30 nM using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. Target sequences of the siRNAs were: occludin (5′-GCAAGAUCACUAUGAGACA-3′), SR-BI (5′-GAGCUUUGGCCUUGGUCUA-3′), CD81 (5′-CUGUGAUCAUGAUCUUCGA-3′), CHC (5′-CUAGCUUUGCACAGUUUAA-3′), Dyn2 (5′-CCCUCAAGGAGGCGCUCAA-3′), Cav1 (5′-CCCUAAACACCUCAACGAU-3′), flotillin-1 (5′-CCUAUGACAUCGAGGUCAA-3′), Arf6 (5′-CAGUUCUUGGUAAAGUCCU-3′), CtBP1 (5′-GACUCGACGCUGUGCCACA-3′) and PAK1 (5′-GCAUCAAUUCCUGAAGAUU-3′). Target sequences of the siRNAs for claudin-1, PI4K and scrambled negative control were as described previously (Suzuki *et al.*, 2013).

Immunoblotting. Cells were washed with PBS and incubated with passive lysis buffer (Promega). Lysates were sonicated for 10 min and added to the same volume of 2× SDS-PAGE sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE and then transferred to PVDF membranes (Merck Millipore). After blocking, membranes were probed with primary antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) in accordance with the manufacturer's protocols.

Flow cytometry. Cultured cells detached by treatment with trypsin were incubated with anti-CD81 antibody or anti-mouse IgG antibody for 1 h at 4 °C. After being washed with PBS containing 0.1 % BSA, cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h at 4 °C, washed repeatedly and resuspended in PBS. Analyses were performed using a FACSCalibur system (Becton Dickinson).

Reagents and antibodies. Bafilomycin A1 was obtained from Wako Pure Chemical Industries. Alexa Fluor 488-conjugated transferrin was obtained from Invitrogen. For immunoblotting, anti-SR-BI (NB400-104; Novus Biologicals), anti-occludin (71-1500; Invitrogen), anti-claudin-1 (51-9000; Invitrogen), anti-Dyn2 (ab3457; Abcam), anti-Cav1 (N-20; Santa Cruz Biotechnology), anti-flotillin (H-104; Santa Cruz Biotechnology), anti-Arf6 (ab77581; Abcam) and anti-PAK1 (2602; Cell Signaling Technology) rabbit polyclonal antibodies; anti-CD81 (JS-81; BD Biosciences), anti-β-actin (AC-15; Sigma-Aldrich), anti-CHC (23; BD Biosciences), anti-GRAF1 (SAB1400439; Sigma-Aldrich) and anti-glyceraldehyde 3-phosphate dehydrogenase (6C5; Merck Millipore) mouse mAb; and anti-CtBP1 goat polyclonal antibody (C-17; Santa Cruz Biotechnology) were used. For immunofluorescence staining, anti-CHC mAb (X22) and anti-HA rat polyclonal antibody (3F10) were obtained from Thermo Scientific and Roche Applied Science, respectively. Anti-NS5A antibody was a rabbit polyclonal antibody against synthetic peptides. Alexa Fluor 488- or 555-labelled secondary antibodies were obtained from Invitrogen.

DNA transfection. Cell monolayers were transfected with plasmid DNA using TransIT-LT1 transfection reagent (Mirus) in accordance with the manufacturer's instructions.

Treatment of cells with bafilomycin A1 and cell viability. Cells were preincubated with various concentrations of bafilomycin A1 for 60 min at 37 °C. Preincubated cells were then infected with HCVtcp. Cells treated with 0.1 % DMSO were used as controls. Cell viability was analysed by the Cell Titre-Glo Luminescent Cell Viability Assay (Promega).

Uptake of transferrin. Cells were grown on glass coverslips. After cells were transfected with HA-tagged Dyn2 expression plasmids, Alexa Fluor 488-conjugated transferrin at 20 μg ml^{-1} was added and incubated for 30 min. Cells were washed with PBS and fixed in 4 % paraformaldehyde.

Immunofluorescence analysis. Huh7.5.1 and Huh-7 cells were fixed with 4 % paraformaldehyde in PBS for 30 min, and were then blocked and permeabilized with 0.3 % Triton X-100 in a non-fat milk solution (Block Ace; Snow Brand Milk Products) for 60 min at room temperature. Samples were then incubated with anti-CHC, anti-Dyn2, anti-Cav1, anti-NS5A or anti-HA for 60 min at room temperature, washed three times with PBS, and then incubated with secondary antibodies for 60 min at room temperature. Finally, samples were washed three times with PBS, rinsed briefly in double-distilled H_2O and mounted with DAPI mounting medium. The signal was analysed using a Leica TCS SPE confocal microscope.

Luciferase assay. For quantification of FLuc activity in HCVtcp-infected cells, cells were lysed with passive lysis buffer (Promega) at 72 h post-infection. FLuc activity of the cells was determined using a luciferase assay system (Promega). For quantification of GLuc activity in supernatants of HCVtcp-infected cells, the *Renilla* Luciferase Assay System (Promega) was used. All luciferase assays were performed at least in triplicate.

Quantification of HCV core protein. HCV core protein was quantified using a highly sensitive enzyme immunoassay (Lumipulse G1200; Fujirebio) in accordance with the manufacturer's instructions.

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Evaluation of single-round infectious, chimeric dengue type 1 virus as an antigen for dengue functional antibody assays

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ABSTRACT

Dengue fever and dengue hemorrhagic fever are endemic throughout tropical and subtropical countries. Four serotypes of dengue viruses (DENV-1 to DENV-4), each with several genotypes including various subclades, are co-distributed in most endemic areas. Infection-neutralizing and -enhancing antibodies are believed to play protective and pathogenic roles, respectively. Measurement of these functional antibodies against a variety of viral strains is thus important for evaluating coverage and safety of dengue vaccine candidates. Although transportation of live virus materials beyond national borders is increasingly limited, this difficulty may be overcome using biotechnology that enables generation of an antibody-assay antigen equivalent to authentic virus based on viral sequence information. A rapid system to produce flavivirus single-round infectious particles (SRIPs) was recently developed using a Japanese encephalitis virus (JEV) subgenomic replicon plasmid. This system allows production of chimeric SRIPs that have surface proteins of other flaviviruses. In the present study, SRIPs of DENV-1 (D1-SRIPs) were evaluated as an antigen for functional antibody assays. Inclusion of the whole mature capsid gene of JEV into the replicon plasmid provided higher D1-SRIP yields than did its exclusion in cases where a DENV-1 surface-protein-expressing plasmid was used for co-transfection of 293T cells with the replicon plasmid. In an assay to measure the balance between neutralizing and enhancing activities, dose (antibody dilution)-dependent activity curves in dengue-immune human sera or mouse monoclonal antibodies obtained using D1-SRIP antigen were equivalent to those obtained using DENV-1 antigen. Similar results were obtained using additional DENV-2 and DENV-3 systems. In a conventional Vero-cell neutralization test, a significant correlation was shown between antibody titers obtained using D1-SRIP and DENV-1 antigens. These results demonstrate the utility of D1-SRIPs as an alternative antigen to authentic DENV-1 in functional antibody assays. SRIP antigens may contribute to dengue vaccine candidate evaluation, understanding of dengue pathogenesis, and development of serodiagnostic systems.

1. Introduction

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most globally important mosquito-borne viral diseases [1,2]. The World Health Organization estimates that 50–100 million infections, including 500,000 DHF cases and approximately 12,500 deaths, occur annually [3]. Vaccines and specific antivirals are currently unavailable. DF and DHF are caused by any of four types of dengue viruses (DENVs) generally designated as serotypes DENV-1 to DENV-4. All of these serotypes are currently co-distributed in most tropical and subtropical areas worldwide [4]. Additionally, each of the four serotypes has 4–6 distinct genotypes with subclades that are locally distributed in various areas and countries [5]. Moreover, introduction of foreign DENV strains occurs in many areas, sometimes accompanied by increases in the number of patients or higher proportions of severe cases (DHF) [6–11]. Furthermore, a new DENV serotype genetically and serologically distinct from the current four serotypes has also recently been discovered [12]. Potential thus exists for human exposure to a variety of DENV strains.

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Several dengue vaccine candidates have been developed and are currently being evaluated in clinical trials [13,14]. All of these candidates are able to induce neutralizing antibody in humans. Neutralizing antibody contributes to reduction in viremia levels and is believed to be an important factor in disease protection [15]. Most neutralizing antibody species against DENVs display infection-enhancing activity in sub-neutralizing doses in vitro [16]; there is consequent concern that neutralizing antibody-inducible dengue vaccine may cause antibody-dependent enhancement (ADE) of infection if insufficient neutralizing antibody levels are induced by vaccination [17,18]. ADE, the mechanism most likely responsible for increased viremia levels, is a process in which monocytes are efficiently infected in an Fc gamma receptor (FcγR)-mediated manner [19]. Measurement of vaccine-induced neutralizing and enhancing antibodies is therefore important for dengue vaccine evaluation. Because of their potential human infectivity, various DENV strains are required as antigens in the antibody assays.

Despite these requirements, transportation of live virus materials beyond national borders is increasingly limited owing to current regulations, such as governmental security export control policies [20] as well as access and benefit-sharing restrictions of the Convention on Biological Diversity [21]. These limitations may be overcome using biotechnology that enables the generation of material equivalent to the authentic virus based on viral full-genome nucleotide sequence information. Although the technique to construct an infectious clone of DENV has already been established [22–25], the method is arduous and thus not practical for preparation of various antigens for antibody assays.

A novel system to generate flavivirus single-round infectious particles (SRIPs) has recently been established [26]. This method exploits a Japanese encephalitis virus (JEV) subgenomic replicon plasmid lacking coding regions of capsid (C), pre-membrane (prM), and envelope (E) structural proteins. SRIPs are produced by co-transfection of this replicon plasmid with a plasmid expressing JEV structural proteins into 293T cells. As a DNA-based production system, this method facilitates simple and rapid antigen generation. Most importantly, chimeric SRIPs have also been produced using a plasmid expressing structural proteins of other flaviviruses (e.g., dengue, yellow fever, and tick-borne encephalitis viruses), although production levels of chimeric SRIPs derived from DENVs have been much lower than those derived from JEV and other flaviviruses. The successful production of flavivirus SRIPs in this system suggests its potential utility for functional antibody assays, as SRIP surface antigens can be theoretically designed based on prM and E coding region nucleotide sequences.

The purpose of the present study was to evaluate the utility of DENV-1 SRIPs (D1-SRIPs) as an antigen for neutralizing and enhancing antibody assays. Results of assays using dengue-immune human serum samples or mouse monoclonal antibodies (MAbs) against DENV-1 demonstrated that antibody levels obtained using D1-SRIP antigen were equivalent to those obtained using authentic DENV-1 antigen. These results indicate that D1-SRIPs can serve as an alternative functional antibody assay antigen to DENV-1. The use of DENV antigens in the form of SRIPs for neutralizing and enhancing antibody assays may thus be suitable for dengue vaccine candidate evaluation.

2. Materials and methods

2.1. Cells

Human embryonic kidney 293T cells (CRL-3216; American Type Culture Collection [ATCC], Manassas, VA) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Vero, C6/36, and K562 cells and their culture media have been described previously [27]. All cells were cultivated in a humidified atmosphere of 5% CO_2–95% air at 37 °C, except for C6/36 cells, which were cultivated at 28 °C.

2.2. Viruses

The Mochizuki strain of DENV-1, New Guinea C (NGC) strain of DENV-2, and H87 strain of DENV-3 were used [28]. Culture fluids harvested from infected C6/36 cells were used as viral antigens in neutralization tests and in an assay to measure the balance between neutralizing and enhancing antibodies.

2.3. Antibodies

Human serum samples previously collected from general patients aged 29–71 years in Indonesia during 1999–2001 and stored at −20 °C [29] were used as antibody specimens for evaluating SRIP antigen. Each of these sera had detectable neutralizing antibody titers against all four DENV serotypes (Supplementary Table 1). As a negative control, we used a human serum sample collected from a residence in a non-dengue-endemic country (Japan) that showed no detectable neutralizing activities against any DENV serotypes. Heat inactivation of sera was performed at 56 °C for 30 min. The use of human serum samples was approved by the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University. MAbs specific for DENV-1 (D1-IV-7F4) or crossreactive to all DENV serotypes (D1-III-9B1, D1-IV-3B8, D1-V-3H12, D1-V-8E8 [30], and JE-10B4 [31]) and D1-4G2 (E-specific, flavivirus group-crossreactive; HB-112, ATCC) in an ascites form were also used for evaluating SRIP antigen. Mouse MAb JE-2D5, specific for JEV non-structural protein 1 (NS1) [32], was used for immunostaining.

2.4. Plasmids

Plasmids pCMV-JErep, pCAG-JEC [26], pcD1ME, pcD2ME, and pcD3ME [28] have been described previously (Fig. 1A). Briefly, pCMV-JErep is a JEV replicon plasmid designed to transcribe viral RNA in transfected cells, and is the full genome of JEV Nakayama strain (GenBank no. EF571853) except lacking 2238 nucleotides (positions 150–2387) corresponding to main portions of C and E and the entire prM gene. pCAG-JEC is an expression plasmid for JEV (Nakayama) mature C, consisting of 105 amino acids, while pcD1ME, pcD2ME, and pcD3ME are expression plasmids for prM and E of DENV-1 (Mochizuki), DENV-2 (NGC), and DENV-3 (H87), respectively. In this study, a portion of C in pCMV-JErep was replaced by the full JEV mature-C region to construct the new replicon plasmid pCMV-JErep-fullC, which was consequently lacking 1971 nucleotides (positions 438–2408) corresponding to a portion of C not responsible for synthesis of mature C, the full prM and a major portion of E (Fig. 1A).

2.5. Preparation of SRIPs

293T cells in a 6-well-plate well were co-transfected with 1 μg each of two plasmids, pCMV-JErep-fullC and pcD1ME (Set II of Fig. 1A), using Lipofectamine LTX and Plus reagent (Invitrogen, Gaithersburg, MD) following the manufacturer's instructions. Culture fluids harvested on days 3–7 served as D1-SRIP antigen in neutralization tests using Vero cells and in an assay to measure the balance between neutralizing and enhancing antibodies using K562 cells. For titration of D1-SRIPs on K562 cells, serial dilutions of D1-SRIPs (50 μl/well) prepared in 96-well poly-L-lysine-coated plates were mixed with 5×10^4 semi-adherent K562 cells (50 μl/well). The mixture was incubated at 37 °C for 2 days, followed by fixation and immunochemical staining (see below). Infective titers

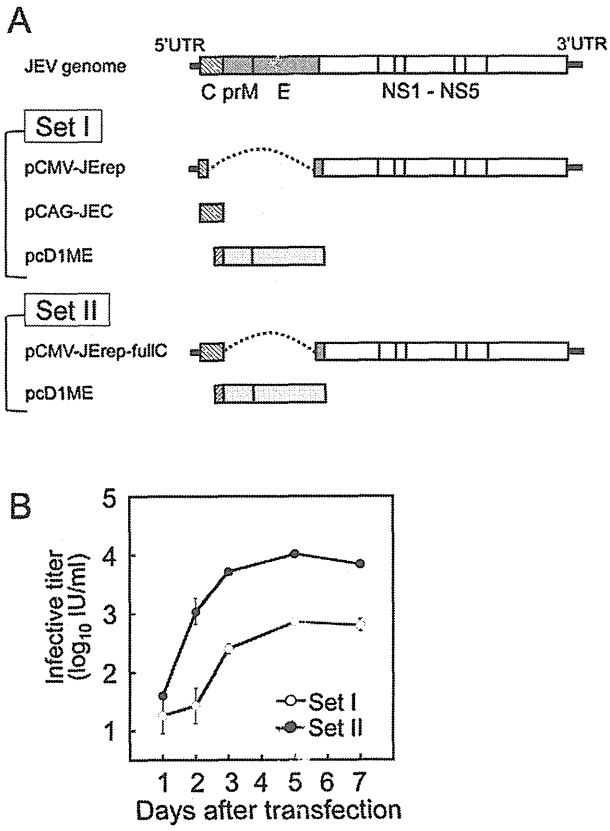

Fig. 1. Generation of D1-SRIPs. (A) Schematic diagram of the JEV genome and DENV-1 genes used to generate D1-SRIPs. pCMV-JErep, pCAG-JEC, and pCMV-JErep-fullC were constructed based on the JEV genome, while pcD1ME was based on DENV-1 genes. Dotted lines show portions deleted from the JEV genome: nucleotide positions 150–2387 for pCMV-JErep and 438–2408 for pCMV-JErep-fullC. Sets I and II refer to combinations of plasmids used for transfection of 293T cells. (B) Time course of D1-SRIP yield. 293T cells grown in 6-well plates were co-transfected with 1 μg of each plasmid in Sets I (3 μg total; open circles) or II (2 μg total; closed circles), and culture fluids were harvested 1, 2, 3, 5, and 7 days after transfection. Infective titers in the culture fluids were measured using K562 cells. Each datum represents an average of values obtained in three wells, with SDs indicated by bars.

were determined by counting the number of infected cells and were expressed as infectious units per ml (IU/ml). SRIPs of DENV-2 (D2-SRIPs) and DENV-3 (D3-SRIPs) were prepared and titrated essentially by the same method used for D1-SRIPs.

2.6. Immunostaining

Immunochemical staining of Vero cells and semiadherent K562 cells was performed essentially as described previously [33]. Briefly, fixed cells were serially incubated with primary antibody (mouse MAb), biotinylated anti-mouse IgG, avidin–biotinylated peroxidase complex (ABC) reagents, and VIP substrate (Vector Laboratories, Burlingame, CA). To determine DENV-infected cells, D1-4G2 was used as a primary antibody to stain DENV E antigen. To determine SRIP-infected cells where no dengue antigens were produced, JE-2D5 was used to stain JEV NS1 antigen produced in cells infected with SRIPs containing all non-structural protein genes of JEV.

2.7. Measurement of the balance between neutralizing and enhancing activities

The balance between neutralizing and enhancing activities was measured using semi-adherent K562 cells essentially in the same way as described previously [33]. Briefly, serial dilutions of antibody specimens (36 μl/well) prepared in 96-well poly-L-lysine-coated plates were mixed with D1-SRIP or DENV-1 antigens (50 μl/well) in the absence or presence of rabbit complement at a final concentration of 5%. Equivalent infectious titers of D1-SRIPs (2×10^3 IU/ml) and DENV-1 (2×10^3 FFU/ml) were used. The virus-antibody mixture was incubated at 37 °C for 2 h and then mixed with 5×10^4 K562 cells (50 μl/well). The virus-antibody-cell mixture was incubated at 37 °C for 2 (for DENV-1) or 3 (for D1-SRIPs) days. After fixation and immunochemical staining, foci (for DENV-1) or infected cells (for D1-SRIPs) were counted under a microscope. Cut-off values for neutralizing and enhancing activities were calculated from the mean plus or minus three times the standard deviation (SD) obtained using eight negative controls adjusted for approximately 1×10^2 infected cells. Assays measuring neutralizing and enhancing antibody balance using DENV-2, DENV-3, D2-SRIP, and D3-SRIP antigens were performed essentially by the same method as described above for DENV-1 and D1-SRIP antigens.

2.8. Neutralization tests

Conventional Vero-cell plaque reduction neutralization tests were performed with DENV-1 essentially as described previously [28], except for the use of a 96-well plate. Serial dilutions of antibody specimens (36 μl/well) prepared in 96-well plates were mixed with D1-SRIP or DENV-1 antigens (50 μl/well) with or without 5% rabbit complement and incubated at 37 °C for 2 h. The virus-antibody mixture was incubated at 37 °C for 2 h and then mixed with 2.5×10^4 Vero cells (50 μl/well). The virus-antibody-cell mixture was incubated at 37 °C for 3 days, fixed, and immunochemically stained. Neutralizing activity was expressed as the percent reduction in plaque number compared with the control without an antibody specimen. Neutralization tests using D1-SRIP antigen were performed exactly by the same method as those using DENV-1 antigen, excepting that the number of infected cells was used for calculating the percent reduction. The antibody dilution showing a 50% reduction in the number of plaques (for DENV-1 antigen) or infected cells (for D1-SRIP antigen) was obtained using the FORECAST function in Microsoft Excel and expressed as PRNT50.

3. Results

3.1. Increased D1-SRIP yield using a modified replicon plasmid

In our previous study [26], chimeric D1-SRIPs were produced using pCAG-JEC expressing JEV C and pcD1ME expressing DENV-1 prM-E to complement a replicon plasmid lacking C-prM-E genes, pCMV-JErep (Set I in Fig. 1A). However, the yield of D1-SRIPs was too low for use in neutralization tests and assays to measure the balance between neutralizing and enhancing antibodies. To seek higher yields, a new replicon plasmid (pCMV-JErep-fullC) was constructed. This plasmid contained the whole mature C gene of JEV and allowed us to produce D1-SRIPs solely by complementation with pcD1ME (Set II in Fig. 1A). Sets I and II were consequently used for transfection of 293T cells and comparison of D1-SRIP yields.

In both Sets I and II, the highest infective titer was detected in culture fluid 5 days after transfection. The titer obtained with Set II ($\approx 10^4$ IU/ml) was higher than that obtained with Set I ($\approx 10^3$ IU/ml; Fig. 1B). This result suggested that the incorporation of the entire JEV C gene into the replicon plasmid increased yields of chimeric D1-SRIPs. Transfection with Set II gave yields of more than 5.2×10^3 IU/ml on days 3, 5 and 7. Culture fluids harvested 3–7 days after the Set II transfection were therefore used for subsequent evaluations. The single-round infection nature of D1-SRIPs

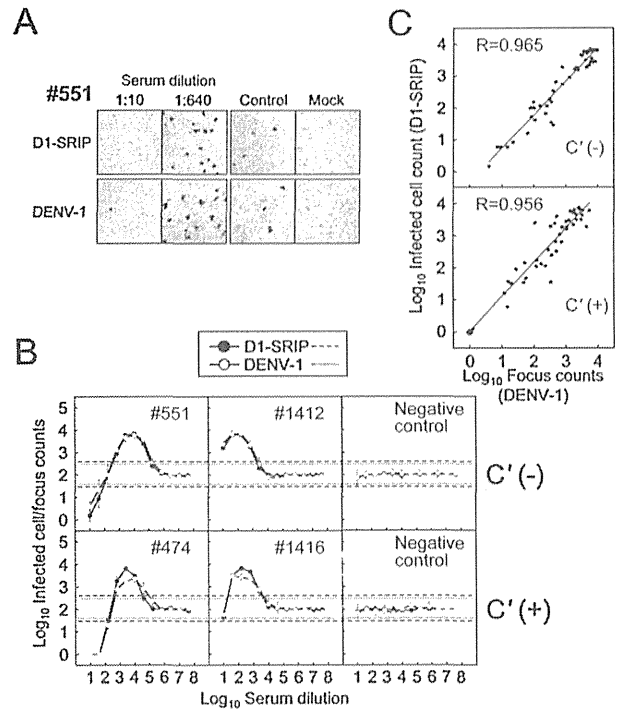

Fig. 2. Evaluation of D1-SRIP antigen in an assay to measure the balance between neutralizing and enhancing antibodies using dengue-immune human serum samples. (A) Micrograph of K562 cells infected with D1-SRIPs or DENV-1. Cells infected with D1-SRIP (upper panels) or DENV-1 (lower panels) were immunostained with monoclonals JE-2D5 and D1-4G2, respectively. D1-SRIP or DENV-1 antigen was incubated with human serum sample no. 551 at a dilution of 1:10 or 1:640 in the absence of complement prior to infection (leftmost and second-left panels) or a control human serum sample negative for neutralizing antibodies against any DENV serotypes (second-right panels). The rightmost panels show results of mock infection. Dark staining is the result of specific immunoreactivity of D1-SRIP-infected cells with JE-2D5 antibody (upper panels) and DENV-1-infected cells with D1-4G2 antibody (lower panels). (B) Dose-dependent antibody activity curves. Curves were obtained using D1-SRIP (closed circles) and DENV-1 (open circles) antigens with four indicated serum samples and a negative control serum in the absence (C′ [−]; upper panels) or presence (C′ [+]; lower panels) of complement. The ordinate indicates numbers of infected cells or foci for D1-SRIP or DENV-1 antigens, respectively. Each datum represents an average of values obtained in two separate assays with SDs indicated by error bars. Dashed and dotted lines indicate cut-off values for differentiating between neutralizing and enhancing activities in assays using D1-SRIP and DENV-1 antigens, respectively. (C) Correlation between D1-SRIP and DENV-1 antigens. Individual data from eight serum samples at dilutions of 1:10–1:10,240 are plotted on the graph, with ordinate and abscissa indicating data obtained using D1-SRIP and DENV-1 antigens, respectively. Upper and lower panels correspond to comparisons in the absence and presence of complement, respectively. Solid lines are linear regression lines, with *R*-values corresponding to correlation coefficients.

was confirmed by the absence of infectivity in culture fluid of Vero or K562 cells infected with D1-SRIPs.

3.2. Neutralizing/enhancing antibody balance-assay evaluation of D1-SRIP antigen

The utility of D1-SRIP antigen in measurement assays of the balance between neutralizing and enhancing antibodies was evaluated using dengue-immune human serum samples. The assay system used semi-adherent K562 cells bearing FcγR, thus allowing us to measure neutralizing or enhancing antibody activities by comparing infected cell (for D1-SRIP antigen) or focus (for DENV-1 antigen) counts with those obtained without antibody. Smaller and larger counts corresponded to neutralizing and enhancing activities, respectively. Fig. 2A shows a micrograph of K562 cells infected with either D1-SRIPs or DENV-1.

As an example, the upper panels of Fig. 2B show dose (antibody dilution)-dependent antibody activity curves observed for two sera (samples no. 551 and no. 1412) in the absence of complement. In sample no. 551, neutralizing and enhancing activities were detected in dilution ranges of 1:10–1:40 and 1:640–1:40,960, respectively, in assays using either D1-SRIP or DENV-1 antigens. Similarly, curves obtained using D1-SRIP and DENV-1 antigens in sample no. 1412 were equivalent to one another. This result suggests that D1-SRIP antigen functions similarly to DENV-1 antigen in this assay system. D1-SRIP and DENV-1 antigens were also compared using another two sera (samples no. 474 and no. 1416) in the presence of complement (lower panels of Fig. 2B). Similar dose-dependent antibody activity curves were also obtained using either antigen. To further evaluate D1-SRIP antigen, four additional human serum samples were used. Data obtained using all eight samples at serum dilutions of 1:10–1:10,240 were plotted on a scatter graph (Fig. 2C). A significant correlation was observed between D1-SRIP and DENV-1 antigens in the absence and presence of complement, with correlation coefficients of 0.965 ($P<0.001$) and 0.956 ($P<0.001$), respectively.

To corroborate the results obtained using human serum samples, mouse MAbs were also used for comparison of D1-SRIP and DENV-1 antigens. Four MAbs reactive to DENV-1 (D1-IV-7F4, D1-III-9B1, D1-V-3H12, and D1-4G2) were subjected to the same assay system in the absence (Fig. 3A) or presence (Fig. 3B) of complement. Equivalent dose-dependent antibody activity curves were generated using D1-SRIP and DENV-1 antigens in all MAbs, both in the absence and presence of complement. Data obtained using six MAbs (including the four above-tested ones) at dilutions of $1:10^1$–$1:10^5$ were plotted on a scatter graph (Fig. 3C). Highly significant correlations were observed between D1-SRIP and DENV-1 antigens in the absence ($R=0.985$; $P<0.001$) or presence ($R=0.986$; $P<0.001$) of complement. These results imply that D1-SRIPs can serve as an alternate antigen to DENV-1 in assays measuring the balance between neutralizing and enhancing antibodies.

3.3. Neutralization-test evaluation of D1-SRIP antigen

The performance of D1-SRIP antigen in a conventional Vero-cell neutralization test was subsequently evaluated. Fig. 4A shows dose-dependent percent inhibition curves obtained with two sera (samples no. 551 and no. 1412) and two MAbs (D1-IV-7F4 and D1-4G2) when complement was absent from the virus-antibody mixture. Equivalent curves were obtained using DENV-1 and D1-SRIP antigens. To further evaluate D1-SRIP antigen, PRNT50s obtained with eight serum samples and six MAbs using DENV-1 and D1-SRIP antigens in the presence or absence of complement were plotted on a scatter graph (Fig. 4B). A significant correlation coefficient ($R=0.919$; $P<0.001$) was obtained between both antigens. This result indicates that D1-SRIP antigen can be used in place of authentic DENV-1 antigen in Vero-cell neutralization tests.

3.4. Evaluation of D2- and D3-SRIP antigens

To expand the utility of SRIP antigens for dengue functional antibody assays, D2- and D3-SRIPs were evaluated for their suitability as antigens in an assay to measure the balance between neutralizing and enhancing antibodies using K562 cells. Infected cell/focus counts obtained from four serum samples (no. 553, no. 1142, no. 1417, and no. 1703) at dilutions of 1:10–1:10,240 and four mouse MAbs reactive to DENV-2 and DENV-3 (D1-III-9B1, D1-V-3H12, D1-4G2, and JE-10B4) at dilutions of 1:10–$1:10^5$ were plotted on scatter graphs (Fig. 5). A significant correlation was observed between D2-SRIP and DENV-2 antigens in serum samples and mouse MAbs, with correlation coefficients of 0.943 ($P<0.001$)

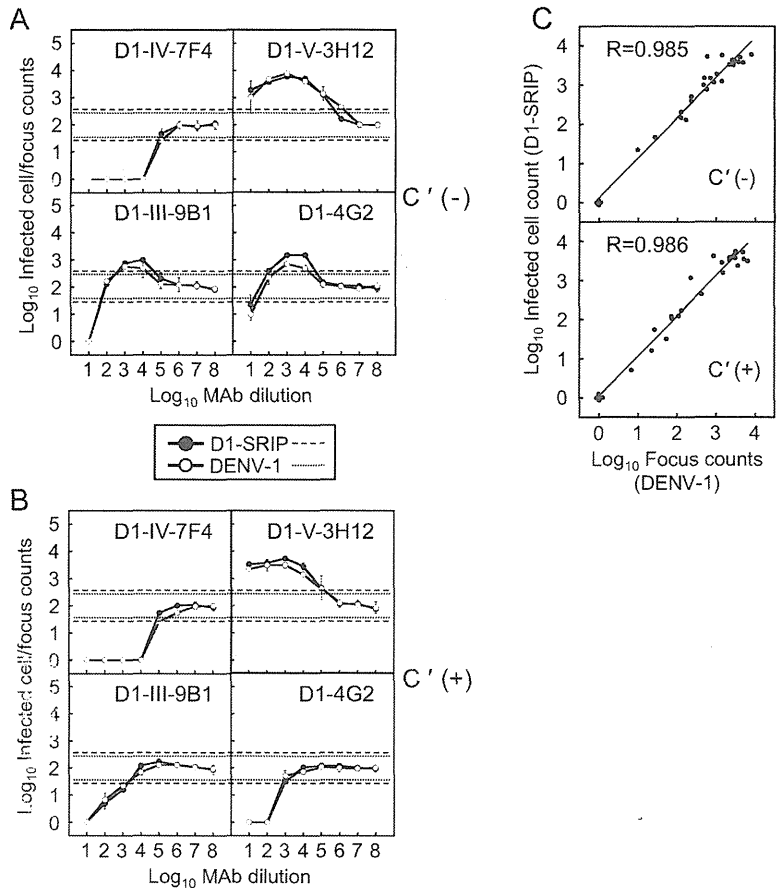

Fig. 3. Evaluation of D1-SRIP antigen in an assay to measure the balance between neutralizing and enhancing antibodies using mouse monoclonal antibodies (MAbs). ((A) and (B)) Dose-dependent antibody activity curves. Curves were obtained using D1-SRIP (closed circles) and DENV-1 (open circles) antigens with four indicated MAbs in the absence (A) or presence (B) of complement. The ordinate indicates numbers of infected cells or foci for D1-SRIP or DENV-1 antigens, respectively. Each datum represents an average of values obtained in two separate assays with SDs indicated by error bars. Dashed and dotted lines indicate cut-off values for differentiating between neutralizing and enhancing activities in assays using D1-SRIP and DENV-1 antigens, respectively. (C) Correlation between D1-SRIP and DENV-1 antigens. Individual data from six mouse MAbs at dilutions of $1:10^1$–$1:10^5$ are plotted on the graph, with ordinate and abscissa indicating data obtained using D1-SRIP and DENV-1 antigens, respectively. Upper and lower panels show comparisons in the absence and presence of complement, respectively. Solid lines are linear regression lines, with *R*-values corresponding to correlation coefficients.

and 0.935 ($P<0.001$), respectively. Furthermore, significant correlations were observed between D3-SRIP and DENV-3 antigens using serum samples ($R=0.975$; $P<0.001$) or mouse MAbs ($R=0.927$; $P<0.001$). These results suggest that D2- and D3-SRIPs can serve as alternate antigens to corresponding authentic viruses (DENV-2 and DENV-3) in assays measuring neutralizing and enhancing antibody balance.

4. Discussion

The first conducted efficacy trial of a dengue vaccine candidate found no significant protection against DENV-2, even though vaccinated volunteers induced neutralizing antibody against DENV-2 according to a conventional Vero-cell neutralization test [34]. To understand the discrepancy between neutralizing antibody levels and protective efficacy, the improvement of antibody assay systems has been suggested as a possible solution [35]. Compared with the use of non-expressing CV-1 cells, the use of Fcγ-receptor-expressing CV-1 cells in an assay system reduced neutralizing antibody titers in dengue-immune sera [36]. Moi et al. [37] further showed that Fcγ-receptor-expressing BHK-21 cells provided better reflection of in vivo antibody activity in patient sera than did a non-expressing control. We have previously used Fcγ-receptor-bearing, semi-adherent K562 cells to measure the balance of neutralizing and enhancing antibody activities [33]. The combination of these antibody assay systems with SRIP antigens should be a powerful tool to evaluate vaccine-induced antibody responses against antigens of a variety of serotype/genotype strains distributed worldwide.

Transfection with a plasmid expressing flavivirus prM-E genes is known to produce nucleocapsid-free, empty subviral extracellular particles (EPs) in mammalian cells [38,39]. EPs are useful as antigens in antibody binding assays, e.g., enzyme-linked immunosorbent assays [40,41]. In contrast, flavivirus SRIPs are generated by transfection with a replicon plasmid in addition to a plasmid expressing prM-E genes. SRIPs, which are infectious antigens, can be used in functional assays, which provide more important information on the protective role of antibodies than do binding assays. Furthermore, an infectious form increases antigen sensitivity; EP antigens, in fact, can be used only when the production level is as high as that of JEV.

Higher yields of D1-SRIPs were obtained using a combination of two (Set II; Fig. 1A) than three plasmids (Set I). Set II included the replicon plasmid pCMV-JErep-fullC, which contained the entire mature C. Potential reasons for this difference are: (i) increased efficiency of co-transfection in the system using a fewer number

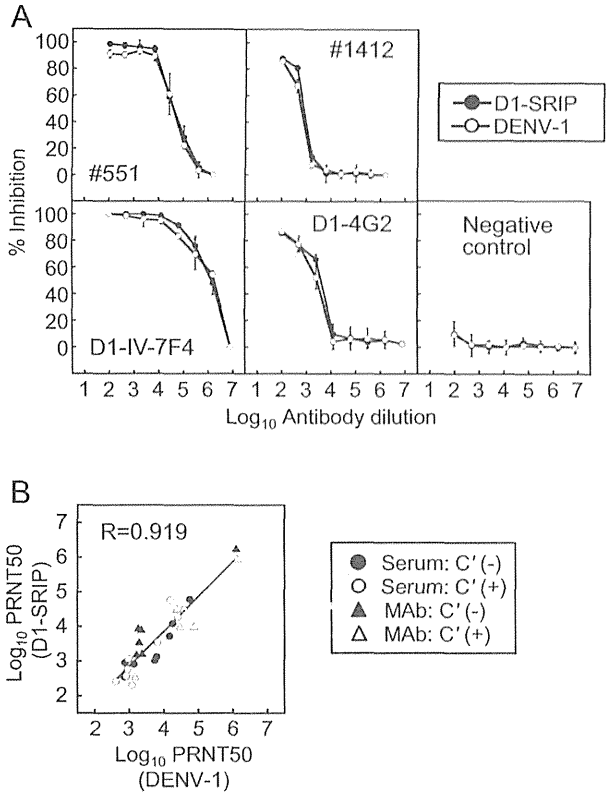

Fig. 4. Evaluation of D1-SRIP antigen performance in a conventional Vero-cell neutralization test. (A) Dose-dependent percent inhibition curves. Curves were obtained using D1-SRIP (closed circles) and DENV-1 (open circles) antigens with two indicated human serum samples (no. 551 and no. 1412), two mouse MAbs (D1-IV-7F4 and D1-4G2), and a human negative control serum (Negative control). All displayed results were obtained in the absence of complement. Each datum represents an average of values obtained in two separate assays, with SDs indicated by error bars. (B) Correlation between D1-SRIP and DENV-1 antigens. Individual PRNT50s obtained from eight human sera (open and closed circles) and six MAbs (open and closed triangles) in the absence (closed circles and triangles) or presence (open circles and triangles) of complement are plotted on the graph, with ordinate and abscissa indicating data obtained using D1-SRIP and DENV-1 antigens, respectively. The solid line is a linear regression line, with the *R*-value corresponding to the correlation coefficient.

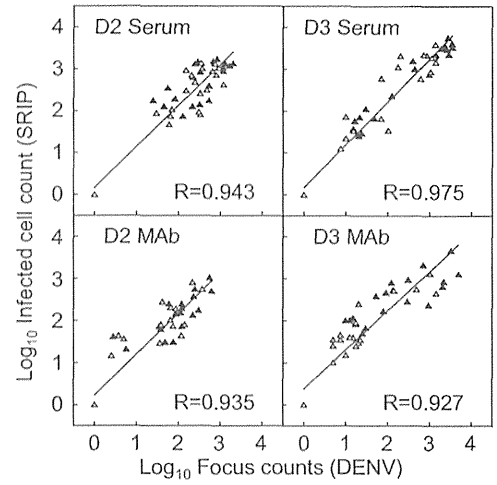

Fig. 5. Correlation between SRIP and DENV antigens in DENV-2 and DENV-3 systems. Individual infected cell/focus counts obtained in an assay to measure neutralizing and enhancing antibody balance using four serum samples at dilutions of 1:10–1:10,240 (upper panels) and four mouse MAbs at dilutions of 1:10–1:10^5 (lower panels) in the absence (closed triangles) or presence (open triangles) of complement are plotted on the graph. Left panels show data obtained using D2-SRIP and DENV-2 antigens; right panels indicate data obtained using D3-SRIP and DENV-3 antigens. Solid lines are linear regression lines, with *R*-values corresponding to correlation coefficients.

of plasmids; and (ii) increased packaging efficacy for production of nucleocapsids in the Set II system, where the C protein is synthesized in close proximity to the subgenomic viral RNA. Detailed investigations should uncover the molecular mechanisms required for efficient packaging and virion particle formation in flaviviruses.

Although dose-dependent antibody activity curves obtained using D1-SRIPs and DENV-1 antigens were equivalent overall, the number of infected cells obtained using D1-SRIP antigen was greater than the number of foci obtained using DENV-1 antigen in some antibody samples. This situation occasionally occurred in an antibody dilution range showing enhancing activity, e.g., sample no. 474 at 1:2560, sample no. 1416 at 1:160–1:640 (Fig. 2B), and D1-4G2 at 1:160–1:640 (Fig. 3A). One potential explanation for this phenomenon is underestimation of the number of plaques formed by DENV-1. While one D1-SRIP infectious unit forms only a single infected cell, a unit of DENV-1 produces a focus composed of more than one infected cell in a well of limited size. In this case, infectious unit counts obtained using D1-SRIP antigen are considered more accurate.

In conclusion, our study has demonstrated the utility of D1-SRIPs as an alternative antigen to authentic DENV-1 in functional antibody assays to measure neutralizing and enhancing activities. These antibody activities are important for evaluating coverage and safety of dengue vaccine candidates. SRIPs that cannot propagate in cells and thus may not be pathogenic to humans can be prepared more safely and securely than infectious clones. Furthermore, SRIP antigens can be easily produced by co-transfection with two plasmids, one of which contains the prM and E genes responsible for virion surface antigens. Based on nucleotide sequence information available in gene databases such as GenBank, production of SRIP antigens of a variety of serotype/genotype/subclades is simpler than production of an infectious clone or even importation of the infectious virus itself. SRIP antigens may not only assist vaccine development, but may also contribute to an understanding of dengue pathogenesis and the development of serodiagnostic systems.

Conflict of interest statement

None of the authors have a conflict of interest in relation to the content of the present work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.06.017.

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Single Strain Isolation Method for Cell Culture-Adapted Hepatitis C Virus by End-Point Dilution and Infection

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Abstract

The hepatitis C virus (HCV) culture system has enabled us to clarify the HCV life cycle and essential host factors for propagation. However, the virus production level of wild-type JFH-1 (JFH-1/wt) is limited, and this leads to difficulties in performing experiments that require higher viral concentrations. As the cell culture-adapted JFH-1 has been reported to have robust virus production, some mutations in the viral genome may play a role in the efficiency of virus production. In this study, we obtained cell culture-adapted virus by passage of full-length JFH-1 RNA-transfected Huh-7.5.1 cells. The obtained virus produced 3 log-fold more progeny viruses as compared with JFH-1/wt. Several mutations were identified as being responsible for robust virus production, but, on reverse-genetics analysis, the production levels of JFH-1 with these mutations did not reach the level of cell culture-adapted virus. By using the single strain isolation method by end-point dilution and infection, we isolated two strains with additional mutations, and found that these strains have the ability to produce more progeny viruses. On reverse-genetics analysis, the strains with these additional mutations were able to produce robust progeny viruses at comparable levels as cell culture-adapted JFH-1 virus. The strategy used in this study will be useful for identifying strains with unique characteristics, such as robust virus production, from a diverse population, and for determining the responsible mutations for these characteristics.

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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality [1,2]. HCV is a positive-stranded RNA virus belonging to the Flaviviridae family. Its genome, about 9.6-kb long, consists of an open reading frame (ORF) encoding a large polyprotein that is cleaved by cellular and viral proteases into at least 10 structural and non-structural (NS) proteins [3,4]. The structural proteins include core, E1 and E2, which form virus particles. The NS proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, which are associated with viral replication.

For research into the HCV life cycle and development of antivirals, *in vitro* models of this virus are indispensable. First, an HCV subgenomic replicon system was used to examine HCV replication in cell culture [5,6]. The HCV infectious step has been assessed by an HCV pseudo-particle (HCVpp) system harboring E1 and E2 glycoproteins [7,8]. This system enabled us to identify several HCV receptors. Finally, to investigate other steps in the HCV life cycle, an HCV cell culture system was developed with a unique genotype 2a strain JFH-1 [9]. This strain is able to replicate efficiently in cultured cells, and its characteristics enabled us to observe the whole life cycle of this virus in cell culture by using cell-culture generated HCV (HCVcc) [10–12].

By modifying this system with CD81-lacking HuH-7-derived cells, we established a novel system designated the single cycle virus production assay, and this enabled us to estimate the efficiency of each step of viral replication, infectious virus production, secretion and infection [13–16]. However, virus production levels of wild-type JFH-1 (JFH-1/wt) in these systems are limited, and this shortage sometimes leads to difficulties in experiments that require high viral concentrations. To overcome these shortcomings, recent studies have identified several adaptive or compensatory mutations that enhance viral production of JFH-1 [17–24]. The contributions of these mutations to the viral life cycle are not well defined. In this study, we isolated the cell culture-adapted JFH-1 virus, which that can efficiently produce progeny viruses by serial passaging of JFH-1 transfected Huh-7.5.1 cells, and evaluated the affected steps in the viral life cycle.

Materials and Methods

Cell Culture

The HuH-7-derived cell lines Huh-7.5.1, provided by Francis Chisari (Scripps Research Institute, La Jolla, CA), and Huh7-25, which lacks CD81 expression, were cultured at 37°C in a 5% CO2 environment using Dulbecco's Modified Eagle's Medium contain-

ing 10% fetal bovine serum [11,25]. 293T cells were also kept under the same conditions.

Plasmid Construction and RNA Transfection

Mutation-introduced JFH-1 variants were prepared by site-directed mutagenesis with appropriate primers. The methods of *in vitro* RNA synthesis and electroporation were described previously [26,27].

Quantification of HCV RNA and Core Antigen

Total RNA was extracted from 140 μL of culture medium or from harvested cell pellets, and the real-time quantitative RT-PCR was performed to determine the HCV RNA titer as described previously [28]. The concentration of total RNA in the cells was also measured. The concentration of HCV core antigen (Ag) in culture medium and cell lysates were measured by the Lumipulse Ortho HCV Ag kit (Ortho Clinical Diagnostics, Tokyo, Japan) [29].

Titration of HCV Infectivity

The infectivity titers of HCV were measured by indirect immunostaining as described previously [27]. The infectivity titer was expressed as focus-forming units (FFU) per mL. The intracellular infectivity and specific infectivity titer were determined as described previously [14].

HCV Pseudo-Particles Assay

HCV pseudo-particles (HCVpp) containing E1 and E2 glycoproteins of wild-type or mutation-introduced JFH-1 were produced as described previously [7,8]. To adjust the amount of virus, copy number of packaged luciferase reporter RNA was quantified by real-time detection PCR with primers and probe as reported previously [30].

HCV Trans-complemented Particles Assay

Generation and infection of HCV trans-complemented particles (HCVtcp) has been reported elsewhere [31–33]. Briefly, the RNA polymerase I-driven JFH-1 reporter replicon plasmid (pHH/SGR-Luc) and the CAG promoter-driven JFH-1 core – NS2 expression plasmid (pCAGC-NS2_JFH1) or T416N mutation in the E2 region introduced pCAGC-NS2/JFH1 (pCAGC-NS2_JFH1/T416N) were co-transfected into Huh-7.5.1 cells. Culture medium was harvested at 6 days after transfection, and was passed through a 0.45- μM pore-size filter for infection. To adjust the amount of virus, RNA in culture medium was extracted with the QIAamp Viral RNA kit, treated with DNase (TURBO DNase; Ambion, Austin, TX), and purified with an RNeasy Mini kit using on-column DNase digestion (QIAGEN). Copy number of HCV was then measured as described previously [34]. Generated viruses were infected into naïve Huh-7.5.1 cells, and cells were harvested at 72 h for analysis of luciferase activity.

HCV Sequencing

Total RNA was extracted from culture medium, and cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, CA) with random 6-mer primer. Synthesized cDNA was subsequently amplified by nested-PCR covering almost the entire open reading frame and part of the 5′-untranslated region with TaKaRa LA *Taq* DNA polymerase (Takara Bio, Shiga, Japan), as described previously [14], and the sequence of amplified fragments was determined directly.

Density gradient analysis

The culture medium of JFH-1 and variants -transfected cells were layered on top of 10–40% iodixanol gradient and centrifuged for 16 h at 40,000 rpm, 4°C in an SW-41 rotor. Fractions were collected from the top of gradient, and the density, HCV core Ag and infectivity titer in each fraction was measured.

Statistical Analysis

Experiments were performed in triplicate, and obtained data are expressed as means ± standard deviation. Statistical analysis was performed by Student's t-test. The p values of less than 0.05 are considered to be statistically significant.

Results

Isolation of Cell Culture-adapted JFH-1

In order to obtain cell culture-adapted JFH-1, we passaged full-length JFH-1 RNA-transfected Huh-7.5.1 cells and monitored extra- and intra-cellular HCV RNA and infectivity of culture medium. At 25 days after transfection, HCV RNA and infectivity titer in culture medium peaked (Figure 1A). To assess cell-culture adaptation, we compared the progeny virus production levels by infection with the same amount of viruses harvested at day 5 (JFH-1/day5) and at day 25 (JFH-1/day25). The intra- and extra-cellular HCV RNA titers of JFH-1/day25-infected cells were $1.42\times10^7\pm3.49\times10^6$ copies/ μg RNA and $7.66\times10^7\pm3.61\times10^7$ copies/mL, respectively, which was 3 log-fold higher than those of JFH-1/day5-infected cells (Figure 1B).

Responsible Mutations in Cell Culture-adapted JFH-1 virus

In order to identify the responsible mutations introduced in the cell culture-adapted virus (JFH-1/day25), we directly sequenced the virus ORF. As indicated in Table 1, we identified 3 non-synonymous mutations at E2 (T416N), NS3 (K1122R) and NS5B (L2525F). To assess the effects of these mutations on HCV propagation, we generated the JFH-1 full-genome constructs with these mutations solely (JFH-1/T416N, JFH-1/K1122R and JFH-1/L2525F) or in combination (JFH-1/3mut). In the transfection assay with full-length HCV RNAs transcribed from these constructs, HCV core Ag in culture medium of JFH-1/K1122R and JFH-1/3mut transfected cells was approximately 1 log-fold higher than that of JFH-1/wt and other variants transfected cells (Figure 2A). HCV core Ag in cells was highest in JFH-1/3mut RNA-transfected cells, followed by JFH-1/K1122R. In the infection study of these variants (multiplicity of infection (MOI) = 0.1), the HCV RNA titer in culture medium of JFH-1/3mut infected cells was highest among these variants and JFH-1/wt, but was approximately 2 log-fold lower than that of cell culture-adapted JFH-1 virus, JFH-1/day25. The intra-cellular HCV RNA titer of culture-adapted JFH-1 virus infected cell was also higher than that of JFH-1/3mut or other variants infected cells (Figure 2B).

In order to assess the function of these mutations on steps of the virus lifecycle, we used the single cycle virus production assay using Huh7–25 cells, which lacks surface expression of CD81. We compared the intra-cellular HCV RNA titer of these variants transfected Huh7–25 cells in order to assess the effects of mutations on HCV replication. The intra-cellular HCV RNA titer of JFH-1/L2525F was lower than that of other variants and JFH-1/wt (Figure 3A). To assess the effects of mutations on infectious virus production in culture-cells and the efficiency of infection, we compared the specific infectivity of these variants in transfected Huh7–25 cells. The mutations K1122R and L2525F

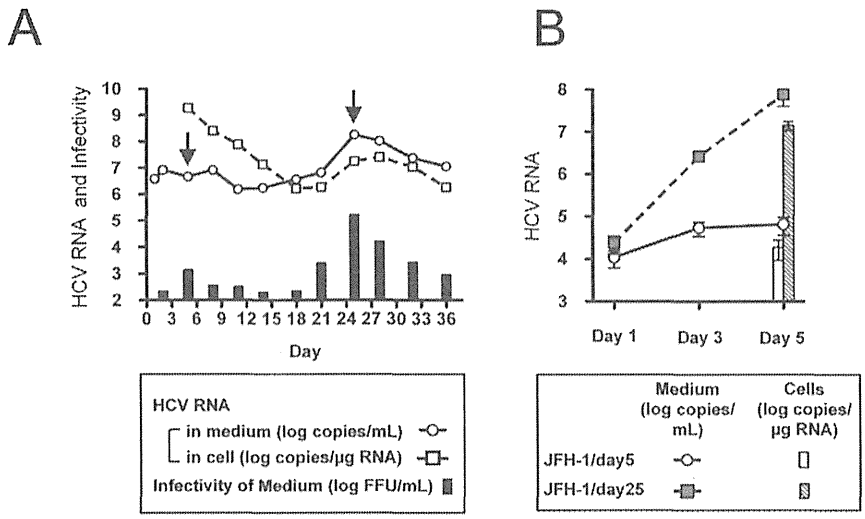

Figure 1. Isolation of cell culture-adapted JFH-1. (A) Long-term culture of JFH-1-transfected cells. The full-genome JFH-1 RNA was transfected into Huh-7.5.1 cells, and transfected cells were passaged for 3 to 4 days. HCV RNA titers in culture medium and cells were monitored. Arrows indicate the harvest points used for the infection study. (B) Production of progeny by infection with viruses harvested at day 5 and day 25 after transfection. The same amount of virus was used for infection at a multiplicity of infection (MOI) of 0.5, and HCV RNA titer was monitored.
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enhanced intra-cellular specific infectivity by 7.2- and 3.7-fold, respectively, although extra-cellular specific infectivity was not affected, thus suggesting their contribution to intra-cellular infectious virus production. JFH-1/3mut containing 3 mutations also showed an 8.5-fold increase in intra-cellular specific infectivity (Figure 3B). On the other hand, JFH-1/T416N-transfected cells showed 2-fold higher intra- and extra-cellular infectivity, as compared with JFH-1/wt and other variants. JFH-1/3mut also showed enhanced extra-cellular infectivity in addition to the effects of K1122R and L2525F (Figure 3B). These data suggest that

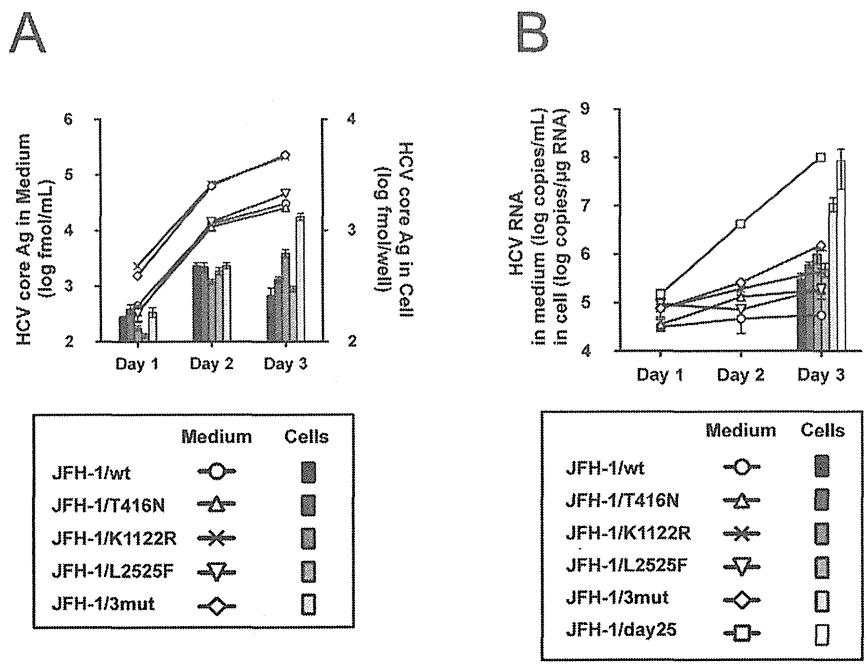

Figure 2. Effects of cell culture-adapted mutations on virus propagation. (A) One million cells were transfected with 2 μg of *in vitro*-transcribed RNA from JFH-1/wt, JFH-1/T416N, JFH-1/K1122R, JFH-1/L2525F and JFH-1/3mut. HCV propagation was monitored by measuring HCV core Ag. (B) The same amounts of JFH-1/wt, JFH-1/T416N, JFH-1/K1122R, JFH-1/L2525F, JFH-1/3mut and JFH-1/day25 viruses were used for infection of naïve Huh-7.5.1 cells (MOI = 1.0), and HCV RNA titers were subsequently monitored.
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Table 1. Mutations Detected in Cell Culture-adapted JFH-1 Variants.

Region	Identified mutation		JFH-1/day25	2G	6B
	Nucleotide	Amino Acid[a]			
E1	C1198T	–	+	+	+
E2	C1587A	T416N	+	+	+
p7	T2612G	L758V		+	
	T2641A	H767Q			+
NS3	A3631G	–			+
	A3705G	K1122R	+	+	+
	G3715A	–			+
	A4294G	I1318M			+
	C5182T	–			+
NS5A	G7069A	–			+
	G7658C	V2440L		+	+
NS5B	C7913T	L2525F	+	+	+
	G8458C	–		+	
	C8932T	–	+	+	+
	A9235G	–	+	+	+

[a]'–' means synonymous mutation.
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T416N enhances the infection step. To assess the biophysical properties of particles with T416N, we analyzed the culture medium of JFH-1/wt- and JFH-1/T416N- transfected cells in the density gradient. However, the density gradient profiles of these strains were similar and we could not detect the mutation specific peak of infectivity in the density gradient of JFH-1/T416N. The peak density of infectivity titer of JFH-1/T416N (1.05 g/mL) was almost identical with that of JFH-1/wt (1.07 g/mL), but the peak infectivity titer of JFH-1/T416N was 1.75-fold higher than that of JFH-1/wt (Figure 4). To confirm the advantage of T416N in the infection step, we exploited the HCVpp system. T416N was introduced into JFH-1 E1 and E2 glycoprotein-expressing vector and generated HCVpp harboring envelope proteins of JFH1/wt and JFH1/T416N. To adjust the amount of HCVpp, the copy number of packaged luciferase reporter RNA was measured. The same copy numbers of HCVpp JFH-1/wt and JFH-1/T416N were infected into naïve Huh-7.5.1 cells and luciferase activities were compared. In contrast to expectations, luciferase activity in JFH-1/T416N HCVpp-infected cells was lower than in JFH-1/wt HCVpp-infected cells (Figure 5A). We also examined the effects of T416N using the recently developed HCVtcp system. This HCVtcp contains the HCV subgenomic replicon and supports single-round infection. In contrast to the HCVpp system, we were able to observe consistent results with single cycle virus production assay in the HCVtcp system. Luciferase activity in JFH-1/T416N HCVtcp-infected cells was 2.8-fold higher than in JFH-1/wt HCVtcp-infected cells (Figure 5B).

Single Strain Isolation Method of Cell Culture-adapted Virus by End-point Dilution and Infection

In order to isolate the JFH-1 variants that can produce more progeny viruses, the JFH-1/day25 virus was diluted and infected into naïve Huh-7.5.1 cells seeded in a 96-well plate at a concentration of 1 FFU per well. After 72-h culture, media were kept in another plate and cells were fixed and stained with anti-HCV core antibody to visualize the foci. Culture media were then harvested from wells that contained a single focus and were used to re-infect naïve Huh-7.5.1 cells. The production of progeny viruses were compared by measuring the HCV RNA titer of infected cells (Figure 6). Inoculation with harvested media resulted in varied progeny virus production. HCV RNA titers in culture medium were 4 to 7 log copies/mL on day 3 after infection.

Among the assessed variants, we selected two strains, 2G and 6B, which showed the highest virus production. Direct sequencing of these isolated strains revealed that they possessed more non-synonymous mutations, in addition to those observed in JFH-1/3mut. The 2G strain had 2 additional mutations; L758V at p7 and V2440L at NS5A. The 6B strain had 3 additional mutations; H767Q at p7, I1318M at NS3 and V2440L at NS5A (Table 1). We generated JFH-1 variants with these mutations and designated them as JFH-1/2G and JFH-1/6B. When transfected with full-genome RNA, extracellular HCV core Ag was approximately 50-fold and 10-fold higher when compared with JFH-1/wt and JFH-1/3mut, respectively, and intracellular HCV core Ag was approximately 10-fold and 2-fold higher when compared with JFH-1/wt and JFH-1/3mut, respectively (Figure 7A).

In an infection study at MOI = 0.1, the variants JFH-1/2G and JFH-1/6B produced more progeny viruses than JFH-1/wt and JFH-1/3mut, and the production levels were comparable to that of JFH-1/day25 (Figure 7B). To assess the effects of additional introduced mutations in these variants on the virus life cycle, we used a single cycle virus production assay. After transfection of full-length RNA from JFH-1/wt, JFH-1/3mut, JFH-1/2G and JFH-1/6B into Huh7–25 cells, the intra-cellular HCV RNA titer was compared. We found that JFH-1/2G-transfected cells showed 1.35-fold higher intracellular HCV RNA titer, thus suggesting enhanced viral replication (Figure 8A).

Next, specific infectivities were compared. Consistent with previous data, JFH-1/3mut showed enhanced intra- and extra-cellular specific infectivity, as compared with JFH-1/wt, and was approximately 8-fold and 2-fold, respectively (Figure 8B). The strains JFH-1/2G and JFH-1/6B also indicated enhanced intra-cellular specific infectivity, 27.8- and 43.7-fold higher when

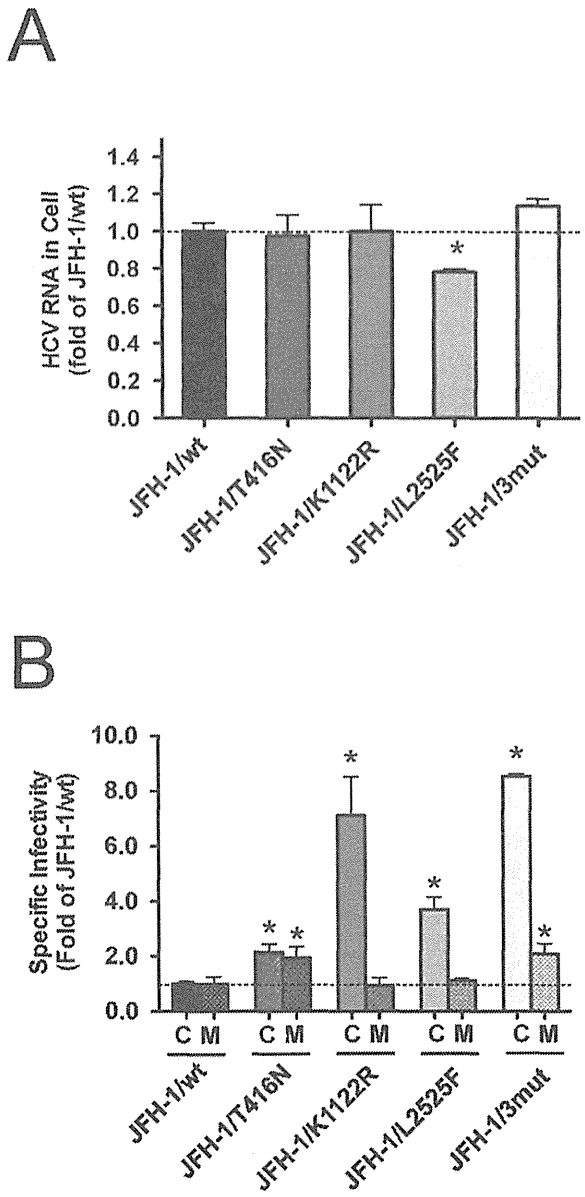

Figure 3. Single cycle virus production assay to assess contribution of mutations on viral life cycle. (A) Intra-cellular HCV RNA titers were assessed in full-length RNA of JFH-1/wt and its variants transfected into Huh7–25 cells. Data are given as fold change vs. JFH-1/wt. (B) Intra- and extra-cellular specific infectivity of JFH-1/wt and its variants transfected into Huh7–25 cells were calculated. Data are given as fold change vs. JFH-1/wt. C; intracellular specific infectivity, M; extracellular specific infectivity, $*p<0.05$.
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compared with JFH-1/wt, respectively (Figure 8A). These enhancements were much higher than in the case of JFH-1/3mut. These strains also showed enhanced extra-cellular specific infectivity (4.92- and 5.83-fold, respectively). To assess the biophysical properties of particles with strains of JFH-1/2G and JFH-1/6B, we analyzed the culture medium of these strains transfected cells in the density gradient (Figure 9). The density

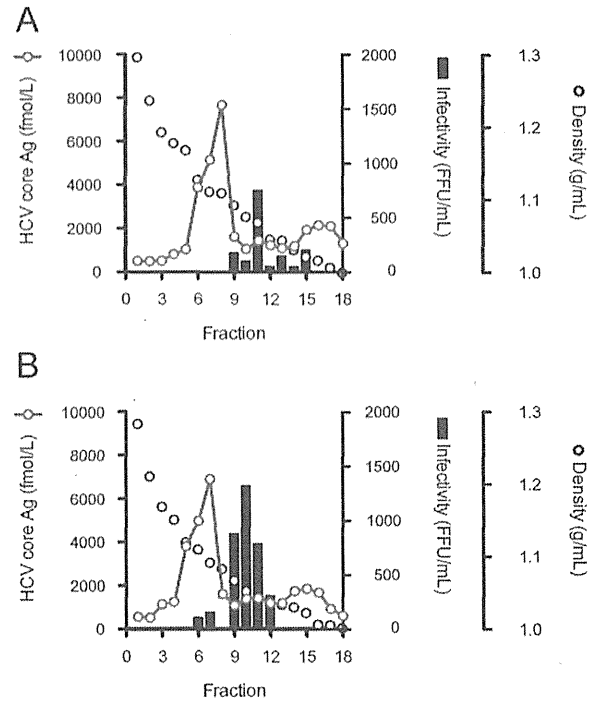

Figure 4. Iodixanol density gradient analysis of JFH-1/wt and JFH-1/T416N. Huh7.5.1 cells were transfected with full-length RNAs of JFH-1/wt and JFH-1/T416N. Culture medium of each strain was collected and analyzed by 10%–40% of iodixinol density gradient. Fractions were collected, and HCV core Ag and infectivity titers JFH-1/wt (A) JFH-1/T416N (B) were measured.
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gradient profiles of these strains were similar to that of JFH-1/wt. The peak densities of infectivity titer of JFH-1/2G (1.06 g/mL) and JFH-1/6B (1.05 g/mL) were almost identical with that of JFH-1/wt (1.07 g/mL), but the peak infectivity titers of these strains were 3 log-fold higher than that of JFH-1/wt.

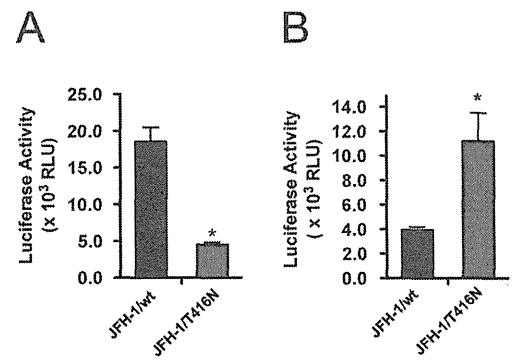

Figure 5. Assessment of T416N on infection step by HCVpp and HCVtcp. (A) Infectivity of JFH-1/wt and JFH-1/T416N with HCVpp envelopes was assessed. Luciferase activity was measured in HCVpp-infected cells. $*p<0.05$. (B) Infectivity of HCVtcp with the structural regions of JFH-1/wt and JFH-1/T416N was assessed. Luciferase activity was measured in HCVtcp-infected cells. $*p<0.05$.
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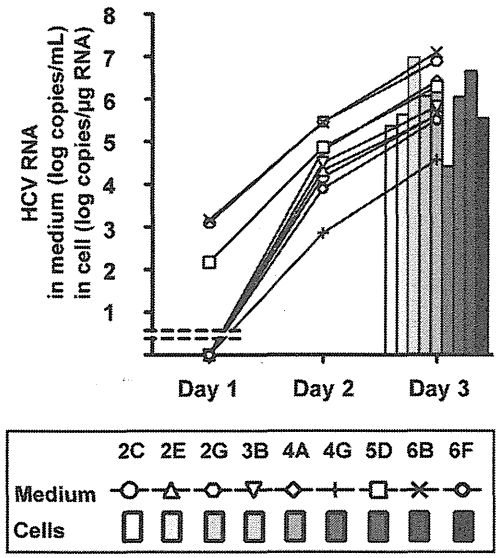

Figure 6. Production of progeny virus after infection of culture media harvested in wells that have a single focus. HCV RNA titers were measured in culture medium and cells.
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Discussion

In this study, we obtained cell culture-adapted virus by serial passage of full-length JFH-1 RNA-transfected Huh-7.5.1 cells for 25 days after transfection. The obtained virus produced 3 log-fold more progeny viruses as compared with JFH-1/wt infected the same amount of FFU. On sequence analysis of this cell culture-adapted virus, 3 amino acid mutations (T416N at E2, K1122R at NS3 and L2525F at NS5B) were identified. On assessment of JFH-1 constructs with these mutations, they were revealed to have advantages in various steps of the viral life cycle. Mutations in K1122R at NS3 and L2525F at NS5B are considered to contribute to efficient infectious virus production. The JFH-1 variants with each mutation, JFH-1/K1122R and JFH-1/L2525F, produced more infectious virus (7.2- and 3.7-fold, respectively), although K1122R slightly reduced intra-cellular viral replication. The mutation T416N at E2 is associated with enhancement of the infection step. JFH-1/T416N showed 2-fold higher efficiency of infection when compared with JFH-1/wt. T416 at E2 is located at the epitope of neutralizing monoclonal antibodies of AP33 and 3/11 and is conserved among genotypes [35–37]. The mutation at this site has been detected in cell culture-adapted J6/JFH-1 chimeric virus, but enhanced infectivity has not been observed in the mutation-introduced chimeric virus [20]. Moreover, substitution of T416A was reported to abolish infectivity of HCVpp with envelopes of H77 strain, genotype 1a [38]. In accordance with these data, we found that mutation T416N also reduces the infectivity in the HCVpp system. In contrast, we confirmed that this mutation enhances infectivity in HCVcc and HCVtcp systems. Such discrepancies between HCVpp and HCVcc on the HCV infection step have been reported previously [33]. Because HCVpp is generated in non-hepatic 293T cells, it is likely that the cell-derived components of HCVpp are different from those of HCVcc and HCVtcp. Thus, we believe that the HCVpp system does not reflect the characteristics of mutations in HCV envelopes and may not be suitable for assessing the effects of mutations in the HCV infection step. On the other hand, the

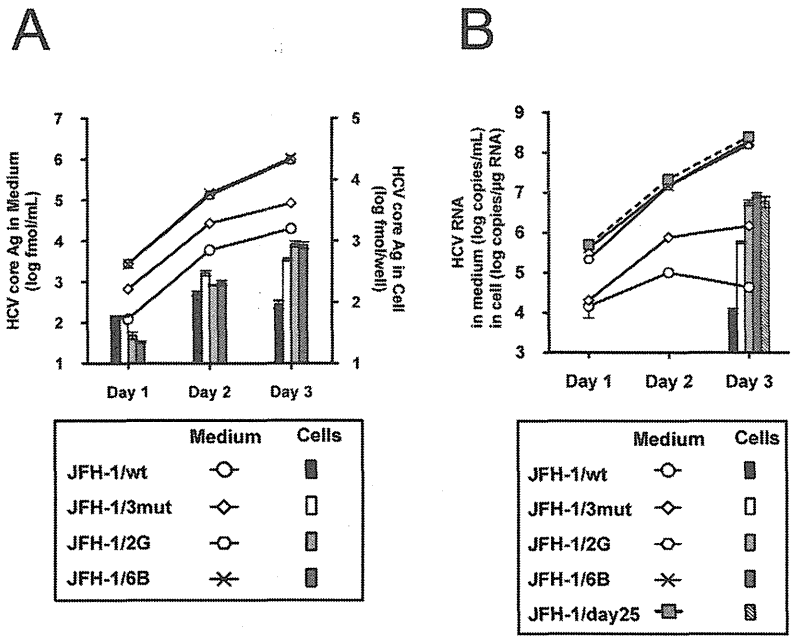

Figure 7. Effects of mutations detected after single-strain isolation method. (A) One million cells were transfected with 2 μg of *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/3mut, JFH-1/2G and JFH-1/6B. HCV propagation was monitored by measuring HCV core Ag. (B) The same amount of JFH-1/wt, JFH-1/3mut, JFH-1/2G, JFH-1/6B and JFH-1/day25 viruses were infected into naïve Huh-7.5.1 cells (MOI = 1.0), and HCV RNA titers were monitored.
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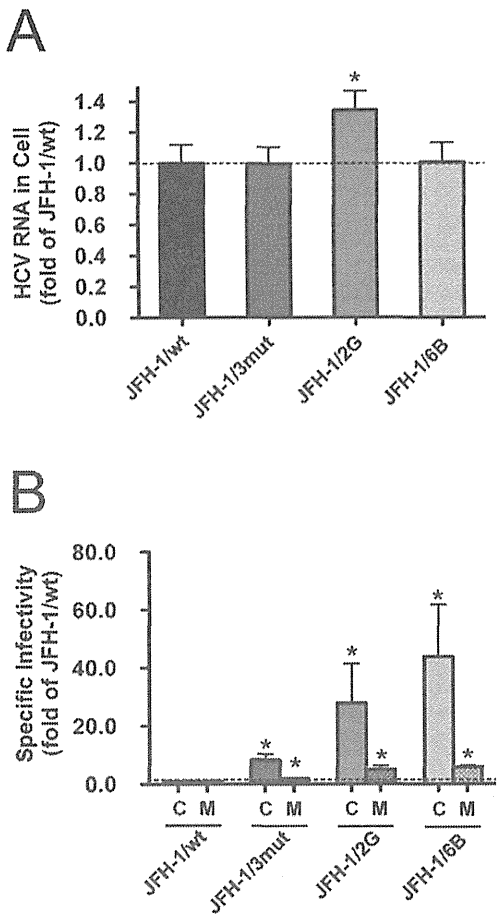

Figure 8. Single cycle virus production assay to assess the contribution of introduced mutations on viral life cycle. (A) Intra-cellular HCV RNA titers were assessed in full-length RNA of JFH-1/wt-, JFH-1/3mut-, JFH-1/2G- and JFH-1/6B-transfected Huh7–25 cells. Data are presented as fold change vs. JFH-1/wt. (B) Intra- and extracellular specific infectivity of JFH-1/wt-, JFH-1/3mut-, JFH-1/2G- and JFH-1/6B-transfected Huh7–25 cells were calculated. Data are given as fold change vs. JFH-1/wt. C; intracellular specific infectivity, M; extracellular specific infectivity, $*p<0.05$.
doi:10.1371/journal.pone.0098168.g008

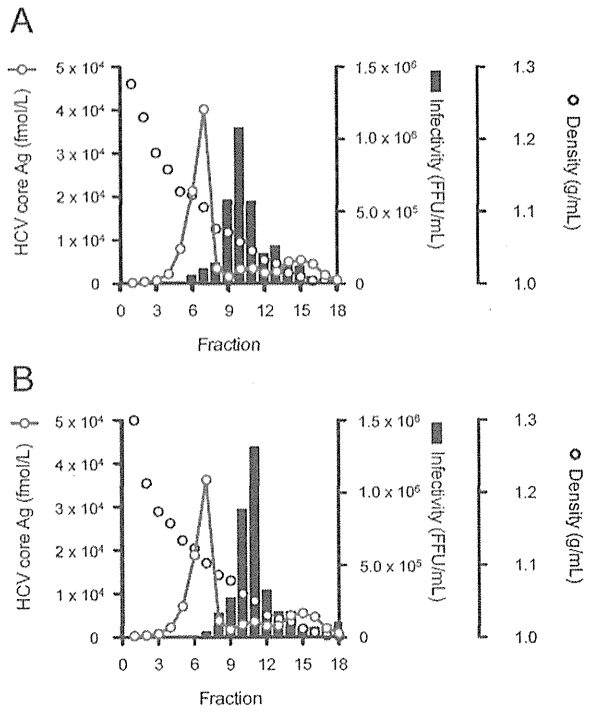

Figure 9. Iodixanol density gradient analysis of JFH-1/2G and JFH-1/6B. Huh7.5.1 cells were transfected with full-length RNAs of JFH-1/2G and JFH-1/6B. Culture medium of each strain was collected and analyzed by 10%–40% of iodixinol density gradient. Fractions were collected, and HCV core Ag and infectivity titers of JFH-1/2G (A) and JFH-1/6B (B) were measured.
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T416N mutation in the HCVtcp system indicated consistent data with HCVcc. Therefore, we conclude that this mutation enhances the infectivity of HCV.

The variant JFH-1/3mut with all three mutations, T416N, K1122R and L2525F, has advantages in infectivity and infectious virus production, and results in the highest efficiency of progeny virus production. However, in the infection study, this JFH-1/3mut could not reach the virus production level of the obtained cell culture-adapted JFH-1 virus, JFH-1/day25. We were puzzled by this, and speculated that JFH-1/day25 was not monoclonal, as the direct sequence method is unable to identify responsible mutations associated mixed viruses. Thus, we exploited another strategy. We isolated a single JFH-1 variant that can produce more progeny virus, and we used a method for single virus isolation by infection with a diluted mixture of cell culture-adapted virus. By infection with cell culture-adapted virus at a concentration of 1 FFU/well, we were able to isolate two variants that showed the highest virus production among the tested strains.

The isolated variants, 2G and 6B strains, have additional mutations at p7, NS3 and NS5A. A reverse-genetics analysis revealed that these variants could produce progeny virus more efficiently than JFH-1/wt and JFH-1/3mut after transfection with full-length RNA. In the infection study, the production of progeny virus of these variants was also superior to the levels of JFH-1/wt and JFH-1/3mut, and was comparable to JFH-1/day25. In order to identify the advantages of these variants in the virus life cycle, we used the single cycle virus production assay. JFH-1/2G was able to replicate 1.35-fold more efficiently in culture cells. Both strains have advantages in the steps of infectious virus production and infection. The intra-cellular specific infectivity of JFH-1/2G and JFH-1/6B was 27.8- and 43.7-fold higher, and the extracellular specific infectivity was 4.92- and 5.83-fold higher than that of JFH-1/wt. This suggests that enhancement of infectious virus production is a major advantage in these strains. These strains included the additional adaptive mutation V2440L. We examined this sequence in JFH-1/day25 retrospectively, and found a mixture of nucleotide G/C at nucleotide 7658 (data not shown). Thus, there may be many strains with mutations other than V2440L and they are able to propagate efficiently as like as clones, JFH-1/2G and 6B, but we might not be able to isolate such strains in this experiment. This mutation, V2440L, has already been reported in cell culture-adapted JFH-1 virus, and to contribute to slow cleavage at the NS5A-NS5B site, increasing the production of

infectious virus [17]. The ability of efficient virus production of JFH-1/2G and JFH-1/6B may be attributable to this mutation.

In conclusion, we were able to successfully isolate 2 cell culture-adapted variants that can produce 3 log-fold more progeny viruses than JFH-1/wt, and identified the responsible mutations. The strategy of single virus isolation by end-point dilution and infection used in this study may be useful for identifying strains with unique characteristics, such as robust virus production, from diverse populations, and for identifying the responsible mutations for these characteristics.

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Author Contributions

Conceived and designed the experiments: TJL TW TK. Performed the experiments: NS AM RS NW MS TK. Analyzed the data: NS AM TK. Wrote the paper: NS AM MS TK.

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