorescence protein-tagged HCV and investigated the life cycles of HCV infection. We have demonstrated the rate of expansion of HCV infection using flow cytometry. Moreover, we have shown the kinetics of co-infection with two virus strains, which differed in their ability to secrete infectious virus particles.

METHODS

Reagents

RECOMBINANT HUMAN IFN-α-2b was from Schering-Plough (Kenilworth, NJ). Protease inhibitor (BILN2061) was from Boehringer Ingelheim (Ingelheim, Germany). BILN2061 is a macrocyclic NS3 protease inhibitor, the antiviral effects of which have been reported in a phase I clinical study.[18] Although further development of BILN2061 has been abandoned, this compound has structural homology with other protease inhibitors that are currently being evaluated in clinical trials, such as TMC435 and MK7009.[7,19]

Cell culture

Huh7.5.1 cells[20] and their derivatives were maintained in Dulbecco's modified minimal essential medium (DMEM; Sigma, St Louis, MO, USA) with 10% fetal bovine serum (FBS; Invitrogen, San Diego, CA, USA) at 37°C under 5% CO_2.

Plasmid constructs (Fig. 1)

In order to produce pJFH1-EYFP, the EYFP gene was amplified from pEYFP-C1 (Clontech, Mountain View, CA, USA) by polymerase chain reaction (PCR). The PCR products were then inserted into the *Cpo*I site (7484) of pJFH1. Similarly, pJFH1-AsRed was produced using pAsRed2 (Clontech). The JFH1-EYFP and JFH1-AsRed mutants, which are JFH1-based mutants with robust virus production capability, have two point mutations (T4209A, C7653T).[17] In order to introduce these mutations into pJFH1-AsRed, the *Avr*II/*Nsi*I and *Bsr*GI fragments of pJFH1-AsRed were amplified by PCR. The PCR products were subcloned into the T-Vector (pGEM-T Easy Vector Systems; Promega, Madison, WI, USA) and the mutations were introduced by site-directed mutagenesis (Quick-Change II Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA), as reported previously.[21] Finally, these *Avr*II/*Nsi*I and *Bsr*GI fragments were reinserted into the parental plasmid, pJFH1-AsRed. The pJFH1-AsRed mutant was digested with *Cpo*I and the DNA fragment was subcloned into pJFH1-EYFP, producing the mutant pJFH1-EYFP. All nucleotide numbers refer to pJFH1.[11]

Constitutive expression of ER-fluorescence protein in Huh7.5.1 cells

Huh7.5.1 cells were seeded into 6-cm diameter dishes and transfected with pDsRed2-ER (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Isolated clones (ER-Huh7.5.1 cells) were maintained under selection with 0.75 mg/mL G418 (Nacalai Tesque, Kyoto, Japan) and

screened for DsRed protein expression using fluorescence microscopy (BZ-8000; Keyence, Tokyo, Japan).

RNA transcription and transfection

Recombinant HCV RNA was synthesized and transfected as previously described.[22,23] Briefly, the plasmids were linearized by digestion with *XbaI* and subjected to *in vitro* transcription using RiboMax Large Scale RNA Production System (Promega). For the RNA transfection, Huh7.5.1 cells were suspended in Opti-MEM (Invitrogen) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and subjected to an electric pulse (1050 μF and 270 V) using the Gene Pulser II apparatus (Bio-Rad, Richmond, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm diameter dish. The transfected cells were split every 3–5 days. The culture media were transferred subsequently onto uninfected Huh7.5.1 cells.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously.[24] Cells were cultured on 18-mm round cover slips (Matsunami, Osaka, Japan) and fixed using 4% paraformaldehyde for 10 min at room temperature. Cells were incubated with the primary antibodies for 1 h at 37°C and with Alexa Fluor 488 goat antimouse immunoglobulin (Ig)G antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature in the dark. Mouse anti-NS5A antibody (Biodesign, Saco, ME, USA) and mouse anti-core antibody (Abcam, Cambridge, MA, USA) were used as primary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by confocal laser fluorescent microscopy (BZ-8000 [Keyence] and FLUOVIEW FV10i [Olympus, Tokyo, USA]).[25]

Flow cytometry

JFH1-EYFP and JFH1-EYFP mutant-transfected Huh7.5.1 cells and uninfected Huh7.5.1 cells were cultured in 12-well plates (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were collected on the days, post-transfection or post-infection, indicated. After washing with phosphate buffered saline (PBS; Nacalai Tesque) supplemented with 3% FBS and staining of dead cells with propidium iodide, the cells were analyzed using a FACS Calibur with CellQuest software (Becton Dickinson).

Quantification of HCV core antigen in the culture medium

The culture media from Huh7.5.1 cells transfected with JFH1 and its derivatives were collected on the days indicated and stored at 80°C. The levels of core antigen in the culture media were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics).

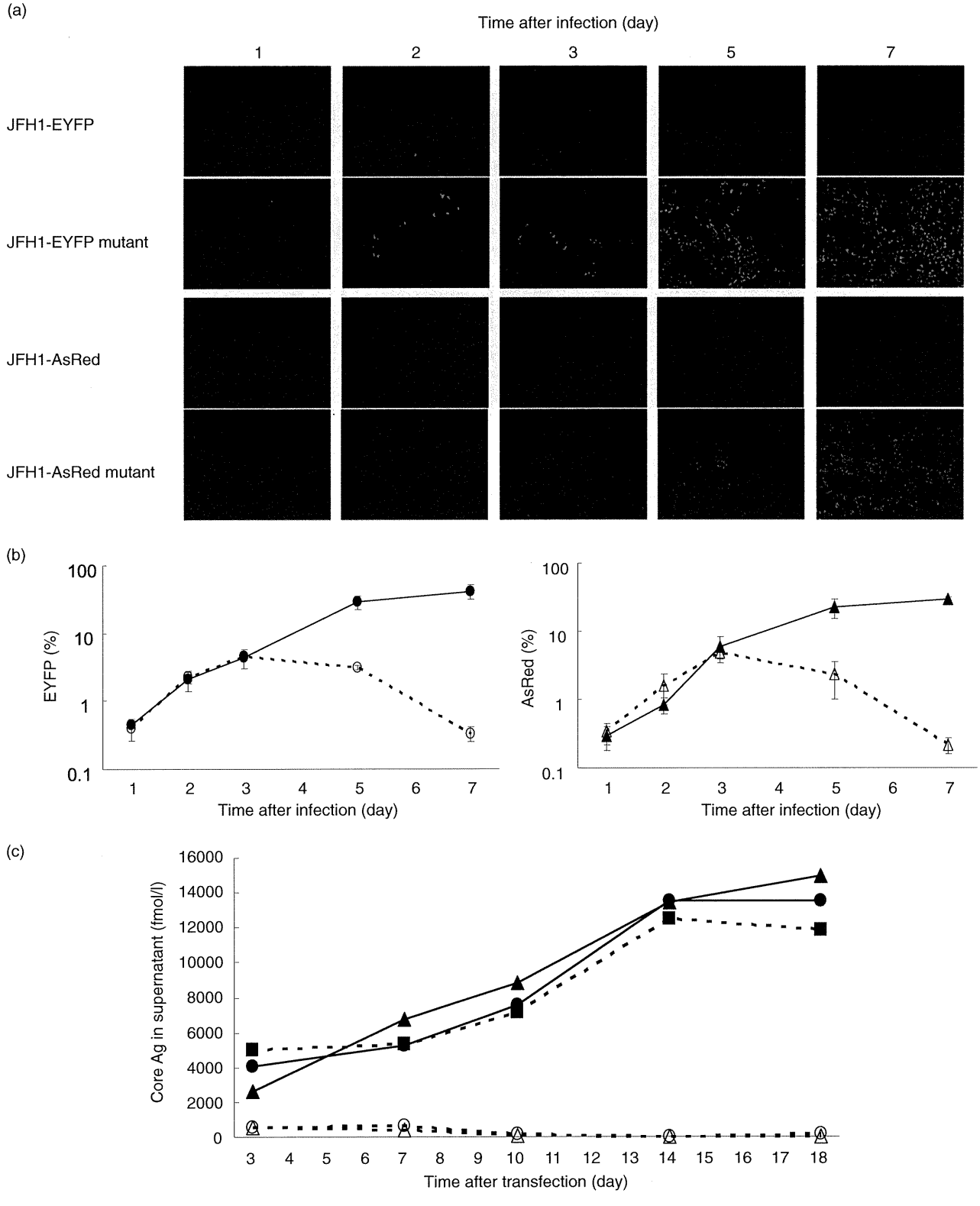
Western blotting

Western blotting was carried out as described previously.[21] Briefly, 10 μg of total cell lysate was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and blotted onto a polyvinylidene fluoride membrane. The membrane was incubated with primary antibodies followed by peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences). The antibodies used were anti-core mouse monoclonal antibody 2H9, anti-NS5A mouse monoclonal antibody 9E10 (provided by Dr Rice), and anti-GFP rabbit polyclonal antibody (Invitrogen). The anti-GFP is able to detect EYFP. To confirm expression of EGFP in Huh 7.5.1 cells, Huh7.5.1 cells were seeded into a 6-cm diameter dish and transfected with pEGFP (Invitrogen) using Lipofectamine 2000 (Invitrogen).

Statistical analyses

Statistical analyses were performed using Welch's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

Figure 2 Infectious hepatitis C virus (HCV) reporter virus with robust virus production capability. (a) Huh7.5.1 cells were transfected with JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed or JFH1-AsRed mutant HCV RNA. At 3 days post-transfection, culture media were collected and added onto uninfected cells. At the days indicated, EYFP or AsRed-directed-fluorescence was visualized directly. (b) The ratio of EYFP or AsRed-positive cells in (a) is counted in each image and plotted vs time. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^*P < 0.05$. —●—, JFH1-EYFP mutant; ··○··, JFH1-EYFP; —▲—, JFH1-AsRed mutant; ··☆··, JFH1-AsRed. (c) The levels of core antigen in the culture medium of JFH1, JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, and JFH1-AsRed mutant-transfected Huh7.5.1 cells collected on the days indicated. Ag, antigen. ··■··, JFH1; ··○··, JFH1-EYFP; —●—, JFH1-EYFP mutant; ··☆··, JFH1-AsRed; —▲—, JFH1-AsRed mutant.



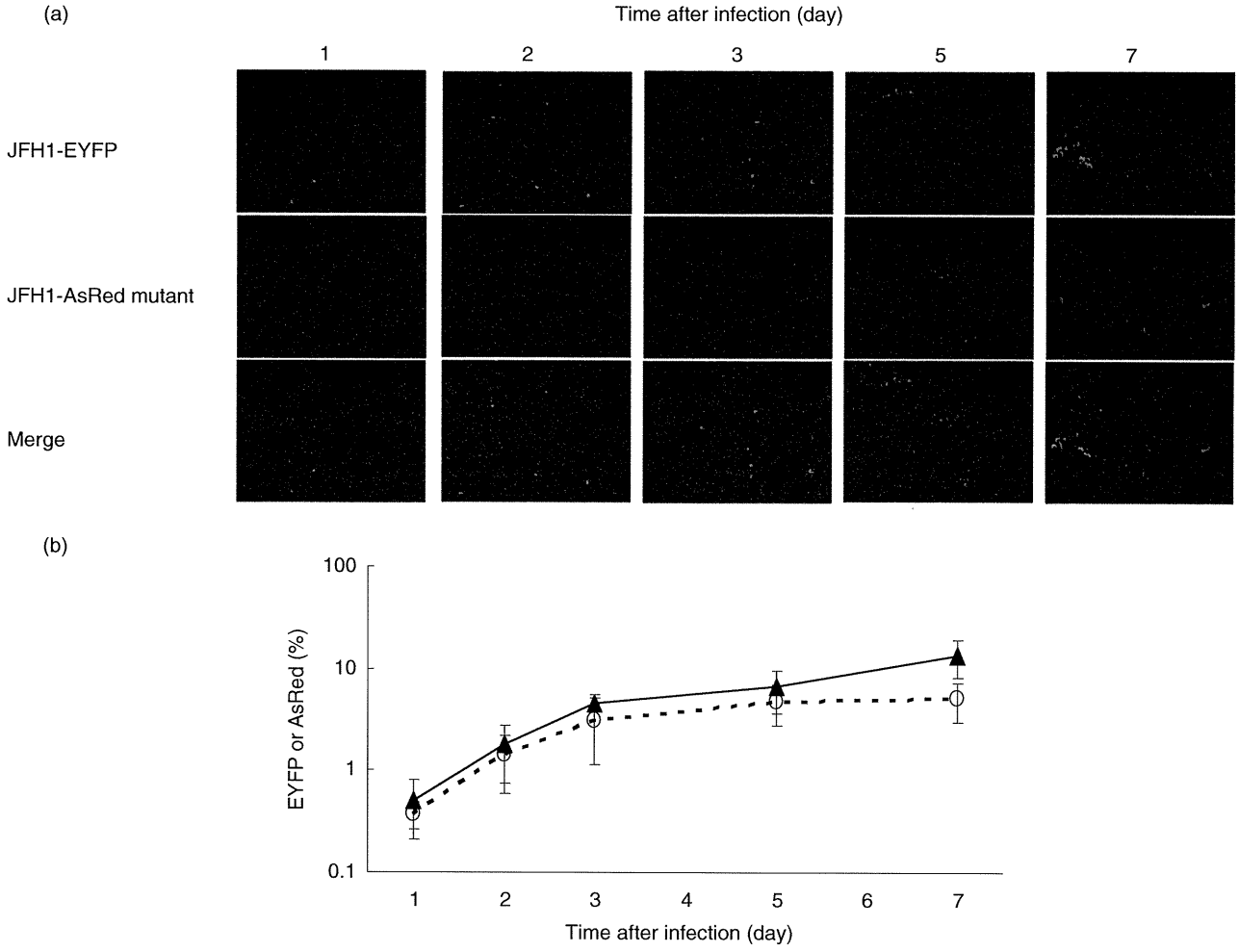


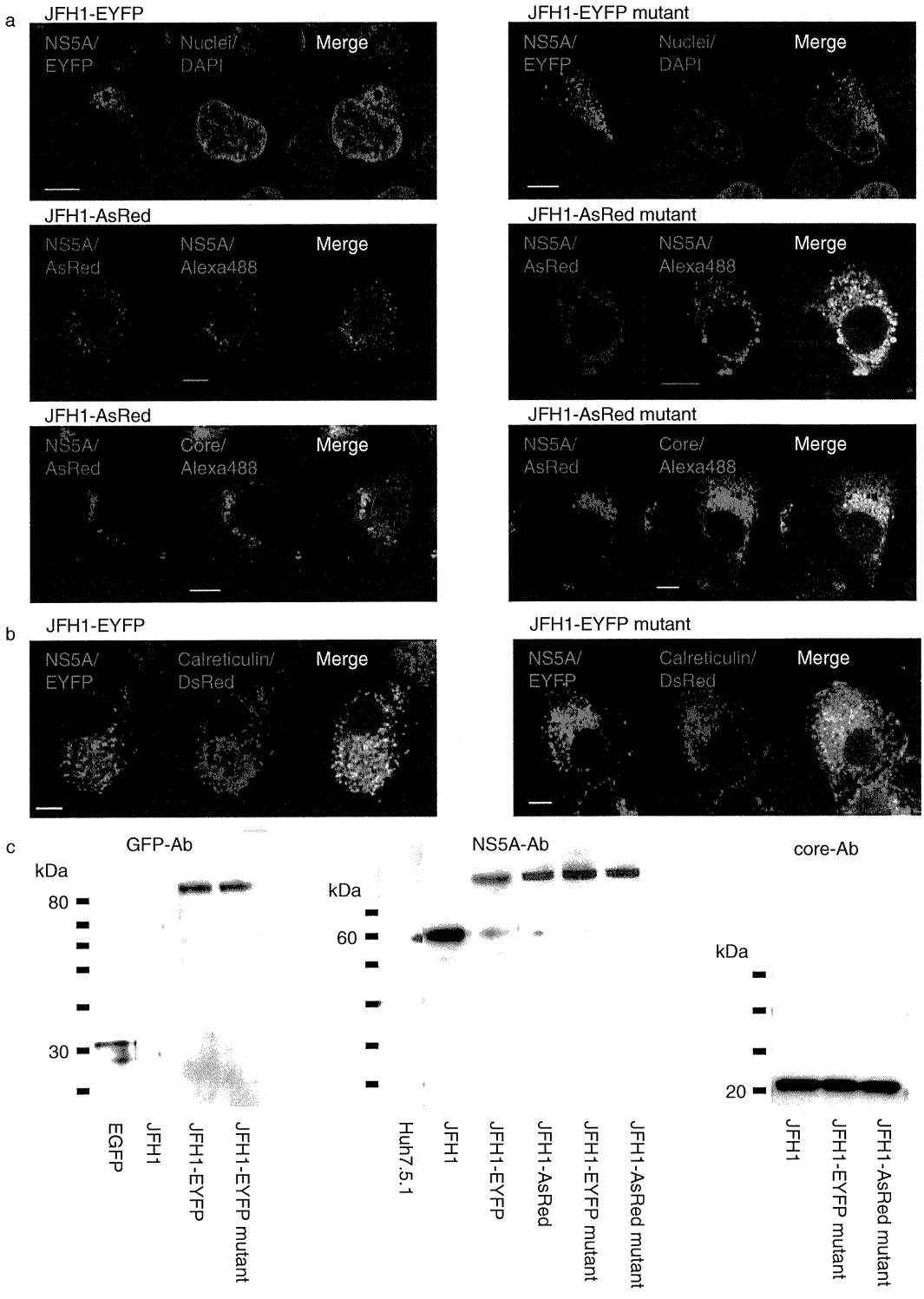
Figure 3 Intracellular *trans*-complementation of virus proteins. (a) Culture media from JFH1-EYFP and JFH1-AsRed mutant-transfected cells at 3 days post-transfection were added onto uninfected Huh7.5.1 cells. At the days indicated, EYFP or AsRed-directed-fluorescence was visualized directly. (b) The ratio of EYFP or AsRed-positive cells in Fig. 2a is calculated and plotted vs time. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. —▲— JFH1-AsRed mutant; ···○··· JFH1-EYFP.

RESULTS

Infectious HCV reporter virus with robust virus production capability

FIRST, WE STUDIED whether the JFH1-EYFP mutant viruses are able to secrete sufficient amounts of infectious virus particles. Full-length HCV RNA was transcribed *in vitro* and transfected into Huh7.5.1 cells. Culture media were collected from cells transfected with JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, or JFH1-AsRed mutant, respectively, and inoculated into uninfected Huh7.5.1 cells. EYFP- or AsRed-positive cells were

Figure 4 Localization and expression of NS5A-EYFP and NS5A-AsRed fusion proteins. (a,b) Huh7.5.1 cells transfected with JFH1-EYFP, AsRed and their mutant RNA genomes were fixed at 3 days post-transfection. NS5A-EYFP and NS5A-AsRed fusion proteins were visualized using EYFP and AsRed, respectively. DsRed auto-fluorescence was observed directly. NS5A and core proteins were immunostained with Alexa Fluor 488-labeled goat antimouse immunoglobulin G (green). 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (blue) staining revealed the nuclear chromatin. Bars represent 10 μm. (c) Cells were transfected with EGFP, JFH1, JFH1-EYFP, JFH1-AsRed, JFH1-EYFP mutant or JFH1-AsRed mutant. Cells were harvested at 3 days post-transfection, and western blotting was performed by using anti-GFP, NS5A or core antibodies.



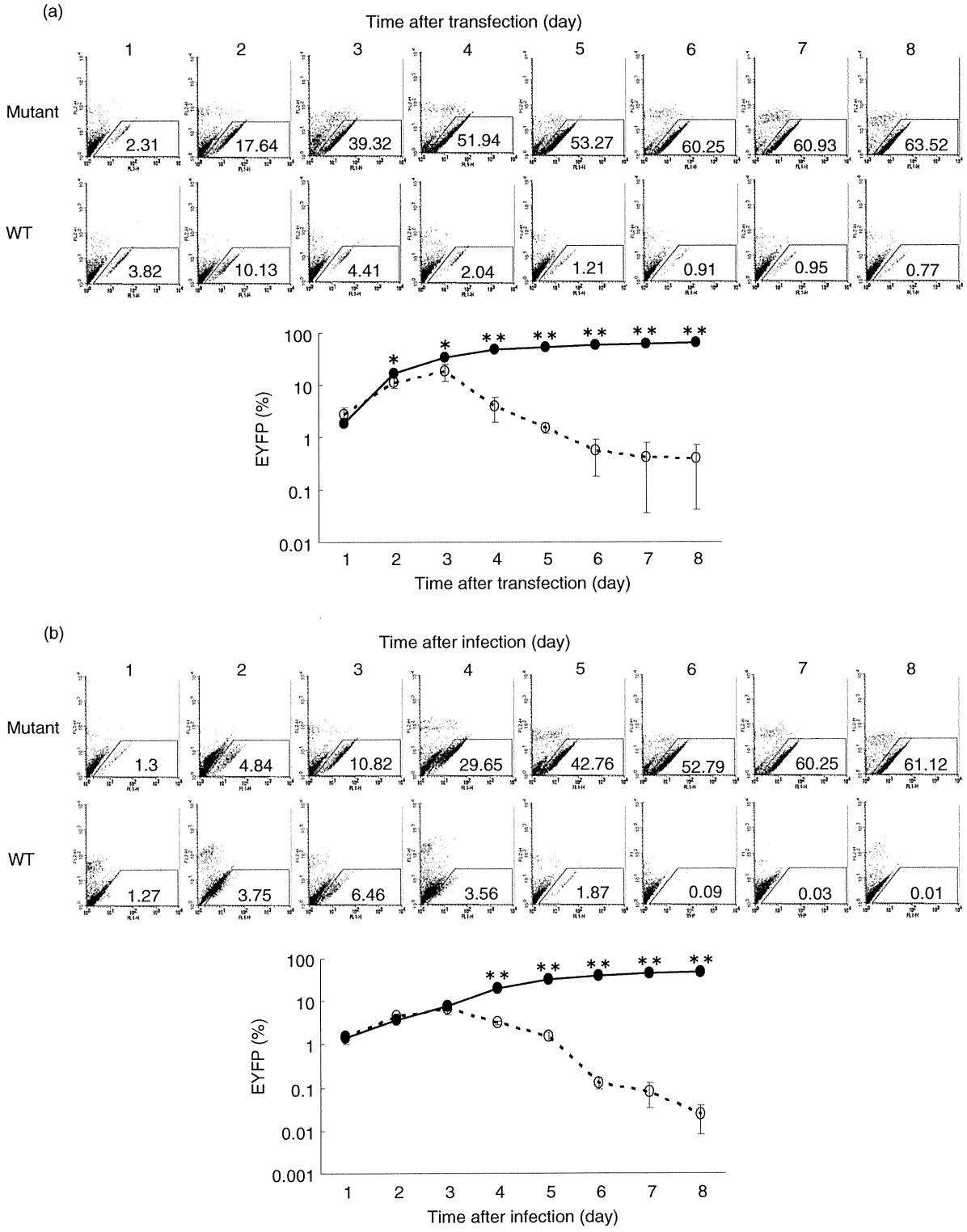
directly visualized by fluorescence microscopy on days 1–7. As shown in Figure 2(a), the number of cells positive for both JFH1-EYFP and JFH1-AsRed mutants, but not for JFH1-EYFP and JFH1-AsRed-infected cells, increased in a time-dependent manner. In JFH1-EFYP mutant-transfected cells, the proportion of EYFP-positive cells on days 3, 5 and 7 post-infection was 4.4%, 29% and 41%, respectively. In contrast, only 4.9% of JFH1-EYFP-transfected cells became EYFP-positive at 3 days post-infection, and the percentage of these fluorescence-positive cells decreased rapidly thereafter (Fig. 2b). Similarly, the percentage of cells infected with JFH1-AsRed mutant but not JFH1-AsRed increased exponentially. These results indicated that the two fluorescence virus clones with mutations are able to secrete infectious virus particles. We next compared levels of HCV core antigen in culture medium of cells infected with JFH1, JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, and JFH1-AsRed mutant viruses. The mutant viruses, but not the wild-type, produced amounts of core protein comparable to that of the parental JFH1 (Fig. 2C). In HCV-JFH1, JFH1-EYFP mutant, and JFH1-AsRed mutant-transfected cells, the core protein reached a peak of 1.25, 1.35 and 1.34 fmol/L, respectively, at 14 days post-transfection, while that of JFH1-EYFP JFH1-AsRed-transfected cells became undetectable at 10 days post-transfection. These results indicated that the mutant type is capable of producing an amount of viral particles comparable to that of the parental JFH1.

Using the two fluorescence-tagged viruses, we conducted co-infection of two virus strains, JFH1-AsRed mutant, which secreted infectious virus particles, and JFH1-EYFP, in which virus particle secretion was impaired. We collected culture media from cells transfected with JFH1-AsRed-mutant or JFH1-EYFP on day 2 post-transfection and infected both media onto uninfected Huh7.5.1 cells at a multiplicity of infection (moi, focus forming unit per cell) of 0.01. The number of JFH1-AsRed mutant-infected cells increased exponentially until day 3 but reached a plateau on days 5 and 7 post-infection. Interestingly, the number of cells positive for viral secretion-impaired JFH1-EYFP also increased in a manner similar to that of the JFH1-AsRed mutant (Fig. 3a). The percentage of AsRed mutant-positive cells was 4.6%, 6.7% and 14.8% at days 3, 5 and 7, respectively, while the percentage of EYFP-positive cells at the corresponding days was 3.1%, 4.8% and 5.1%, respectively (Fig. 3b). These results suggest that, in the co-culture of two HCV clones with and without virus particle secretion, a secretion-impaired virus clone is able to replicate and produce infectious particles possibly through the complementation of the intact virus.

Expression and subcellular localization of NS5A-fluorescence proteins

We next used fluorescence microscopy to study the subcellular localization of fluorescence and viral proteins. In cells transfected with JFH1-EYFP, JFH1-AsRed, and the respective mutants, EYFP and AsRed, were clearly visualized as dot-like structures in the perinuclear area (Fig. 4a). To determine if the NS5A-AsRed fusion protein indicates the subcellular localization of NS5A, we performed immunofluorescence staining of JFH-AsRed- and JFH1-AsRed mutant-infected cells using NS5A and HCV-core antibodies. Fluorescence of AsRed was co-localized precisely with NS5A and partially with core proteins. The fluorescence intensities of the JFH1-EYFP and -AsRed mutants within the cells were equal to that of the wild-type constructs. EYFP-NS5A of wild type and mutant JFH1 were localized in the ER (Fig. 4b). Western blotting was performed by using anti-GFP and anti-HCV-NS5A antibodies. As shown in Figure 4(c), three bands of the expected molecular weights of 27, 58 and 85 kDa, which corresponded to EGFP, NS5A and NS5A-EYFP fusion protein, were detected in EGFP, JFH1, JFH1-EYFP, JFH1-AsRed, JFH1-EYFP mutant, and JFH1-AsRed mutant-transfected cells. The expression levels of core protein in JFH1-EYFP mutant- and JFH1-AsRed mutant-transfected cells were almost the same as those transfected with parental JFH1. These results indicate

Figure 5 Kinetics of hepatitis C virus (HCV)-infected cells. (a) Huh7.5.1 cells were infected with JFH1-EYFP or JFH1-EYFP mutant HCV RNA. At the days indicated, cells were harvested and subjected to flow cytometry. EYFP-positive cells were sorted based on EYFP activating (*x*-axis) and staining with a marker of dead cells (*y*-axis). The results are depicted as density plots. The ratios of EYFP-positive cells vs time are shown below. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $*P < 0.05$. $**P < 0.01$. —•—, JFH1-EYFP mutant; -○-, JFH1-EYFP. (b) Culture media from JFH1-EYFP or mutant-transfected cells were added onto uninfected Huh7.5.1 cells at a moi of 0.01. At the days indicated, infected cells were analyzed using flow cytometry. The results are depicted as density plots. The ratio of EYFP-positive cells vs time are shown below. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $*P < 0.05$. $**P < 0.01$. WT, wild type. —•—, JFH1-EYFP mutant; -○-, JFH1-EYFP.



that the fusion proteins of NS5A and the fluorescent proteins remain intact within cells and serve as accurate markers of infection and as indicators of the subcellular localization of HCV-NS5A proteins.

Kinetics of HCV infection

Using those fluorescence-tagged HCV constructs, we analyzed more precisely the ratio and kinesics of HCV RNA-transfected cells and virus-infected cells by flow cytometry. After HCV RNA transfection, the percentages of JFH1-EYFP and JFH1-EYFP mutant-transfected cells were almost equivalent up to 2 days. Thereafter, JFH1-EYFP-positive cells began to decrease in number, while the mutant-transfected cells increased exponentially until 5 days post-transfection and then reached a plateau, when 52.2% of the cells were EYFP-positive (Fig. 5a). We collected the media from JFH1-EYFP and mutant-transfected cells at 2 days post-transfection, and added it onto uninfected Huh7.5.1 cells at a moi of 0.01. Similar to the results of the transfection assay, the population of JFH1-EYFP mutant-infected cells increased exponentially and reached a stable state at 6 days post-infection, when 39.2% of the cells were EYFP-positive (Fig. 5b). Calculating from the above data, the rate of expansion of HCV-infected cells was $2^{1.5}$/day. The cell-to-cell expansion of the JFH1-EYFP mutant infection was blocked by prior treatment of cells with anti-CD81 antibody (data not shown). This finding indicated that the expansion of the EGFP-positive cells was due to cell–cell spread of EYFP-tagged HCV and not the division of the virus-positive cells.

To further refine the calculation of the rate of cell-to-cell spread of infection, we carried out JFH1-EYFP mutant infection of uninfected Huh7.5.1 cells seeded at various densities from 2×10^3 to 2×10^5 cells/mL (Fig. 6). Flow cytometry showed that the rates of expansion of HCV-infected cells were $2^{1.5}$, $2^{2.3}$ and $2^{2.5}$/day at 2×10^3, 2×10^4 and 2×10^5 cells/cm^2, respectively. The ability of JFH1-EYFP to spread is greater in cells seeded at higher density. The maximum rate of expansion of HCV-infected cells was calculated as $2^{2.5}$/day.

Effects of antiviral drugs on HCV-infected cells

We next investigated the effects of antiviral agents on the infection kinetics of tagged-HCV. Eighteen hours after transfection of EYFP-tagged HCV RNA, the cells were treated with 10, 30 or 50 U/mL of IFN-α-2b or with 10 μM of protease inhibitor, BILN2061. JFH1-EYFP mutant-transfected cells were analyzed using flow

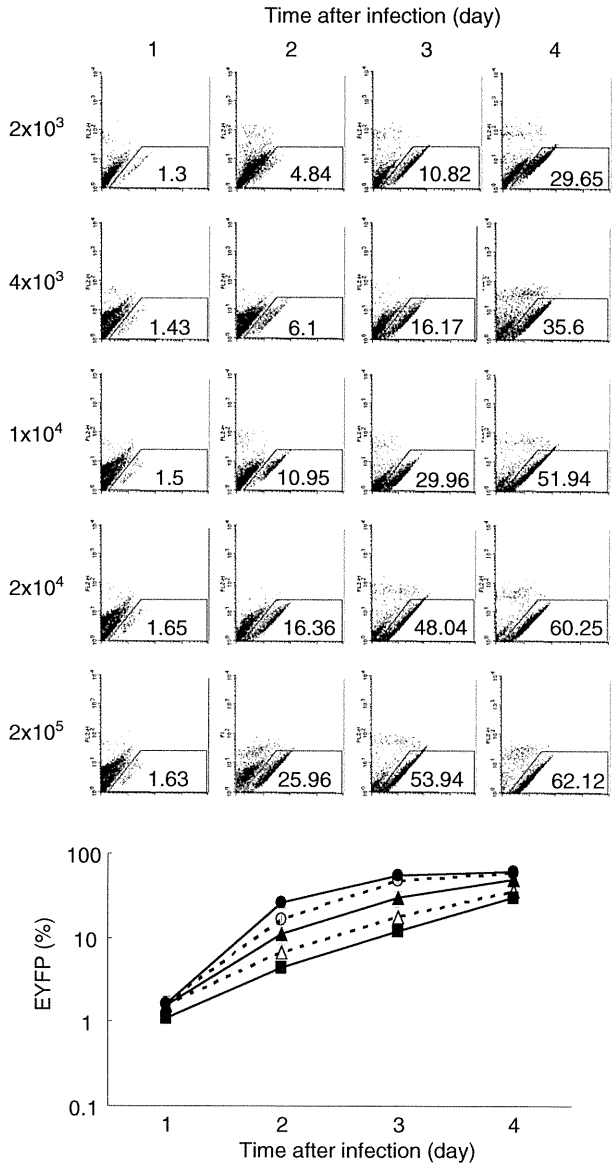


Figure 6 Rate of expansion of hepatitis C virus (HCV)-infected cells. The medium from JFH1-EYFP mutant was inoculated onto Huh7.5.1 cells seeded at different densities (2×10^3, 4×10^3, 1×10^4, 2×10^4 and 2×10^5 cells/cm^2) with core antigen adjusted doses. The results of flow cytometric analysis are depicted as density plots. The ratios of EYFP-positive cells vs time are shown beneath. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. —■—, 2×10^3; - -△- -, 4×10^3; —▲—, 1×10^4; - -◇- -, 2×10^4; —●—, 2×10^5.

cytometry. As shown in Figure 7, treatment of cells with the two compounds suppressed the time-dependent increase of HCV propagation. In addition, IFN-α-2b suppressed the dose-dependent increase of HCV

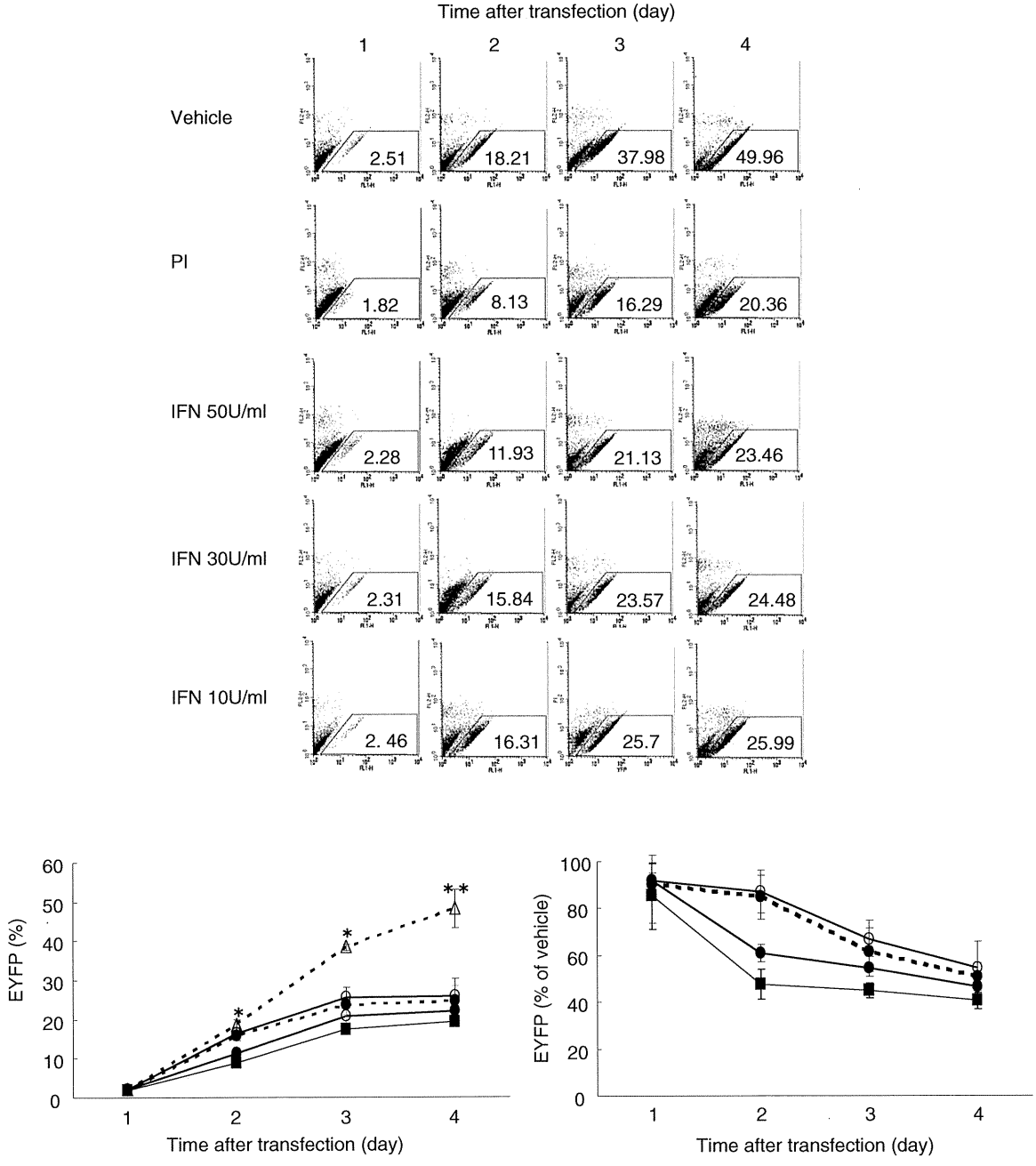


Figure 7 Effect of antiviral drugs on hepatitis C virus (HCV)-infected cells. Huh7.5.1 cells were transfected with JFH1-EYFP mutant RNA. Eighteen hours after transfection, cells were cultured with 10, 30 or 50 U/mL of interferon (IFN)-α-2b or 10 μM of the protease inhibitor BILN-2061. The cells were harvested at the days indicated and flow cytometry was performed. Ratios of EYFP-positive cells over time are shown at lower left. Plot values of 100% in each curve represent the EYFP expression levels in untreated cells (lower right). Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^*P < 0.05$. $^{**}P < 0.01$. PI, protease inhibitor. -☆-, vehicle; —●—, 50 U/mL; -●-, 30 U/mL; —○—, 10 U/mL; —■—, PI.

propagation. At all time points, the number of infected cells was significantly lower in the culture treated with the two compounds than in the untreated culture. The protease inhibitor suppressed infection faster than did IFN-α-2b. These data indicate that IFN and the protease inhibitor were not only able to suppress intracellular HCV replication levels but also to inhibit virus particle secretion and expansion of HCV-infected cell populations.

DISCUSSION

IN THIS STUDY, we used fluorescence-tagged HCV, in which virus assembly, particle secretion and re-infection functions are fully preserved (Fig. 1).[17] Utilizing the fluorescence-tagged HCV, we analyzed the rate of expansion of HCV infection using live-cell flow cytometric analyses (Figs 5–7). In the early periods of infection, the expansion of the virus-positive cell population increased exponentially and the maximum rate of expansion was calculated as $2^{2.5}$/day. It is not clear why HCV propagation reaches a plateau, but this observation, where HCV replication is limited in confluent cells, has been made previously.[26] Possible explanations include cell death due to over-confluence, depletion of the nucleoside triphosphate pools in resting cells, and/or cell cycle-dependent effects on virus RNA replication and translation.

Co-infection of the two virus clones, EYFP-JFH1 and AsRed mutant JFH1, showed that viruses with impaired particle secretion were able to replicate and expand virus-infected cells (Fig. 3). Although we have found no clear mechanism to explain those effects, we speculate that the secretion-defective virus (JFH1-EYFP) may assemble into infectious virus through *trans*-complementation of virus proteins via co-infection in a single cell or recombination of mutant and wild-type virus genomic RNA. The co-infection experiment showed that the increase of JFH1-AsRed mutant-positive cells was slower than in the single clone infection experiment (Fig. 2a). These findings suggest that viruses with impaired particle secretion (JFH1-EYFP) partially suppressed expansion of viruses with intact particle secretion (JFH1-AsRed mutant) through *trans*-suppression of cellular virus replication or competitive binding to cellular virus entry receptors.

After the development of HCV-JFH1 cell culture,[11] many variations of HCV cell culture systems have been developed. Lindenbach *et al.* developed a genotype 2a intragenotypic chimera, J6/JFH, in which the JFH1 structural region was replaced with that of J6, isolated from a patient with chronic hepatitis.[27] The J6/JFH chimera is able to produce virus particles more efficiently than JFH1 but does not produce virus-induced cytopathic effects (CPE). Several marker protein-tagged viruses have been reported, in which viral infection could be visualized readily in living cells. A subgenomic replicon that expressed an NS5A-GFP fusion protein was reported first.[12] However, the clone lacked the structural regions that are required for virus propagation. Subsequently, full-length HCV reporter viruses were developed in which the EGFP gene was inserted into the NS5A-C-terminus of JFH1[13] or JC-1.[14] Jones *et al.* inserted the *renilla* luciferase gene into P7 of J6/JFH.[15] Unfortunately, the efficiency of virus production by the recombinant reporter viruses was greatly reduced compared to wild-type viruses. Very recently, it has been reported that a JFH1-based adaptive strain of a HCV reporter virus can produce infectious HCV particles as robustly as the JFH1 wild-type strain.[17] This virus system has overcome the serious limitations associated with the use and application of other reporter viruses.

Compared with the other HCV reporter viruses, the JFH1-EYFP/AsRed mutant is capable of producing amounts of HCV virus equivalent to that of the parental JFH1, which enables continuous passage of infection in cell culture and analyses using various research modalities, including flow cytometry and live-cell microcopy. Considering the current situation regarding the lack of singly effective, proven antiviral agents against HCV, other than IFN formulations, the search for potential antiviral agents will continue to be a dominant goal of research to improve clinical anti-HCV chemotherapeutics. This tagged HCV culture system may provide a very convenient tool for studies of the complete virus life cycle in live cells and of virus–host interactions, and it may be useful for high-throughput screening of drugs.

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Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

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Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

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2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. ***J. Med. Virol.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon-λ3

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon-λ3 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN-α2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μg/kg subcutaneously once a week) or PEG-IFN-α2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μg subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w: 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the *IL28B* locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P*-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a ($P = 0.036$) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with $\geqq 80\%$ and $<80\%$ of RBV dose, $P = 0.021$, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years)[a]	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m^2)[a] (N = 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N = 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/μl)[b] (N = 94)	5,115 ± 1,630
Neutrophils (/μl)[b] (N = 94)	2,765 ± 1,131
Hemoglobin (g/dl)[b] (N = 95)	14.2 ± 1.3
Platelet count ($\times 10^{-3}$/μl)[b] (N = 98)	187 ± 95
ALT (IU/L)[b] (N = 95)	82 ± 78
Serum HCV-RNA level (log(IU/ml))[a,c]	6.2 (3.6–7.4)
Treatment duration (>16, ≦24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.
[a]Data are shown as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log(IU/ml)).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR = 0.170, $P = 0.019$).

TABLE II. Response Rates to Therapy

Character			Number/total number (%)
Overall			
RVR			68/112 (61)
ETR			125/129 (97)
SVR			98/129 (76)
Genotype	2a	2b	*P*-value
RVR	46/67 (69)	22/45 (49)	**0.036**
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.
Bold indicated *P*-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≧80%	<80%	*P*-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	**0.021**

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin.
The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy.
Bold indicated *P*-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	*P*-value
Genotype (2a/2b)		56/42	21/10
IL28B SNPs (rs8099917)			
TT/TG + GG	81/17	19/12	**0.024**
Age (years)[a]	56 (20–73)	61 (40–72)	**0.002**
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m^2)[a]	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/μl)[b]	5,318 ± 1,617	4,489 ± 1,540	**0.032**
Neutrophils (/μl)[b]	2,913 ± 1,139	2,278 ± 983	**0.021**
Hemoglobin (g/dl)[b]	14.2 ± 1.4	14.1 ± 1.1	NS
Platelet count (×10^{-3}/μl)[b]	193 ± 105	171 ± 54	NS
ALT (IU/ml)[b]	79 ± 73	94 ± 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml))[a,c]	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≧80%/<80%)	75/23	21/10	NS
RBV adherence (≧80%/<80%)	61/37	12/19	**0.024**
RVR/non-RVR	57/24	11/20	**0.001**

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.
[a]Data are show as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log (IU/ml)).
Bold indicated *P*-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	*P*-value
Regression analysis			
RVR	RVR	1	**0.019**
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≧80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG + GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.
Bold indicated *P*-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, $P = 0.002$, sustained virological response: 88% and 44% with IL28B major and hetero/minor, $P = 0.009$).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II), In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, $P = 0.044$), and no one achieved a rapid virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, $P = 0.630$, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, $P = 0.552$).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	*P*-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

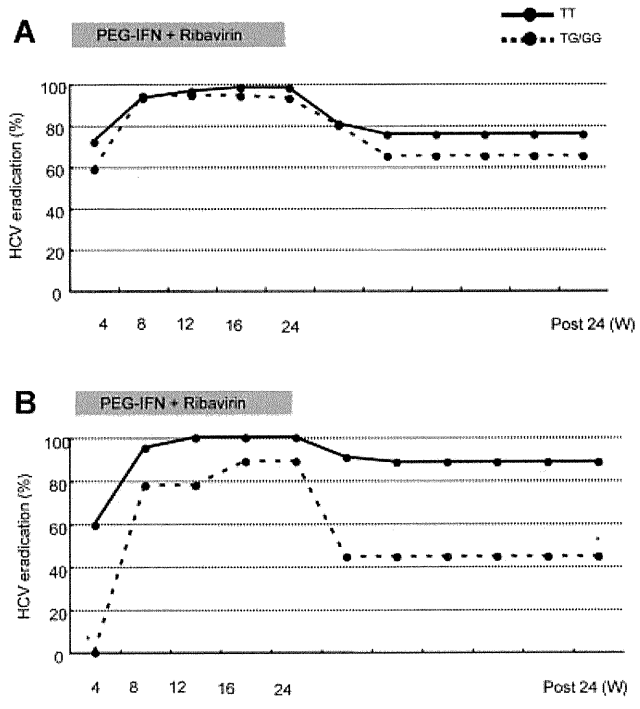

Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. *P*-values were two-tailed and those of less than 0.05 were considered to be statistically significant. $^{*}P < 0.01$.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment (n = 2), general fatigue (n = 2), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent in vitro study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate

analysis (Table IV). Regardless of the drug adherence, end of treatment response rates of patients infected with genotype 2 were around 94–99%, but the sustained virological response rates of the patients who received a total cumulative treatment dose of RBV of <80% was reduced significantly. As reported previously, increased RBV exposure during the treatment phase was associated with an increased likelihood of a sustained virological response [McHutchison et al., 2009] and these results confirm the importance of RBV in order to prevent relapse. Furthermore, host genetic variation leading to inosine triphosphatase (ITPA) deficiency protects against hemolytic anemia in chronic hepatitis C patients receiving RBV as revealed recently [Fellay et al., 2010]. We have reported also that the *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of RBV-induced anemia in Japanese patients and that the incidence of early dose reduction was significantly higher in patients with ITPA-major (CC) variant as expected and, more importantly, that a significant higher sustained virological response rate was achieved in patients with the *ITPA-hetero*/minor (CA/AA) variant with non-genotype 1 or low viral loads [Sakamoto et al., 2010].

A rapid virological response was extracted in this study as a factor associated with sustained virological response only by multivariate analysis. It has been reported recently that a rapid virological response is an important treatment predictor and that drug adherence, which is reported to affect the therapeutic efficacy in patients infected with genotype 1, had no impact on the both sustained and rapid virological responses in combination therapy for patients infected with genotype 2 [Inoue et al., 2010]. The reasons why several host factors useful for predicting the response to therapy in patients with genotype 1, such as gender, age, progression of liver fibrosis and IL28B polymorphism had no influence on the efficacy in patients with genotype 2, can be attributed to IFN-sensitive genotypes. Similarly, the other viral factors useful for predicting the response to therapy, such as viral load and amino acid substitutions in the Core and NS5A regions had no influence on treatment outcomes. In this study, patients who achieved a rapid virological response had a high sustained virological response rate, regardless of IL28B polymorphism in patients with genotype 2a but, interestingly, none of the IL28B-TG and -GG patients with genotype 2b achieved a sustained virological response (although there were nine IL28B-TG and -GG patients with genotype 2b, two could not be determined as rapid virological response because the times at which they became HCV-negative were not recorded clearly, being described as 4–8 weeks.) These results also suggest that patients with both genotype 2b and IL28B minor allele are refractory cases.

IL28B encodes a protein also known as IFN-λ3 [O'Brien, 2009]. *IL28A* (IFN-λ2) and *IL29* (IFN-λ1) are found adjacent to *IL28B* on chromosome 19. These three IFN-λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al., 2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN-λ, and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN-λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN-α induces expression of IFN-λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN-λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN-α and that the anti-HCV activity of either IFN-α or IFN-λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN-α and IFN-λ may play a key role in the suppression of HCV [O'Brien, 2009].

In conclusion, IL28B polymorphism is predictive of PEG-IFN plus RBV treatment outcomes in patients infected with genotype 2, and more remarkably with genotype 2b. These results suggest that IL-28B polymorphism affects responses to IFN-based treatment in more difficult-to-treat subpopulations of HCV patients, and that intersubgenotypic differences between genotype 2a and 2b are revealed by responses to PEG-IFN plus RBV treatment according to IL28B variants.

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The ESCRT System Is Required for Hepatitis C Virus Production

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Abstract

Background: Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for hepatitis C virus (HCV) production. However, the mechanisms of HCV assembly, budding, and release remain poorly understood. Retroviruses and some other enveloped viruses require an endosomal sorting complex required for transport (ESCRT) components and their associated proteins for their budding process.

Methodology/Principal Findings: To determine whether or not the ESCRT system is needed for HCV production, we examined the infectivity of HCV or the Core levels in culture supernatants as well as HCV RNA levels in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, expressing short hairpin RNA or siRNA targeted to tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2 interacting protein X (Alix), Vps4B, charged multivesicular body protein 4b (CHMP4b), or Brox, all of which are components of the ESCRT system. We found that the infectivity of HCV in the supernatants was significantly suppressed in these knockdown cells. Consequently, the release of the HCV Core into the culture supernatants was significantly suppressed in these knockdown cells after HCV-JFH1 infection, while the intracellular infectivity and the RNA replication of HCV-JFH1 were not significantly affected. Furthermore, the HCV Core mostly colocalized with CHMP4b, a component of ESCRT-III. In this context, HCV Core could bind to CHMP4b. Nevertheless, we failed to find the conserved viral late domain motif, which is required for interaction with the ESCRT component, in the HCV-JFH1 Core, suggesting that HCV Core has a novel motif required for HCV production.

Conclusions/Significance: These results suggest that the ESCRT system is required for infectious HCV production.

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Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. HCV Core, a highly basic RNA-binding protein, forms a viral capsid and is targeted to lipid droplets [2–6]. The Core is essential for infectious virion production [7]. NS5A, a membrane-associated RNA-binding phosphoprotein, is also involved in the assembly and maturation of infectious HCV particles [8,9]. Intriguingly, NS5A is a key regulator of virion production through the phosphorylation by casein kinase II [9]. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [4]. Indeed, NS5A is known to colocalize with the Core on lipid droplets [5], and the interaction between NS5A and the Core is critical for the production of infectious HCV particles [3]. However, the host factor involved in HCV assembly, budding, and release remains poorly understood.

Budding is an essential step in the life cycle of enveloped viruses. Endosomal sorting complex required for transport (ESCRT) components and associated factors, such as tumor susceptibility gene 101 (TSG101, a component of ESCRT-I), charged multivesicular body protein 4b (CHMP4b, a component of ESCRT-III), and apoptosis-linked gene 2 interacting protein X (ALIX, a TSG101- and CHMP4b-binding protein), have been found to be involved in membrane remodeling events that accompany endosomal protein sorting, cytokinesis, and the budding of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1) [10–12]. The ESCRT complexes I, II, and III are sequentially, or perhaps concentrically recruited to the endosomal membrane to sequester