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Received: July 23, 2014. Accepted: September 8, 2014
Published online: December 24, 2014
DOI: 10.7883/yoken.JJID.2014.328

Advance Publication articles have been accepted by JJID but have not been copyedited or formatted for publication.
Development of an Infectious Surrogate Hepatitis C Virus Based on a Recombinant Vesicular Stomatitis Virus Expressing Hepatitis C Virus Envelope Glycoproteins and Green Fluorescent Protein

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Keywords: HCV, VSV, Surrogate virus, E1E2, GFP
Summary

To develop surrogate viruses for hepatitis C virus (HCV), we previously produced recombinant vesicular stomatitis viruses (rVSVs) lacking glycoprotein G, but instead expressing chimeric HCV E1/E2 fused to G, which were not infectious in HCV-susceptible liver cells. In this study, to develop an infectious surrogate HCV based on an rVSV (VSV/HCV), we generated a novel rVSV encoding the native E1/E2 (H77 strain) and green fluorescent protein (GFP) instead of G. We show that this VSV/HCV efficiently entered human liver cells, including Huh-7 cells, expressed GFP in them, and propagated, but did not do so in nonsusceptible BHK-21 cells. The infectivity of the VSV/HCV, which was measured as the number of foci of GFP-positive cells, was specifically reduced by the addition to the cultures of chimpanzee anti-HCV serum, anti-E2 antibody, or anti-CD81 antibody. When sera obtained from HCV-infected or uninfected patients were added, infection was selectively inhibited only by the sera of HCV-infected patients. These data together suggest that this infectious GFP-expressing VSV/HCV could be a useful tool for studying the mechanisms of HCV entry into cells and for assessing potential inhibitors of viral entry, including neutralizing antibodies.
Introduction

Hepatitis C virus (HCV) belongs to the genus *Hepacivirus* in the family *Flaviviridae*, and is a major cause of non-A and non-B viral hepatitis (1). HCV is a positive-strand RNA virus with a genome of ~9.6 kb, encoding a polyprotein precursor consisting of structural and nonstructural proteins. It is classified into six major genotypes and multiple subtypes (2). The structural proteins include the envelope glycoproteins (E1 and E2), which form a noncovalently associated heterodimer on the envelope (3) and are responsible for viral attachment to the cell surface and viral entry into cells (4,5). The C-terminal hydrophobic regions of both envelope proteins contain signals responsible for retaining the proteins in the endoplasmic reticulum (ER) (6,7).

Vesicular stomatitis virus (VSV) is a nonsegmented negative-strand RNA virus and prototype of the family *Rhabdoviridae*. VSV has an 11.121-kb genome encoding five structural proteins, designated N, P, M, G, and L (8). The G protein is the single transmembrane glycoprotein of VSV and is responsible for viral attachment and entry. Recombinant VSVs (rVSVs) can be recovered from plasmid DNA and foreign genes can be expressed at high levels from multiple sites in the genome (9,10). These rVSVs show targeting specificity that is dependent on the foreign protein expressed on the envelope of the viral particles in place of G (11,12).

One major obstacle to HCV research has been the lack of a cell culture system to analyze viral infection and replication. Recently, several assay systems for the study of HCV–host cell interactions have been developed: recombinant HCV envelope glycoproteins (13,14), HCV pseudotyped particles
(4,5), and cell-culture-derived infectious HCV particles (15,16). These systems have allowed the identification of cell-surface molecules involved in HCV entry, including CD81 (13), scavenger receptor class B type I (14), claudin-1 (17), occludin (18), dendritic cell/liver-specific ICAM-3 grabbing nonintegrin (19), low-density lipoprotein receptor (20), and heparan sulfate (21).

HCV infectivity assays based on VSV pseudotypes or recombinants have been developed (22,23). VSV pseudotypes or recombinants were generated that express chimeric E1G/E2G, in which the extracellular domains of E1/E2 were fused to the transmembrane and cytoplasmic domains of G. Both E1G and E2G were expressed at the cell surface because their ER-retention signals were deleted, and they were consequently incorporated at high levels into the VSV particles that budded from the cell surface. However, these recombinant and pseudotyped particles do not specifically infect liver cells, indicating that these VSV-based assay systems might not be appropriate for the study of cell entry by HCV.

To develop a more relevant HCV infection assay model using VSV, we generated a novel rVSV lacking the G protein but instead expressing full-length unmodified E1/E2. This rVSV also encodes green fluorescent protein (GFP) and GFP expression was used as an indicator of viral infectivity.

Materials and Methods

Cells

BHK-21 (hamster kidney), Huh-7 (human hepatoma), PLC/PRF/5
(human hepatoma), and HepG2 cells (human hepatocarcinoma) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen) (10%FBS–DMEM). BHK-G cells (24) were cultured in 10%FBS–DMEM supplemented with 750 μg/ml geneticin (G418) and 0.5 μg/ml tetracycline (Invitrogen). The expression of VSV G was induced by transferring the BHK-G cells to tetracycline-free 10%FBS–DMEM ~18 h before infection. All cells were cultured at 37 °C in 5% CO₂.

HCV-specific antibodies and sera

A monoclonal antibody directed against E1 (A4), a polyclonal antibody against E2 (WU105), and a monoclonal antibody against E2 (3/11) (4,5) were kindly provided by Dr. J. A. McKeating (University of Birmingham).

Chimpanzee anti-HCV serum and uninfected chimpanzee control serum (25) were generously provided by Dr. S. M. Feinstone (U.S. Food and Drug Administration). Sera from HCV-infected and uninfected patients were generously provided by Dr. M. L. Landry (Yale University School of Medicine). The use of patient sera was approved by Yale New Haven Hospital/Yale University School of Medicine before the initiation of the present study, and carried out in compliance with the relevant laws and guidelines in accordance with the ethical standards of the Declaration of Helsinki.

Construction and recovery of rVSV
The DNA sequence encoding the E1E2 polyprotein (including the N-terminal signal sequence derived from the C-terminus of the capsid gene) was amplified with PCR from the infectious clone BRTM/HCV1-3011 (H77 strain; generously provided by Dr. C. M. Rice, The Rockefeller University) (26,27), using the forward primer 5′-CACGCTCGACACGCGTACCATGGG TTGCTCTTTCTCTATCTTCC-3′ and the reverse primer 5′-CGATCCCCCC GGGCTAGCCTACGCCTCCGCTTGGGATATGAGTAAC-3′. The primers introduced *Mlu*I (underlined) and *Nhe*I (bold) restriction enzyme sites upstream and downstream, respectively, from the coding sequence. The PCR product was digested with *Mlu*I and *Nhe*I, purified, and ligated into the pVSV-XNGFP vector (22) digested with the same enzymes. The resulting plasmid was designated pVSVΔG–E1E2–GFP and used for virus recovery. The recombinant virus was recovered with established methods (9, 28) and designated VSVΔG–E1E2–GFP. Because the virus lacked the VSV G gene (ΔG), the virus was initially recovered in the presence of complementing G protein and then propagated in BHK-G cells that had been induced to express G (24). This G-complemented virus was isolated from a single plaque, and grown in BHK-G cells expressing G. The supernatant containing the virus (~5 × 10⁶ pfu/ml) was stored at –80 °C.

VSVΔG–E1340G–E2711G–GFP (referred to as VSVΔG–E1GE2G–GFP), which lacks the G gene and instead expresses chimeric E1G/E2G and GFP, was constructed previously (22), and its infectivity was compared with that of VSVΔG–E1E2–GFP in this study. VSV–GFP, expressing GFP (11), was also used as a control.
The protocols were approved by the Committee on Recombinant DNA Experiments of the National Institute of Infectious Diseases before the initiation of the present study.

**Infection of cells by rVSVs**

To prepare non-G-complemented VSVΔG–E1GE2G–GFP and VSVΔG–E1E2–GFP by removing the G protein from G-complemented stocks, the G-complemented viruses were initially grown in BHK-21 cells (not expressing G) for a single round of replication. The viruses were then incubated with neutralizing anti-G antibodies (I1, I14) (11,12) at 37 °C for 30 min to eliminate any remaining infectivity attributable to traces of G carried through the procedure. These non-G-complemented viruses were used in all subsequent experiments (except the immunofluorescence assay).

To determine the infectivity of VSV–GFP, VSVΔG–E1GE2G–GFP, and/or VSVΔG–E1E2–GFP, 5–10 × 10^5 cells were seeded in each well of a 24-well plate. The viruses were then added to the wells, and 2–3 h later, the inocula were replaced with 1% methylcellulose-containing medium. After infection for 24–48 h, the infectivity of each virus was quantified by counting the numbers of isolated areas of GFP-expressing cells under a fluorescence microscope. Titers are expressed as infectious units (IU)/ml. The cells were photographed with an Olympus C-3040 Zoom digital camera on an Olympus CK40 microscope (Olympus) equipped with a 25× objective.

**Inhibition of rVSV infection with various antibodies and sera**
Huh-7 cells (5 × 10^5 per well) were seeded on a 24-well plate. Chimpanzee or human normal control and anti-HCV sera were heat-inactivated at 56 °C for 30 min. VSV–GFP and/or VSVΔG–E1E2–GFP (~500 IU) were then incubated with or without serially diluted (1:50–1:50,000) or a 1:50 dilution of chimpanzee anti-HCV serum, a 1:50 dilution of uninfected chimpanzee control serum, ~10 μg/ml anti-E1 antibody (A4), ~10 μg/ml anti-E2 antibody (WU105, 3/11), or a 1:50 dilution of serum from HCV-infected or -uninfected patients at 37 °C for 30 min. These mixtures were then added to Huh-7 cells. Huh-7 cells were also incubated with or without ~10 μg/ml anti-CD81 antibody (JS-81) (BD Pharmingen) at 37 °C for 30 min, after which VSVΔG–E1E2–GFP (~500 IU) was added to the cell–antibody mixture. Following incubation for 2–3 h, the inocula were replaced with 1% methylcellulose-containing medium. After infection for 24–48 h, the GFP expression in the cells was observed with fluorescence microscopy, and the percentage infectivity (percentage of the control value) was calculated from the counted numbers of foci of GFP^+ cells in the samples relative to the number in an untreated control or a control treated with uninfected patient serum.

Results

We previously generated an rVSV-based surrogate virus for HCV (VSV/HCV), VSVΔG–E1GE2G–GFP, expressing chimeric E1G/E2G (22). However, because this VSV/HCV could not infect liver-derived cells, we expressed an unmodified E1/E2 in this study to determine whether it might
confer the correct specificity of infection on a surrogate virus. Therefore, we inserted genes encoding native E1/E2 (H77 strain) and GFP into a plasmid from which an rVSV expressing E1/E2 and GFP instead of G, designated VSVΔG–E1E2–GFP (a novel VSV/HCV), could be recovered. Figure 1 shows the gene order of the VSV constructs, including VSV/HCVs.

Initially, we investigated whether BHK-21 cells infected with G-complemented VSVΔG–E1E2–GFP expressed E1/E2 in the cytoplasm and at the cell surface. The infected cells were tested with an indirect immunofluorescence assay with anti-E1 (A4) and anti-E2 (WU105) antibodies. In the infected cells, labeled E1 and E2 were readily detected in the cytoplasm in an ER-like pattern, but both envelope proteins appeared to be expressed at only very low levels on the cell surface (data not shown).

We next investigated whether VSVΔG–E1E2–GFP infected cells reported to be susceptible to HCV infection (4,5). VSVΔG–E1E2–GFP was used in parallel with VSV–GFP (a control) to infect human liver-derived Huh-7, PLC/PRF/5, HepG2, and unrelated BHK-21 cells. The infectivity of each virus was quantified and the results are given as titers (Fig. 2A). As expected, the VSV–GFP control virus infected all cell lines, with titers in the range $10^8$–$10^9$ IU/ml. VSVΔG–E1E2–GFP significantly infected Huh-7 cells with a titer of approximately $0.8 \times 10^4$ IU/ml and PLC/PRF/5 cells with a lower titer. It also infected HepG2 cells at very low levels but did not infect BHK-21 cells at all. These results demonstrate that this novel VSV/HCV is only infectious to HCV-susceptible cells.

To clarify the differences between the two VSV/HCVs that we have constructed, we compared the infection efficiencies of VSVΔG–E1E2–GFP...
and VSVΔG–E1GE2G–GFP. We tested whether Huh-7 and BHK-21 cells were infected by either VSVΔG–E1E2–GFP or VSVΔG–E1GE2G–GFP. The infection efficiency of each virus was determined (as titers), and the cells tested were imaged. VSVΔG–E1E2–GFP did not infect BHK-21 cells, but formed many areas with GFP-expressing cells on Huh-7 cells, with titers of \(~0.8 \times 10^4\) IU/ml (Fig. 2B and 2C). The formation of GFP-expressing areas/foci indicated viral spread from an initially infected cell. However, VSVΔG–E1GE2G–GFP infected neither cell line, suggesting that replacement of the transmembrane and cytoplasmic domains of E1/E2 with those of G interfered with the correct folding and/or interaction of E1/E2 and eliminated HCV-specific infectivity. These data for VSVΔG–E1GE2G–GFP are consistent with those of another study of a similar chimeric E1/E2 (5). Our results clearly indicate that the VSVΔG–E1E2–GFP particles incorporated native E1/E2, which specifically mediated the infection of HCV-susceptible cells.

Because the novel VSV/HCV, VSVΔG–E1E2–GFP, is clearly infectious, we investigated whether infection by this VSV/HCV is dependent on the expression of E1/E2 from the genome. We examined whether infection by VSVΔG–E1E2–GFP or VSV–GFP (control) was inhibited by a chimpanzee neutralizing antiserum (Fig. 3A). Each virus was incubated with the indicated dilutions of chimpanzee anti-HCV serum, and then added to Huh-7 cells. The percentage infectivity was calculated in the serum-treated samples relative to that in an untreated control. As shown in Fig. 3A, infection by VSV–GFP was slightly inhibited by low dilutions of anti-HCV serum (probably a nonspecific effect), whereas infection by VSVΔG–E1E2–GFP was markedly inhibited by the same serum in a dose-dependent manner and almost completely at a
To determine the dependence of the infectious VSV/HCV on functional E1/E2 expression at the viral surface, we tested the inhibitory effects of antibodies directed against E1/E2 and the well-known HCV receptor, CD81, on VSV/HCV infection. We incubated VSVΔG–E1E2–GFP with anti-E1 (A4), anti-E2 (WU105), or anti-E2 (3/11) antibody and the mixture was added to Huh-7 cells. Huh-7 cells were also incubated with anti-CD81 antibody (JS-81) before the cell–antibody mixture was inoculated with VSVΔG–E1E2–GFP. Percentage infectivity was calculated in each antibody-treated sample relative to the no-addition sample. Figure 3B shows that both the anti-E1 (A4) and anti-E2 (WU105) antibodies inhibited less than ~50% infection and that the anti-E2 (3/11) antibody blocked around 90% infection. As expected, chimpanzee anti-HCV serum (diluted 1:50) caused a > 95% reduction in infection, whereas control chimpanzee serum did not reduce infection at all. Anti-CD81 antibody (JS-81) caused a reduction in infection of > 95%. These data confirm the important roles of E1/E2 and CD81 in viral entry.

To determine whether the present assay could detect HCV-neutralizing antibodies in human sera, we tested 11 coded human serum samples obtained from either chronically HCV-infected or -uninfected patients with our assay for the infectious VSV/HCV. Thus, VSVΔG–E1E2–GFP was incubated with the serum samples and then added to Huh-7 cells. Percentage infectivity was calculated for each serum-treated sample relative to that of a sample treated with serum no. 1 (Fig. 3C). Infection was 95% blocked or more by sera nos 8–11, but not with sera nos 1–7. The study was unblinded after the completion of this assay. All four neutralizing sera were confirmed to be from HCV-positive
patients (with HCV RNA RT–PCR and/or recombinant immunoblot assay). The seven remaining nonneutralizing sera were from HCV-uninfected patients. Therefore, these data demonstrate that our present assay using VSVΔG–E1E2–GFP can correctly detect neutralizing antibodies in HCV-infected patient sera, but not in uninfected patient sera. Some of the HCV-negative serum samples also enhanced the infection, by as much as two-fold, compared with serum no. 1, suggesting that this enhanced infectivity might be mediated by serum factor(s) such as high-density lipoproteins, as shown in other studies (29,30).

**Discussion**

In this study, we developed a novel VSV/HCV encoding native E1/E2 and GFP, VSVΔG–E1E2–GFP, and verified that the infectivity of this VSV/HCV requires the expression of intact E1/E2 on the viral envelope. In our previous study (22), a chimeric E1G/E2G was engineered so that both proteins, whose unmodified native forms are assembled on the ER membrane, were expressed at the cell surface and efficiently incorporated into the VSVΔG–E1GE2G–GFP particles when the virions budded from the plasma membrane. However, despite the presence of E1G/E2G at high levels on the virions, VSVΔG–E1GE2G–GFP did not infect any cells, including those derived from the human liver. It remains to be clarified how the substitution of the G domains within the chimeric proteins alters the conformations of the molecules.

Both native E1/E2 proteins contain ER retention signals (6,7). However,
at least a small fraction of these proteins appear to be transported to the cell surface, where they can be incorporated into budding retrovirus pseudotypes (4,5). VSV particles budding at the cell surface presumably obtain low levels of the native E1/E2 expressed at the cell surface, resulting in infectious virions.

Other groups have previously generated retroviruses pseudotyped with the native HCV envelope proteins expressed transiently in cells (4,5), with results similar to our results presented here. However, one significant difference is that our infectious VSV/HCV actually replicates in and propagates on the liver cells because it encodes E1/E2 in its genome, whereas those pseudotypes do not. Another group generated an rVSV encoding native E1/E2 and the p7 ion channel protein in place of G (31). Although this recombinant virus is similar to our infectious VSV/HCV, a major difference is that ours does not encode or express p7, but expresses GFP. Importantly, the present results indicate that the infectivity of a VSV/HCV expressing unmodified E1/E2 does not always require the expression of p7. Moreover, the expression of GFP upon infection by our VSV/HCV allowed its easy and rapid detection in infected cells without immunostaining (Figs 2 and 3). Consequently, our infection assay should be more useful than other assays using surrogate viruses that do not encode GFP in their genomes. A cell culture system for the growth of HCV has also been developed using unique clones derived from HCV strains (15,16). The genetic basis for the propagation of these specific clones and the lack of propagation of most other HCVs in culture are not understood in detail. Therefore, infectivity assays using this system might not reflect the common mechanisms of HCV infection.

In conclusion, the assay model presented here, with an infectious
VSV/HCV, has major advantages (summarized in Fig. 4), as follows. (i) It is possible to efficiently analyze the mechanisms of HCV infection, including the interactions between a variety of E1/E2s (from different genotypes and strains) and receptors, by detecting GFP-expressing infected cells (Fig. 4A). (ii) Potential inhibitors of the entry of HCV into cells could be found and assessed efficiently by the inhibition of GFP expression, because VSV/HCV infection can be specifically blocked with an anti-E1/E2 antibody (Fig. 4B) or an anti-CD81 receptor antibody (Fig. 4C). This advantage might facilitate the development of new therapeutic agents. Therefore, our infectious, replication-competent, and GFP-expressing VSV/HCV provides a useful and valuable assay system for basic studies of HCV.

Acknowledgments

We thank Drs C. M. Rice, M. L. Landry, and S. M. Feinstone for generously providing the plasmid encoding E1E2, patient sera, and chimpanzee sera, respectively. We also thank Drs J. A. McKeating and B. D. Lindenbach for kindly providing the antibodies against E1/E2 and suggestions on the manuscript, respectively. We are also grateful to Drs T. Wakita, H. Tani, and T. Shimoike for their discussions and support on this work.

Conflict of interest

None to declare.
References


Figure legends

**Fig. 1. Diagrams of VSV/HCVs expressing chimeric E1G/E2G or native E1/E2 and GFP.** The gene orders in the two VSV/HCVs, VSV\(\Delta\)G–E1GE2G–GFP and VSV\(\Delta\)G–E1E2–GFP, are illustrated. Genes encoding chimeric E1G/E2G or native E1/E2 and GFP were substituted for the gene encoding G in the VSV genome. The gene orders in VSV wt (wild type), VSV\(\Delta\)G (lacking G), and VSV–GFP (expressing GFP) are aligned. The gene encoding L in the VSV genome is shortened by slant lines.

**Fig. 2. Infection of cells by a VSV/HCV expressing native E1/E2 and GFP.** (A) VSV–GFP (dashed bar) and VSV\(\Delta\)G–E1E2–GFP (black bar) were added to the indicated cells. Titers of both viruses were determined and are shown as log10 IU/ml. The data are presented as the mean + standard error. (B) To compare their infectivity, VSV\(\Delta\)G–E1GE2G–GFP and VSV\(\Delta\)G–E1E2–GFP were added to BHK-21 and Huh-7 cells. The titers of each virus in BHK-21 (white bar) and Huh-7 cells (black bar) were determined and are shown as log10 IU/ml. (C) Photographs of cells incubated with VSV\(\Delta\)G–E1E2–GFP were taken (100× magnification). ND, not detected. These data are representative of three independent experiments.

**Fig. 3. Neutralization of VSV/HCV infection with various antibodies and sera.** (A) VSV–GFP (open circles) and VSV\(\Delta\)G–E1E2–GFP (closed circles)
were incubated with or without serially diluted chimpanzee anti-HCV serum, as indicated, and then added to Huh-7 cells. (B) VSV\(\Delta\)G–E1E2–GFP was incubated with or without the indicated chimpanzee sera and antibodies (except anti-CD81) and then used to inoculate Huh-7 cells. Anti-CD81 antibody was added to Huh-7 cells and incubated, and then VSV\(\Delta\)G–E1E2–GFP was added directly to the cell–antibody mixture. (C) VSV\(\Delta\)G–E1E2–GFP was incubated with the indicated human sera and then used to inoculate Huh-7 cells. The data show the percentage infectivity, calculated from the viral titers of samples relative to the titer of a control sample. The data are presented as the mean ± standard error. ND, not detected; +, HCV-infected; –, uninfected. These results represent two independent experiments.

Fig. 4. Schematic representation of VSV/HCV infection and its inhibition.

(A) An infectious VSV/HCV (VSV\(\Delta\)G–E1E2–GFP) enters its target cells after specific binding of E1/E2 on the virions to receptors on the cell surface. VSV/HCV then releases the viral genome into the cytoplasm and expresses GFP (shown in green) from its genome. After complete replication, the progeny VSV/HCV, which carry the same viral genome as the parental VSV/HCV, bud from the cell surface. The viruses produced enter the surrounding target cells and express GFP again. Because the progeny virus remains infectious and replication competent, the life cycle represented here is repeated for as long as target cells exist. Furthermore, (B) anti-E1/E2 or (C) anti-CD81 receptor antibody specifically blocks the entry of VSV\(\Delta\)G–E1E2–GFP into the cells.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.