5′-GAGAATTCGTAGACCGTGCACCATG-3′ and antisense primer 5′-AAGAATTCTTAGGCATAAGCCTGCCGGGGCA-3′. Core to NS2 coding region of JFH-1 was amplified using pJFH-1 as a template and sense primer 5′-GAGAATTCGTAGACCGTGCACCATG-3′ and antisense primer 5′-AAGAATTCTTAAAGGAGCTTCCACCCCTTGG-3′. Amplified fragments were inserted into EcoRI site of pEF4 (Invitrogen) to generate pEFJFH/c-p7 and pEFJFHc-NS2, respectively.

Establishment of cell lines capable of packaging JFH-1 replicon RNA into VLPs. Huh7 cells were transfected using Lipofectamine (Invitrogen) with either pEFJFH/c-p7 or pEFJFHc-NS2 and were cultured with 0.2 mg/ml of zeocin (Invitrogen). Zeocin-resistant colonies were collected 3 weeks after transfection. The cell lines, Huh/c-p7 and Huh/c-NS2 (expressing pEFJFH/c-p7 and pEFJFHc-NS2, respectively) were then electroporated with 1 μg of JFH-1 subgenomic replicon (SGR-JFH1) RNA and were cultured with 0.375 mg/ml of G418 (Nacalai Tesque). Expression of core, E2 and NS5A was confirmed by Western blotting using anti-HCV core and anti-HCV E2 monoclonal antibodies [22] and anti-HCV NS5A polyclonal antibody [23]. The total RNA of culture media for each cell line (Huh/c-p7/SGR and Huh/p-NS2/SGR) was extracted using the QIAampViral RNA Mini spin column (Qiagen). Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously [24,25]. The HCV core antigen in the culture media was measured by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnotics), following the manufacturer's instructions. Culture medium was centrifuged at 8000g for 30 min to remove all cellular debris, after which the supernatant was concentrated to 1 ml by centrifugation using Amicon Ultracel 100k (Amicon). The concentrated medium was then layered on top of a continuous 10–60% (wt/vol) sucrose gradient in phosphate buffered saline (PBS) and then centrifuged at 40,000 rpm at 4℃ for 16 h (SW41E rotor, Beckman). Fractions (1 ml each) were collected from the top of the tube (12 fractions in total) and the density for each fraction was determined. The concentrations of replicon RNAs and core proteins of each fraction were measured as described above.

Infectivity of HCV-LPs. To determine whether these cell lines produced infectious HCV-LPs, we performed a colony formation assay using neomycin-resistant gene of SGR-JFH1 RNA. Naive Huh7 cells were infected with pooled fractions of 1.12–1.20 g/ml of both cell lines and were cultured for 3 weeks with G418 at 0.375 mg/ml. Formed colonies were stained with crystal violet and counted.

We also performed an immunofluorescence study in order to analyze the infectivity of the HCV-LPs. Following 3 days of incubation, the cells were fixed and immunostained for NS5A with anti-NS5A rabbit polyclonal antibody as described previously (Murakami et al., in press). Ffu (focus forming units) was calculated essentially based on the method as described previously [7,26]. Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1×10^4 cells/well. Samples were serially diluted 5-fold in complete growth medium and used to infect the seeded cells (six wells per dilution). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI).

Results

Establishment of cell lines capable of packaging JFH-1 replicon RNA into VLPs

Stable cell lines expressing JFH-1 structural proteins were generated by transfecting with either pEFJFH/c-p7 or pEFJFHc-NS2. Zeocin-resistant colonies were collected 3 weeks after transfection and the expression of JFH-1 structural proteins was confirmed by Western blotting using anti-HCV core and anti-HCV E2 monoclonal antibodies [22] (Fig. 1A, lanes 1 and 2). The cell lines, Huh/c-p7 and Huh/c-NS2 (expressing pEFJFH/c-p7 and pEFJFHc-NS2, respectively) were then electroporated with 1 μg of SGR-JFH1 RNA. Six G418-resistant colonies were selected 3 weeks after electroporation and were termed Huh/c-p7/SGR (1-6) and Huh/c-NS2/SGR (1-6) cells. Expression of core, E2 and NS5A of Huh/c-p7/SGR-1, and Huh/c-NS2/SGR-3 was confirmed by Western blotting (Fig. 1A, lanes 3 and 4).

To investigate whether HCV-LPs were secreted from Huh/c-p7/SGR and Huh/c-NS2/SGR cells, we analyzed the culture medium of these cell lines 6 days postinfection. As shown in Fig. 1B, HCV replicon RNA and core protein were secreted from both cell lines. Fifty milliliters of culture medium from one Huh/c-NS2/SGR-1 and Huh/c-p7/SGR-3 cell line was concentrated, layered on top of a continuous 10–60% (wt/vol) sucrose gradient in PBS and then centrifuged at 40,000 rpm at 4℃ for 16 h. Fractions were collected from the top of the tube and the concentrations of replicon RNAs and core proteins of each fraction were measured. HCV RNA and core protein were predominantly detected in the 1.15–1.20 g/ml fractions, with a peak fraction of 1.16 g/ml fraction (Fig. 2A). HCV-LPs were

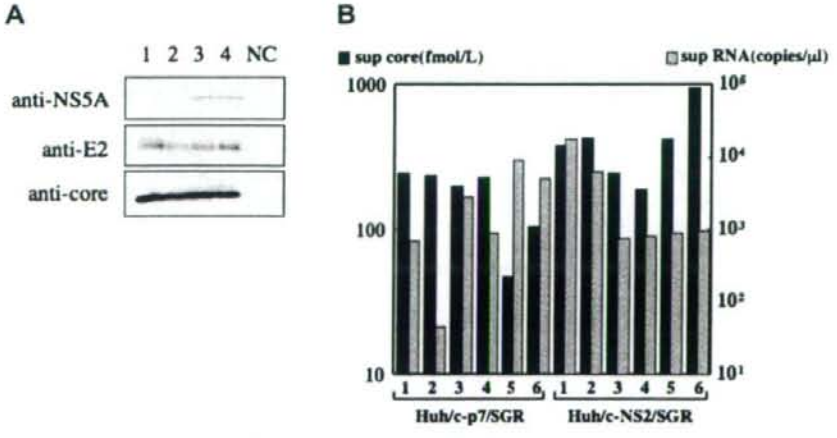

Fig. 1. (A) Western blot analysis of established cell lines. Huh/c-p7/SGR (1), Huh/c-NS2/SGR (2), Huh/c-p7/SGR-1 (3), and Huh/c-NS2/SGR-3 (4) cells were analyzed using anti-core, anti-E2, and anti-NS5A antibodies, respectively. Huh7 cells were used as a negative control. (B) Screening of G418-resistant cell lines. HCV replicon RNA and core protein of culture media of six colonies from Huh/c-p7/SGR or Huh/c-NS2/SGR cells were measured by real-time RT-PCR and ELISA, respectively. Black bars represented the concentration of core protein (fmol/l), dotted bars represented the concentration of replicon RNA (copies/μl).

observed by electron microscopy and these resembled previously reported particles (Fig. 2B)[27]. The secretion of HCV-LPs from these cell lines was maintained at almost the same level for more than 1 year (data not shown).

Infectivity of HCV-LPs

To determine whether these cell lines produced infectious HCV-LPs, we performed a colony formation assay using neomycin-resistant gene of SGR-JFH1 RNA. If HCV-LPs were infectious, SGR-JFH1 that was encapsidated in the particles would be introduced into infected cells, thus would confer neomycin resistance to the cells. To exclude the possibility that subgenomic replicon RNA in culture medium was captured by inoculated cells, Huh7 cells were also inoculated with concentrated culture medium of SGR-JFH1 cells. As shown in Fig. 3A, Huh7 cells infected with the fraction of Huh/c-NS2/SGR cells formed visible colonies 10–14 days after infection. Calculated colony forming units (cfu) of the culture medium of Huh/c-NS2/SGR cells were in the order of $5.54 \pm 2.92 \times 10^1$ cfu/ml similar to those of culture medium of JFH-1-infected cells [28]. The cells inoculated with concentrated medium of SGR-JFH1 cells formed no colonies (Fig. 3A). On the other hand, cells infected with Huh/c-p7/SGR formed no colonies, suggesting that NS2 protein was required for the infectivity of HCV-LPs. Infectivity of HCV-LPs from other cell lines of Huh/c-NS2/SGR, shown in Fig. 1, were also confirmed by colony formation assay, whereas HCV-LPs from other cell lines of Huh/c-p7/SGR showed no infectivity (data not shown).

In order to analyze the infectivity of the HCV-LPs, an immunofluorescence study was also performed. Huh7 cells infected with the Huh/c-NS2/SGR culture medium peak fraction (Fig. 2A) were positive for NS5A at 72 h postinfection (Fig. 3B), whereas the cells infected with the Huh/c-p7/SGR culture medium peak fraction were negative for NS5A (Fig. 3B), suggesting that the expression of NS2 protein in infected cells was critical for the infectivity of the HCV-LPs. The infectivity of the Huh/c-NS2/SGR culture medium was calculated to be $3.4 \pm 0.6 \times 10^2$ ffu/ml. The Cfu of this culture medium was determined to be approximately 16% of ffu, likely because only a portion of introduced replicon could render neomycin resistance to the infected cells. The cells infected with JFH-1 showed spread of infection 72 h postinfection. On the other hand, the cells infected with the Huh/c-NS2/SGR culture medium peak fraction showed very limited or no spread of infection (Fig. 3B). Moreover, no NS5A-positive cells were observed when we inoculated new Huh7 cells with the concentrated culture medium from Huh7 cells that were infected the Huh/c-NS2/SGR culture medium peak fraction (Fig. 3B, reinfection), suggesting that HCV-LPs produced by Huh/c-NS2/SGR cells supported only a single-round of infection.

We also measured the infectivity of the 12 sucrose density gradient fractions of the culture medium of Huh/c-NS2/SGR cells. The density of the peak of infectivity was lower than the peak densities of the core protein and replicon RNA (Fig. 2A), however this result agreed with a previous observation [29].

Neutralization of HCV-LPs infection by CD81-specific antibody

CD81 was shown to be involved in HCV entry. To determine whether HCV-LPs formed in Huh/c-NS2/SGR cells were infected in a CD81-dependent fashion, we incubated Huh7 cells with the peak fractions of Huh/c-NS2/SGR and Huh/c-p7/SGR cells in the presence of 10 μg/ml of CD81 specific monoclonal antibody or non-specific mouse antibody and cultured in the presence of 0.375 mg/ml of G418. After 3 weeks postinfection, colonies were fixed and the numbers of colonies were counted. CD81-specific antibody reduced the number of colonies from 132.3 ± 32.3 to 13.0 ± 11.5 ffu/

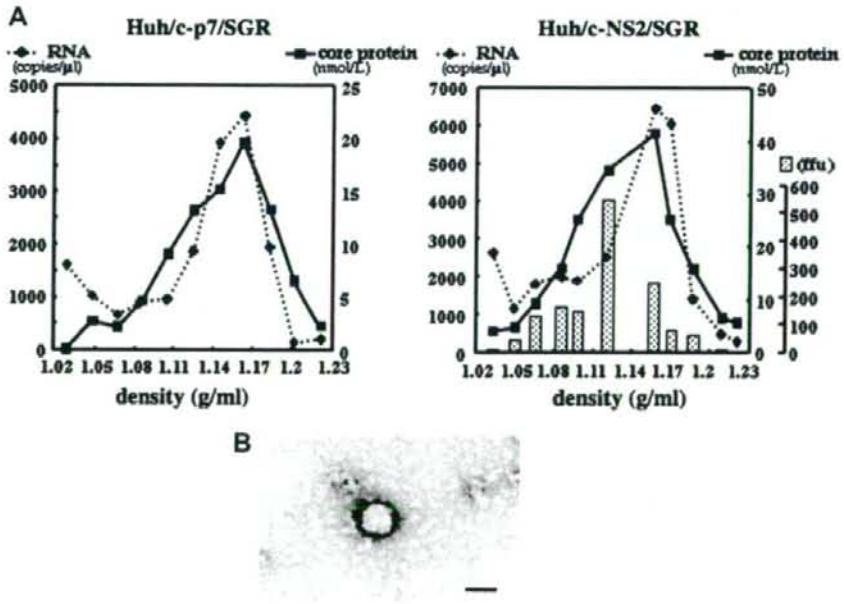

Fig. 2. (A) Sucrose density gradient analysis of culture supernatants of Huh/c-p7/SGR and Huh/c-NS2/SGR cells. Fifty milliliters of culture media collected from Huh/c-p7/SGR or Huh/c-NS2/SGR cells was concentrated to 1 ml and fractionated by ultracentrifugation at 40,000 rpm for 16 h by continuous 10–60% (wt/vol) sucrose gradient in PBS. Fractions (1 ml each) were collected from the top of the tube (12 fractions in total). HCV replicon RNA and core protein were measured by real-time RT-PCR and ELISA. The infectivity of each fraction of culture supernatant of Huh/c-NS2/SGR cells (right, lower panel) was determined by immunostaining of NS5A. (B) Electron microscopy analysis. Samples were prepared from the 1.12–1.20 g/ml fractions of culture media collected from Huh/c-NS2/SGR cells. Bar: 50 nm.

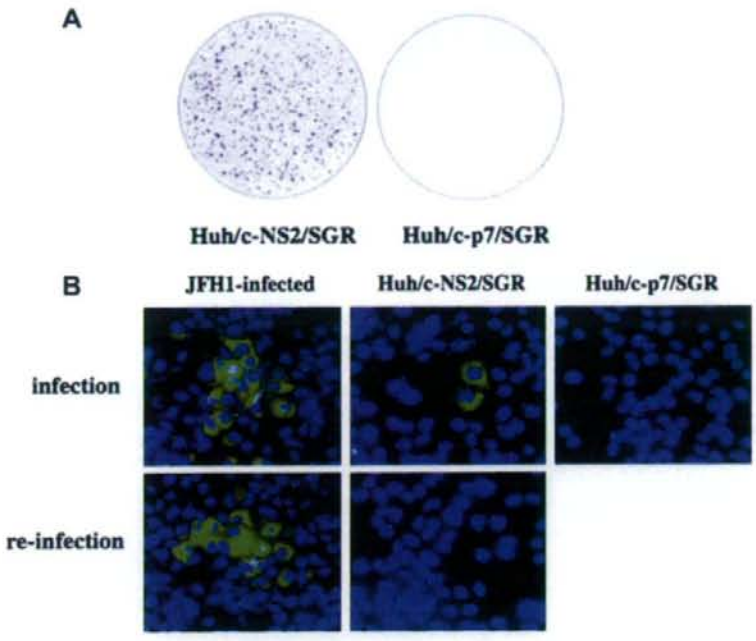

Fig. 3. (A) G418-resistant colony formation. Naive Huh7 cells were infected with 1.12–1.20 g/ml fractions of either Huh/c-p7/SGR or Huh/c-NS2/SGR cells and were cultured for 3 weeks with G418 at 0.375 mg/ml working concentration before staining with crystal violet. Experiments were performed in triplicate, and representative staining examples are shown. (B) Immunostaining experiments. Samples were serially diluted 5-fold in complete growth medium and used to infect the seeded cells (six wells per dilution). Huh7 cells in 96-well plates infected with the peak fraction of culture medium. Three days postinfection, infected cells were fixed, permeabilized with 0.3% Triton X-100 in Block Ace (Yukijirushi) and stained with anti-NS5A rabbit polyclonal antibody and Alexa488-conjugated goat anti-rabbit IgG as described previously (Murakami et al., in press). NS5A protein was shown in green. Nuclei were labeled with DAPI and were shown in blue. Re-infection shows the immunostaining of naive Huh7 cells infected with either culture media of JFH1-infected cells or that of Huh/c-NS2/SGR cells.

ml (Fig. 4), confirming that the infection of HCV-LPs to target cells is CD81-dependent and an important role of CD81 in HCV entry.

Discussion

Here we describe the development of cell lines selected to persistently harbor noncytopathic subgenomic replicons of HCV encoding neomycin resistant gene and the HCV core to NS2 cassette. The HCV-LPs secreted by this cell line are not proliferative and exhibit morphological, biophysical and antigenic properties similar to those of the putative HCV virions [27]. Jeong et al. suggested that HCV-LP is a potent immunogen for the induction of HCV-specific humoral and cellular immune responses by using baboon as a primate model [30]. Recently, replicon-based vectors of positive-stranded RNA viruses were recognized as a desirable choice of highly efficient and safe vaccines. Recent comparative analyses of vaccine potential of Kunjin virus replicons delivered as plasmid DNA, as naked RNA, and as VLPs showed a significantly better induction of immune responses to an encoded immunogen after VLP delivery than with other delivery modalities [31]. These studies suggested that HCV-LPs encapsidating its subgenomic replicon RNA are an attractive candidate for a hepatitis C vaccine. We are now constructing cell lines that secrete HCV-LPs of genotype 1a and 1b strains with this trans-packaging system and analyzing the HCV-LPs infectivity. We also showed that the expression of NS2 region is essential for infectious

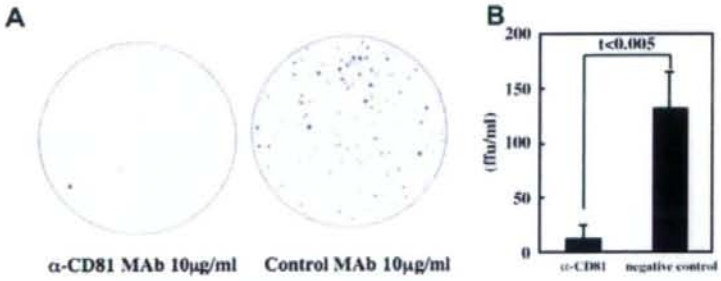

Fig. 4. Neutralization of HCV-LPs infection by CD81-specific antibody. Naive Huh7 cells were infected with peak fraction of either Huh/c-p7/SGR or Huh/c-NS2/SGR cells in the presence of 10 μg/ml of CD81 specific monoclonal antibody or nonspecific mouse antibody, then cultured 3 weeks with 0.375 mg/ml of G418. Colonies were stained with crystal violet and colony numbers were counted. (A) Colony formation. Experiments were performed in triplicate, and representative staining examples are shown. (B) Cfu of culture media per 1 ml was calculated and means ± SD was shown.

HCV-LPs production. NS2 is dispensable for RNA replication, since subgenomic replicons that lack the entire core to NS2 coding region replicate autonomously. The HCV NS2/3 protein is a highly hydrophobic protease responsible for the cleavage of the viral polypeptide between nonstructural proteins NS2 and NS3. However, many aspects of the NS2/3 protease's role in the viral life cycle and mechanism of action remain unknown. By using intergenotypic chimeras, Pietschmann et al. showed that NS2 plays an important role in the HCV morphogenesis by interacting with other NS proteins during the process of virion assembly [32]. Jones et al. reported that NS2 was required for infectious virus production and acts early in virion morphogenesis prior to the accumulation of infectious intracellular virus and indicated that the NS2 protease domain may form important interactions with other NS proteins during the process of virion assembly [33]. The results presented here also showed the importance of NS2 protein expression for the production of infectious particles, coincided with these previous observations. The mechanism NS2 plays in the process of virion morphogenesis is still unclear and remains to be determined.

In summary, we have generated a stable packaging cell line allowing production of large amounts of HCV-LPs in which the subgenomic replicon was encapsidated. The packaging cell line proved to be useful both for the production of HCV-LPs and for the encapsidation of HCV replicons for a single-round of infection.

Acknowledgments

The authors gratefully acknowledge Mami Matsuda, Mami Sasaki, and Sayaka Yoshizaki for technical assistance, Tomoko Mizoguchi for secretarial work and Grant S. Hansman for critical reading. This work was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan; by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

References

[1] Q.L. Choo, K.H. Richman, J.H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P.J. Barr, et al., Genetic organization and diversity of the hepatitis C virus, Proc. Natl. Acad. Sci. USA 88 (1991) 2451–2455.
[2] N. Kato, M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, K. Shimotohno, Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis, Proc. Natl. Acad. Sci. USA 87 (1990) 9524–9528.
[3] A. Takamizawa, C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, H. Okayama, Structure and organization of the hepatitis C virus genome isolated from human carriers, J. Virol. 65 (1991) 1105–1113.
[4] A. Grakoui, C. Wychowski, C. Lin, S.M. Feinstone, C.M. Rice, Expression and identification of hepatitis C virus polyprotein cleavage products, J. Virol. 67 (1993) 1385–1395.
[5] M. Hijikata, N. Kato, Y. Ootsuyama, M. Nakagawa, K. Shimotohno, Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis, Proc. Natl. Acad. Sci. USA 88 (1991) 5547–5551.
[6] M. Hijikata, H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, K. Shimotohno, Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus, Proc. Natl. Acad. Sci. USA 90 (1993) 10773–10777.
[7] B.D. Lindenbach, M.J. Evans, A.J. Syder, B. Wolk, T.L. Tellinghuisen, C.C. Liu, T. Maruyama, R.O. Hynes, D.R. Burton, J.A. McKeating, C.M. Rice, Complete replication of hepatitis C virus in cell culture, Science 309 (2005) 623–626.
[8] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.R. Burton, S.F. Wieland, S.L. Uprichard, T. Wakita, F.V. Chisari, Robust hepatitis C virus infection in vitro, Proc. Natl. Acad. Sci. USA 102 (2005) 9294–9299.
[9] T.F. Baumert, S. Ito, D.T. Wong, T.J. Liang, Hepatitis C virus structural proteins assemble into virus like particles in insect cells, J. Virol. 72 (1998) 3827–3836.
[10] H.J. Ezelle, D. Markovic, G.N. Barber, Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector, J. Virol. 76 (2002) 12325–12334.
[11] E. Blanchard, D. Brand, S. Trassard, A. Goudeau, P. Roingeard, Hepatitis C virus-like particle morphogenesis, J. Virol. 76 (2002) 4073–4079.
[12] A.A. Khromykh, M.T. Kenney, E.G. Westaway, Trans-complementation of flavivirus RNA polymerase gene NS5 by using Kunjin virus replicon-expressing BHK cells, J. Virol. 72 (1998) 7270–7279.
[13] A.A. Khromykh, A.N. Varnavski, E.G. Westaway, Encapsidation of the flavivirus Kunjin replicon RNA by using a complementation system providing Kunjin virus structural proteins *in trans*, J. Virol. 72 (1998) 5967–5977.
[14] T.J. Harvey, I. Anraku, R. Linedale, D. Harrich, J. Mackenzie, A. Suhrbier, A.A. Khromykh, Kunjin virus replicon vectors for human immunodeficiency virus vaccine development, J. Virol. 77 (2003) 7796–7803.
[15] C.T. Jones, C.G. Patkar, R.J. Kuhn, Construction and applications of yellow fever virus replicons, Virology 331 (2005) 247–259.
[16] R. Gehrke, M. Ecker, S.W. Aberle, S.L. Allison, F.X. Heinz, C.W. Mandl, Incorporation of tick-borne encephalitis virus replicons into virus-like particles by a packaging cell line, J. Virol. 77 (2003) 8924–8933.
[17] S.L. Hanna, T.C. Pierson, M.D. Sanchez, A.A. Ahmed, M.M. Murtadha, R.W. Doms, N-linked glycosylation of West Nile virus envelope proteins influences particle assembly and infectivity, J. Virol. 79 (2005) 13262–13274.
[18] F. Puig-Basagoiti, T.S. Deas, P. Ren, M. Tilgner, D.M. Ferguson, P.Y. Shi, High-throughput assays using a luciferase-expressing replicon, virus-like particles, and full-length virus for West Nile virus drug discovery, Antimicrob. Agents Chemother. 49 (2005) 4980–4988.
[19] T.J. Harvey, W.J. Liu, X.J. Wang, R. Linedale, M. Jacobs, A. Davidson, T.T. Le, I. Anraku, A. Suhrbier, P.Y. Shi, A.A. Khromykh, Tetracycline-inducible packaging cell line for production of flavivirus replicon particles, J. Virol. 78 (2004) 531–538.
[20] T.C. Pierson, M.D. Sanchez, B.A. Puffer, A.A. Ahmed, B.J. Geiss, L.E. Valentine, L.A. Altamura, M.S. Diamond, R.W. Doms, A rapid and quantitative assay for measuring antibody-mediated neutralization of West Nile virus infection, Virology 346 (2006) 53–65.
[21] T. Kato, T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, T. Wakita, Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon, Gastroenterology 125 (2003) 1808–1817.
[22] M. Shirakura, K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K. Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Morilshi, Y. Matsuura, T. Wakita, T. Suzuki, P.M. Howley, T. Miyamura, I. Shoji, E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein, J. Virol. 81 (2007) 1174–1185.
[23] K. Murakami, T. Kimura, M. Osaki, K. Ishii, T. Miyamura, T. Suzuki, T. Wakita, I. Shoji, Virological characterization of HCV JFH-1 strain in lymphocytes. J. Gen. Virol. (in press).
[24] H. Aizaki, K.J. Lee, V.M. Sung, H. Ishiko, M.M. Lai, Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts, Virology 324 (2004) 450–461.
[25] T. Suzuki, K. Omata, T. Satoh, T. Miyasaka, C. Arai, M. Maeda, T. Matsuno, T. Miyamura, Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival crevicular fluid of HCV-infected patients, J. Clin. Microbiol. 43 (2005) 4413–4417.
[26] M. Yi, R.A. Villanueva, D.L. Thomas, T. Wakita, S.M. Lemon, Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells, Proc. Natl. Acad. Sci. USA 103 (2006) 2310–2315.
[27] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.
[28] T. Date, M. Miyamoto, T. Kato, K. Morikawa, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, M. Mizokami, T. Wakita, An infectious and selectable full-length replicon system with hepatitis C virus JFH-1 strain, Hepatol. Res. 37 (2007) 433–443.
[29] Y. Miyanari, K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, K. Shimotohno, The lipid droplet is an important organelle for hepatitis C virus production, Nat. Cell. Biol. 9 (2007) 1089–1097.
[30] S.H. Jeong, M. Qiao, M. Nascimbeni, Z. Hu, B. Rehermann, K. Murthy, T.J. Liang, Immunization with hepatitis C virus-like particles induces humoral and cellular immune responses in nonhuman primates, J. Virol. 78 (2004) 6995–7003.
[31] I. Anraku, T.J. Harvey, R. Linedale, J. Gardner, D. Harrich, A. Suhrbier, A.A. Khromykh, Kunjin virus replicon vaccine vectors induce protective CD8+ T-cell immunity, J. Virol. 76 (2002) 3791–3799.
[32] T. Pietschmann, A. Kaul, G. Koutsoudakis, A. Shavinskaya, S. Kallis, E. Steinmann, K. Abid, F. Negro, M. Dreux, F.L. Cosset, R. Bartenschlager, Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras, Proc. Natl. Acad. Sci. USA 103 (2006) 7408–7413.
[33] C.T. Jones, C.L. Murray, D.K. Eastman, J. Tassello, C.M. Rice, Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus, J. Virol. 81 (2007) 8374–8383.

JOURNAL OF VIROLOGY, June 2008, p. 5715–5724
0022-538X/08/$08.00+0 doi:10.1128/JVI.02530-07

Vol. 82, No. 12

Critical Role of Virion-Associated Cholesterol and Sphingolipid in Hepatitis C Virus Infection▽

Hideki Aizaki,[1] Kenichi Morikawa,[1] Masayoshi Fukasawa,[2] Hiromichi Hara,[1] Yasushi Inoue,[1] Hideki Tani,[3] Kyoko Saito,[2] Masahiro Nishijima,[2] Kentaro Hanada,[2] Yoshiharu Matsuura,[3] Michael M. C. Lai,[4] Tatsuo Miyamura,[1] Takaji Wakita,[1] and Tetsuro Suzuki[1]*

Department of Virology II[1] and Department of Biochemistry and Cell Biology,[2] National Institute of Infectious Diseases, Tokyo 162-8640, and Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871,[3] Japan, and Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California 90033-1054[4]

Received 27 November 2007/Accepted 17 March 2008

In this study, we establish that cholesterol and sphingolipid associated with hepatitis C virus (HCV) particles are important for virion maturation and infectivity. In a recently developed culture system enabling study of the complete life cycle of HCV, mature virions were enriched with cholesterol as assessed by the molar ratio of cholesterol to phospholipid in virion and cell membranes. Depletion of cholesterol from the virus or hydrolysis of virion-associated sphingomyelin almost completely abolished HCV infectivity. Supplementation of cholesterol-depleted virus with exogenous cholesterol enhanced infectivity to a level equivalent to that of the untreated control. Cholesterol-depleted or sphingomyelin-hydrolyzed virus had markedly defective internalization, but no influence on cell attachment was observed. Significant portions of HCV structural proteins partitioned into cellular detergent-resistant, lipid-raft-like membranes. Combined with the observation that inhibitors of the sphingolipid biosynthetic pathway block virion production, but not RNA accumulation, in a JFH-1 isolate, our findings suggest that alteration of the lipid composition of HCV particles might be a useful approach in the design of anti-HCV therapy.

Hepatitis C virus (HCV) is recognized as a major cause of chronic liver disease, including chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma. It presently affects approximately 200 million people worldwide (26). HCV is an enveloped positive-strand RNA virus belonging to the *Hepacivirus* genus of the family *Flaviviridae*. Its genome of ~9.6 kb encodes a polyprotein precursor of ~3,000 residues, and the structural proteins (core, E1, and E2) reside in its N-terminal region.

Little is known about the assembly of HCV and its virion structure, because efficient production of authentic HCV particles has only recently been achieved. Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. This process is thought to occur upon interaction of the core protein with viral RNA, and this core-RNA interaction may induce a change from RNA replication to packaging. As with related viruses, the mature HCV virion likely consists of a nucleocapsid and an outer envelope composed of a lipid membrane and envelope proteins. Expression of the structural proteins in mammalian cells has been observed to generate virus-like particles with ultrastructural properties similar to those of HCV virions (5, 29). Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the endoplasmic reticulum (ER) has also been observed (8, 34). However, HCV structural proteins are observed both in the ER and in the Golgi apparatus (45). Moreover, complex N-linked glycans have been detected on the surfaces of HCV particles isolated from patient sera, suggesting that the glycans transit through the Golgi apparatus (44). Interactions between the core and E1/E2 proteins are thought to determine viral morphology and are mediated through a cytoplasmic loop present in the polytopic form of E1 (35). Recently, we and others have identified a unique HCV genotype 2a isolate, JFH-1, that is able to replicate and produce high levels of infectious virus in culture (HCVcc) (54, 56), enabling us to investigate new aspects of the HCV life cycle.

In this study, we examine the importance of cholesterol and sphingolipid in association with the HCV membrane in virion maturation and virus infectivity. Mature HCV particles are rich in cholesterol. Cholesterol depletion or hydrolysis of sphingolipid from HCV particles results in a loss of infectivity. We further demonstrate a requirement for virion-associated cholesterol and sphingolipid for viral entry.

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line Huh-7, which is permissive to HCV infection, was obtained from Francis V. Chisari (The Scripps Research Institute). Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM)–10% fetal bovine serum. Huh-7 cell lines, which carry subgenomic replicon RNA of either the JFH-1 (20) or the N (11, 17) strain, were cultured as previously described (21, 46).

Reagents. The primary antibodies used in this study were mouse monoclonal antibodies against vesicular stomatitis virus glycoprotein (VSV-G) (Sigma, St. Louis, MO), HCV E1 (54) and E2 (Biodesign International, Saco, ME), caveolin-2 (New England Biolabs, Beverly, MA), and CD81 (BD Pharmingen, Franklin Lakes, NJ), as well as rabbit polyclonal antibodies against calnexin (Stressgen, Ann Arbor, MI) and HCV core (48). ISP-1/myriocin, cholesterol, and

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111. Fax: 81 3 5285 1161. E-mail: tesuzuki@nih.go.jp.

▽ Published ahead of print on 26 March 2008.

heparinase I were purchased from Sigma, and recombinant *Bacillus cereus* sphingomyelinase (SMase) was obtained from Higeta Shoyu (Tokyo, Japan). (1*R*,3*R*)-*N*-(3-Hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12), which was synthesized as described elsewhere (24), was a gift from Shu Kobayashi (University of Tokyo).

Plasmids. pCAE1 and pCAE2 contain HCV cDNAs spanning the E1 region (amino acids 192 to 383) with a FLAG tag at the N terminus and the E2 region (amino acids 384 to 809) with a Myc tag at the N terminus of strain NIHJ1 (1), respectively, under the control of the CAG promoter (38). pCAV340V and pCAV711V consist of the ectodomains of E1 and E2, respectively, with the N-terminal signal sequences, transmembrane domains, and cytoplasmic domains derived from VSV-G, as described elsewhere (50) (see Fig. 4D).

Virus production. Plasmid pJFH1, containing full-length cDNA of the JFH-1 isolate, was used to generate HCVcc as described elsewhere (23, 33, 34, 54). pJ6/JFH was obtained from JFH1 by replacement of the 5′ untranslated region to the p7 region (EcoRI-BclI) of J6. In vitro-transcribed RNA from linearized pJFH1 or pJ6/JFH1 was delivered to Huh-7 cells by electroporation. Culture supernatants were collected at 72 h posttransfection, clarified by low-speed centrifugation, passed through a 0.45-mm-pore-size filter, and concentrated using an Amicon Ultra-15 unit (Millipore, Bedford, MA) or by ultracentrifugation (23). Infectious titers, HCV RNA copies, and core protein concentrations of the viral stocks were $\sim 5 \times 10^3$ focus-forming units per ml, $\sim 1 \times 10^7$ copies/ml, and $\sim 1 \times 10^4$ fmol/liter, respectively. HCVcc was isolated by a combination of ultrafiltration, ion-exchange chromatography, heparin affinity chromatography, and sucrose density ultracentrifugation (33; K. Morikawa and T. Wakita, unpublished data). Pseudotyped VSV containing E1 and E2 proteins of the HCV genotype 1a isolate H77c (HCVpv) was generated as previously described (51). Briefly, 293T cells transiently expressing E1 and E2 proteins (strain H77) were infected with VSVdelG-GFP/G, in which the G envelope gene was replaced with green fluorescent protein (GFP) and pseudotyped with VSV-G.

Determination of cholesterol and phospholipid contents of HCVcc and infected cells. Cellular and viral lipids were extracted from isolated HCVcc and from uninfected and infected Huh-7 cells. Cholesterol content was determined using the cholesterol oxidase method as previously described (14). Total phospholipid content was determined using the method of Rouser et al. (42).

Cholesterol depletion and replacement. To remove cholesterol from the HCV envelope, stock samples of HCVcc were treated with *methyl*-β-cyclodextrin (B-CD) in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma) and nonessential amino acids (Invitrogen, Carlsbad, CA) for 1 h at 37°C, followed by centrifugation at 100,000 × *g* for 3 h to form a pellet, which was resuspended in 0.5 ml of the medium. In order to replenish cholesterol, the medium of HCVcc treated with 5 mg/ml B-CD was replaced with DMEM containing various concentrations of exogenous cholesterol (Sigma) and incubated for 1 h, followed by centrifugation to form a pellet. In order to perform HCVcc infection assays, Huh-7 cells were infected with HCVcc, with or without the treatment described above, for 1 h at 37°C and then washed as described above. Viral core protein levels in the cells and in the supernatant were quantified 72 h later using an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan).

SMase treatment. HCVcc was treated with SMase at various concentrations in DMEM for 1 h at 37°C and was then centrifuged at 100,000 × *g* for 3 h to form a pellet, which was resuspended in 0.5 ml of medium for the infection assays.

HCVcc binding and internalization assays. To monitor binding, cells grown in a 6-well plate were preincubated for 1 h at 4°C, after which B-CD- or SMase-treated HCVcc was bound to the cells for 1 h at 4°C. As a measure of virus internalization, following the virus binding procedure, the cells were warmed to 37°C and maintained for 2 h, after which they were treated with 0.25% trypsin for 10 min at 37°C. Huh7-25, a CD81-negative Huh-7 subclone (3), was used to ensure removal of surface-bound virus by trypsin treatment. For both the binding and internalization assays, the resulting cells, as described above, were washed with ice-cold phosphate-buffered saline, followed by lysis with TRIzol reagent (Invitrogen). Cell-associated virus was quantified by measuring the amount of HCV RNA in the cell lysate by the real-time reverse transcription-PCR method (2, 34). Cells were treated with heparinase as previously described (33).

HCV replication assay in HCVcc-infected or replicon cells. HCV subgenomic replicon cells or cells infected with HCVcc were treated with various concentrations of inhibitors for 72 h. Total RNA was isolated from replicon cells using TRIzol reagent (Invitrogen), followed by quantification of HCV RNA by real-time reverse transcription-PCR as previously described (2, 34). Levels of core protein in the culture supernatants of HCVcc-infected cells were tested as described above.

Detection of cholesterol content of HCVcc. For [^{3}H]cholesterol labeling of viruses, HCVcc-infected or uninfected cells were incubated with 50 mCi of [1α,2α-^{3}H]cholesterol in DMEM for 24 h. Culture supernatants of the cells were incubated in the presence or absence of B-CD at 5 mg/ml for 1 h at 37°C, followed by ultracentrifugation on a 60% sucrose cushion. The virus-containing fractions and corresponding fractions from an uninfected culture were lysed in the buffer containing 1% Triton X-100 (TX-100), and radioactivity was quantified by scintillation counting. Radioactivities (in counts per minute) of HCVcc samples were determined by subtracting the radioactivity of uninfected cells from that of HCVcc-infected cells.

Metabolic labeling analysis of sphingolipid content. After 2 h of incubation with [^{14}C]serine (0.5 mCi/ml) in Opti-MEM (Invitrogen), the cells were lysed with 0.1% sodium dodecyl sulfate, and total lipid was extracted with chloroform-methanol (1:2, vol/vol). The extracts were spotted onto silica gel 60 plates (Merck, Darmstadt, Germany) and chromatographed with methyl acetate–1-propanol–chloroform–methanol–0.25% KCl (25:25:25:10:9, vol/vol). Radioactive spots were quantitatively detected by BAS 2000 (Fuji Film, Japan).

Membrane flotation assay. The membrane flotation assay was performed as previously described (46).

TABLE 1. Cholesterol and phospholipid contents of HCVcc and cells

Cell type or virus	Content (nmol/mg of protein)[a]		Chol/PL ratio
	Chol	PL	
Cells			
Uninfected	105.9 ± 10.4	253.2 ± 10.6	0.42
JFH-1 infected	116.5 ± 10.0	292.0 ± 18.4	0.40
Virus			
JFH-1	43.6 ± 2.4	33.8 ± 1.8	1.29
J6/JFH-1[b]	28.7 ± 4.8	22.7 ± 2.9	1.26

[a] Data are averages of three independent measurements ± standard deviations. Chol, cholesterol; PL, phospholipids.

[b] J6/JFH1 virus was produced from the pJ6/N2X-JFH1 construct and has structural proteins from the J6CF strain.

RESULTS

Critical role of virion-associated cholesterol. A role of virion-associated cholesterol in infectivity has been demonstrated for several enveloped viruses (4). However, little is known about the role of lipids associated with the virions of flaviviruses, including HCV, and their contribution to the viral life cycle. To determine the lipid composition of mature HCV virions, we extracted total lipid from HCVcc (JFH-1 and chimeric J6/JFH-1) prepared from the culture supernatants of cells infected with HCV, as well as the total cellular membrane fractions of uninfected and infected Huh-7 cells. The cholesterol and phospholipid contents were quantified, because these are the two major lipid constituents of biological membranes. The cholesterol-to-phospholipid molar ratio, which is known as a parameter of membrane viscosity (47), was significantly higher in virus samples (1.29 and 1.26 for JFH-1 and J6/JFH-1, respectively) than in cell membrane samples (0.40 and 0.42 for JFH-1-infected and uninfected cells, respectively) (Table 1). The ratios in viral samples were similar to or greater than those in mammalian plasma membranes, where most cellular cholesterol is found. Minimal contamination of the viral samples with extracellular microvesicles likely occurred, since only a small amount of lipid was detected in a sample prepared from the culture medium of uninfected cells (data not shown). Thus, it is likely that HCV virions are enriched with cholesterol during assembly and maturation.

To investigate a potential role for the particular lipid composition of HCV particles, HCVcc was treated with

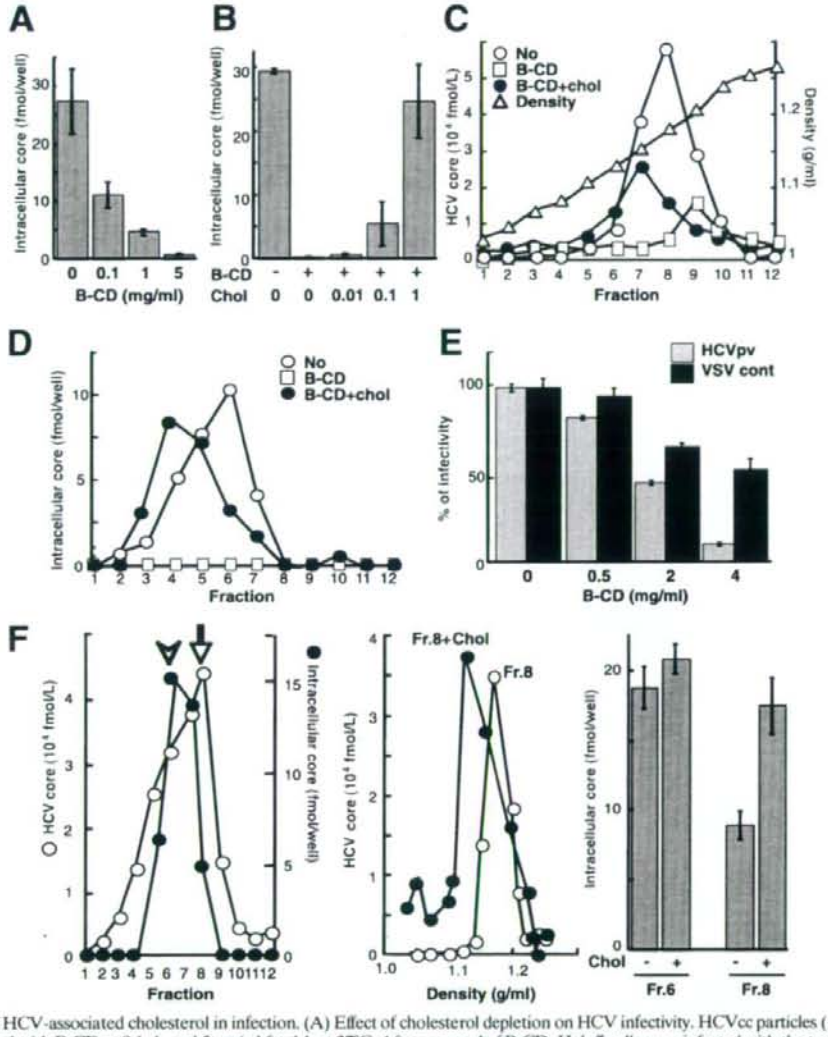

FIG. 1. Role of HCV-associated cholesterol in infection. (A) Effect of cholesterol depletion on HCV infectivity. HCVcc particles (~2 fmol of the core protein) were treated with B-CD at 0.1, 1, and 5 mg/ml for 1 h at 37°C. After removal of B-CD, Huh-7 cells were infected with the treated virus particles, after which the core protein content of infected cells at 72 h p.i. was determined as an indicator of infectivity, as previously established (24). (B) Effect of cholesterol replenishment on infectivity. After treatment with 5 mg/ml B-CD, virus was treated either with medium alone or with medium containing exogenous cholesterol for 1 h at 37°C. (C) Effect of cholesterol depletion and replenishment on density gradient profiles of the viral particles. The HCVcc treated with 5 mg/ml B-CD was replenished with exogenous cholesterol (1 mM) and then separated by 10-to-60% sucrose gradient ultracentrifugation. The core protein in each fraction was measured. The density of each fraction was determined by refractive index measurement. (D) Effects of cholesterol depletion and replenishment on viral infectivity. Each fraction (see panel C) was infected, and then the core proteins in the cells were measured at 72 h p.i. (E) Effect of cholesterol depletion on the infectivity of HCVpv (genotype 1a) (shaded bars) or the control, VSVdelG-GFP/G (solid bars). The viruses were preincubated with B-CD for 1 h at 37°C before infection. (F) (Left) The culture medium from HCVcc-producing cells was fractionated as described above. For each fraction, the amounts of core and intracellular core (infectivity) are plotted. Peaks of the core (arrow) and infectivity (arrowhead) are indicated. (Center) An aliquot of fraction 8 (peak of the core) was treated with 1 mM cholesterol for 1 h at 37°C. The resultant aliquot and an untreated aliquot of the fraction were subjected to sucrose gradient ultracentrifugation. The core in each fraction was plotted. (Right) The infectivities of fractions (Fr.) 6 and 8 (see the left panel) with or without cholesterol treatment were determined as shown above. Data are means from four independent experiments. Error bars, standard deviations.

increasing concentrations (0.1 to 5 mg/ml) of B-CD, which is known to extract cholesterol from membranes (40). The viral samples were then used to inoculate Huh-7 cells after removal of B-CD by ultracentrifugation. Infectivity was evaluated by quantifying the viral core protein in cells at 72 h postinfection (p.i.). Using an immunoassay that provides results indicative of HCV infectivity (25), we also confirmed a good correlation between the core level and

TABLE 2. Depletion of virion-associated cholesterol by B-CD

Treatment	Radioactivity (cpm) of HCVcc[a]		Avg (%[b])
	Expt 1	Expt 2	
None	5,327	5,573	5,450 (100)
B-CD (5 mg/ml)	3,643	1,646	2,644 (48.5)

[a] Determined by subtracting the radioactivity of uninfected cells from that of HCVcc-infected cells in two experiments.
[b] Percentage of the radioactivity of the untreated sample.

infectious titers (data not shown). As shown in Fig. 1A, core protein levels following B-CD treatment at 0.1, 1, or 5 mg/ml were reduced by 60, 83, or 98%, respectively, from the levels with the untreated virus. The cholesterol level of HCVcc treated with 5 mg/ml B-CD was found to be ~50% of that of untreated virions (Table 2).

To demonstrate that the reduced infection efficiency of B-CD-treated virus was caused by the reduced cholesterol content of the viral envelope, we attempted to reverse the inhibitory effect by adding exogenous cholesterol. Following treatment of HCVcc with 5 mg/ml B-CD, the drug was washed out, and increasing concentrations of cholesterol were added in an attempt to reconstitute the normal virion cholesterol content. The addition of 1 mM cholesterol completely reversed the virus infectivity (Fig. 1B). After cholesterol was replenished, the viral RNA was restored to a level similar to that in the untreated control.

To investigate the effect of cholesterol on the density of infectious HCV virions, B-CD-pretreated or untreated viral samples, as well as cholesterol-replenished treated viral samples, were subjected to sucrose density gradient centrifugation (Fig. 1C). The density of HCVcc core protein at its peak concentration in untreated virus samples was ~1.17 g/ml. When virion-associated cholesterol was removed by B-CD, the density of HCVcc core protein at its peak concentration was shifted to 1.20 g/ml. Addition of exogenous cholesterol to this cholesterol-depleted sample restored a lower-density fraction (1.15 g/ml). Figure 1D illustrates the infectivity of each gradient fraction. Untreated virus had maximum infectivity at ~1.13 g/ml (fraction 6), while, as expected, fractions from B-CD-treated viral samples exhibited minimal to no infectivity. Replenishment of depleted virus with cholesterol returned infectivity to untreated-control levels, and cholesterol-replenished virus had a buoyant density of ~1.07 g/ml (fraction 4), suggesting that HCV-associated cholesterol is crucial for viral infectivity and that the effect of a cholesterol-depleting drug is reversible. We further observed that B-CD treatment of a pseudotyped VSV containing the E1 and E2 proteins of the HCV genotype 1a isolate H77c (HCVpv) resulted in a progressive loss of infectivity, while B-CD had significantly less impact on the infectivity of the control virus VSVdelG-GFP/G (Fig. 1E).

The results described above raise the possibility that the infectivity of HCV virions with relatively low levels of incorporated cholesterol might be enhanced by supplementation with exogenous cholesterol. Density gradient fractions of culture supernatants collected from HCV-infected cells were analyzed with regard to the presence of core protein and infectivity (Fig. 1F, left). As indicated above, maximum infectivity was obtained with fraction 6 (1.13 g/ml). In contrast, a major fraction of core protein banded at a higher density (1.17 g/ml) in fraction 8. We hypothesized that fraction 8 contains lipids at lower levels than those in fraction 6. However, quantification of lipids, including cholesterol, in the fractions obtained failed, presumably due to a low sensitivity of detection. Thus, to extend our findings on the involvement of cholesterol, we added exogenous cholesterol to fraction 8, followed by ultrafiltration to remove unincorporated cholesterol. A subsequent density gradient profile demonstrated a shift in the core protein peak to 1.13 g/ml (Fig. 1F, center). A concomitant increase in the infectivity of the fraction, approaching that of untreated fraction 6, was observed (Fig. 1F, right). In contrast, supplementation of fraction 6 with exogenous cholesterol did not alter its infectivity (Fig. 1F, right) or change its density gradient (data not shown). These results suggest that exogenous cholesterol supplementation can reverse deficits in the infectivity of HCV virions due to low cholesterol content.

Sphingolipid dependence of HCV infectivity. In addition to cholesterol, sphingolipid is a major component of eukaryotic lipid membranes. We therefore investigated the functional significance of sphingomyelin (SM), the most abundant sphingolipid, with regard to HCV infectivity. HCVcc was treated for 1 h with increasing concentrations (0.1 to 10 U/ml) of bacterial SMase, which is known to hydrolyze membrane-bound SM to ceramide. Following ultracentrifugation to remove the SMase, Huh-7 cells were inoculated with the HCVcc. The amount of HCV core protein within the cells was quantified at 72 h p.i. Figure 2A shows 50 and 90% reductions in HCV infectivity after incubation of the virion with 0.1 and 1 U/ml SMase, respectively. We further observed that SMase treatment of HCVpv resulted in a progressive loss of infectivity, while SMase had no effect on the infectivity of the control virus (Fig. 2B). This demonstrates that sphingolipid, like cholesterol, plays an essential role in HCV infectivity.

Requirement for virion-associated cholesterol and sphingolipid during HCV cell entry. These findings support the idea that virion-associated cholesterol and sphingolipid may influence viral entry into host cells by altering the interaction between viral particles and a host cell factor(s). Viral entry is a multistep process including binding of the virion to the cell surface and internalization into the cytoplasm by endocytosis. To examine whether virion-associated cholesterol and SM might play a role in cell binding or postbinding events during viral entry, we used a binding assay in which Huh-7 cells preincubated for 1 h at 4°C were infected with B-CD- or SMase-treated HCVcc. Total RNA was extracted after a 1-h addition of the virions at 4°C, followed by quantification of HCV RNA. As shown in Fig. 3A, treatment of the virions with either B-CD or SMase had little influence on their ability to bind to cells.

It has been shown that CD81 plays an important role in HCV internalization but is not correlated with viral attachment (7, 33). An anti-CD81 antibody was used as a negative control for reduced viral attachment. It is likely that heparan sulfate proteoglycan on the target cell surface is needed for the initial attachment of HCV (33). Thus, heparinase I was used as a positive control for reduced HCV attachment to the cells. To examine the roles of cholesterol and sphingolipid on the HCVcc membrane in viral internalization, a virus-cell mixture

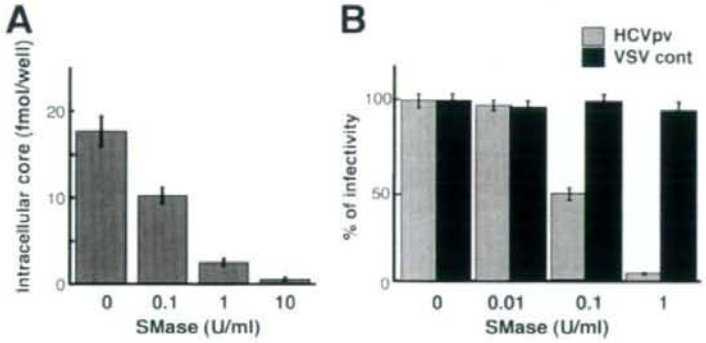

FIG. 2. Effect of SM hydrolysis on viral infectivity. (A) Effect on the infectivity of HCVcc. HCVcc was treated with 0.1, 1, or 10 U/ml SMase for 1 h at 37°C, after which SMase was removed by ultracentifugation. Huh-7 cells were infected with the treated virus, and the core protein content of infected cells was determined at 72 h p.i. (B) Effect on the infectivity of HCVpv (genotype 1a) (shaded bars) or the control, VSVdelG-GFP/G (VSV cont) (solid bars). The viruses were preincubated with SMase for 1 h at 37°C before infection. Data are means from four independent experiments. Error bars, standard deviations.

prepared at 4°C as described above was incubated for 2 h at 37°C, followed by trypsinization to remove virions that were surface bound but not internalized (Fig. 3B). We verified that 94% of surface-bound-viruses were removed by trypsinization using CD81-negative Huh-7 subclones. A marked reduction in viral RNA levels within cells was detected after pretreatment of the virus with either B-CD or SMase. These results strongly suggest that virion-associated cholesterol and sphingolipid function as key determinants of internalization but not of cell attachment.

Association of HCV structural proteins with lipid rafts. Cholesterol and sphingolipid are major components of lipid rafts, which can be isolated as detergent-resistant membranes (DRMs) by treatment with cold TX-100, followed by equilibrium flotation centrifugation. Matto et al. (30) reported that HCV core protein is associated with DRMs in cells carrying the full-length HCV replicon. To investigate whether HCV structural proteins are associated with DRMs in HCVcc-producing cells, lysates from cells infected with HCVcc were subjected to membrane flotation analysis. In the absence of detergent treatment, the majority of the core (Fig. 4A) and E1 (Fig. 4B) proteins were detected in the membrane fractions. After treatment with cold TX-100, significant amounts of both viral proteins were recovered from the DRM fraction. However, after treatment with TX-100 at 37°C, the majority of the E1 and core proteins had shifted to the detergent-soluble fractions. We also found that HCV genotype 1b E1 and E2 can be associated with the lipid raft in 293T cells transfected with an E1 or E2 expression plasmid (Fig. 4C) and that the cytoplasmic tails of envelope

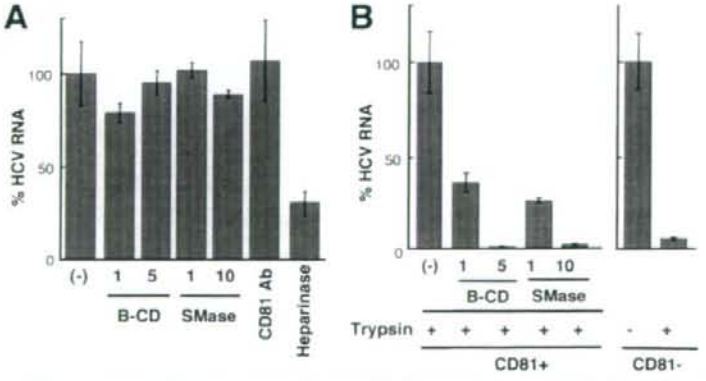

FIG. 3. Effects of B-CD or SMase on virus attachment and internalization. (A) Virus attachment to Huh-7 cells was determined at 4°C after treatment of HCVcc with B-CD (1 or 5 mg/ml) or SMase (1 or 10 U/ml). An antibody (Ab) against CD81 was used, in order to ensure that the antibody did not inhibit HCVcc binding (7, 33). Heparinase was used to reduce HCV attachment to the cell. Viral RNA copies were normalized to total cellular RNA, and the normalized RNA copies in the mock-treated sample (−) were arbitrarily set at 100%. (B) Virus internalization was measured in Huh7-25, a CD81-negative subclone (CD81$^-$) (3), and Huh7-25-CD81, which stably expresses CD81 (CD81$^+$), after treatment of the virions with B-CD or SMase. After internalization for 2 h at 37°C, cells were exposed to trypsin (trypsin +) or phosphate-buffered saline (trypsin −). Huh7-25 was used to ensure that surface-bound virus would be removed by trypsin treatment. The amounts of HCV RNA in Huh7-25 and Huh7-25-CD81 cells infected with untreated HCVcc were assigned the arbitrary value of 100%, respectively. Results are representative of four independent experiments.

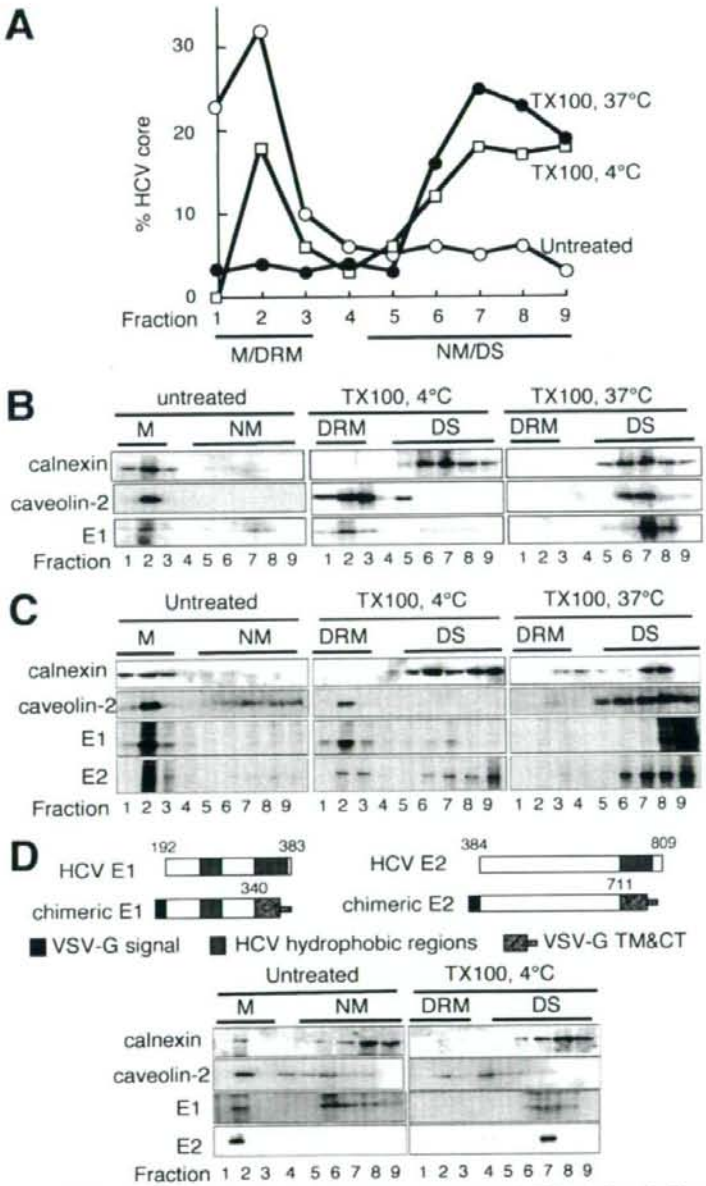

FIG. 4. Compartmentation of HCV structural proteins within DRM fractions. Lysates of HCVcc-infected cells were either treated with 1% TX-100, either on ice or at 37°C, or left untreated, followed by sucrose gradient centrifugation. (A and B) For each fraction, the amount of core protein was determined by an enzyme-linked immunosorbent assay (A), and E1, calnexin, and caveolin-2 were analyzed by Western blotting (B). The amount of core protein in each lysate (TX-100, 37°C; TX-100, 4°C; Untreated) was assigned the arbitrary value of 100%. M, membrane; NM, nonmembrane; DS, detergent soluble. (C) Lysates of 293T cells expressing HCV E1 or E2 protein were either treated with 1% TX-100, either on ice or at 37°C, or left untreated, followed by discontinuous sucrose gradient centrifugation. Each fraction was concentrated in a Centricon YM-30 filter unit and subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with antibodies against calnexin, caveolin-2, Myc (E1), or FLAG (E2). (D) (Top) Structures of HCV envelope genes used. Amino acid positions of HCV are indicated. Signal sequence, transmembrane (TM), and cytoplasmic tail (CT) domains of VSV G protein are shown. (Bottom) Cell lysates expressing chimeric HCV E1 or E2 protein were treated with 1% TX-100 on ice or left untreated, followed by discontinuous sucrose gradient centrifugation. It has been reported that VSV-G is not associated with lipid (39). Calnexin, caveolin-2, and chimeric glycoproteins (chimeric E1 and chimeric E2) were analyzed by immunoblotting. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

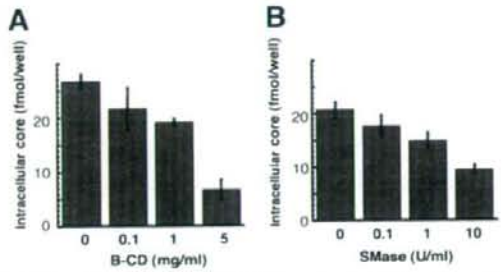

FIG. 5. Effects of B-CD or SMase treatment of cells on HCV infectivity. Huh-7 cells were either left untreated or treated with B-CD at 0.1, 1, or 5 mg/ml (A) or with SMase at 0.1, 1, or 10 U/ml (B) prior to HCVcc infection. Intracellular core levels were quantitated 72 h p.i. Data are means from four independent experiments. Error bars, standard deviations.

proteins are important for their interaction (Fig. 4D). These data suggest that subpopulations of HCV structural proteins are associated with lipid rafts in cells generating the HCV particles.

Moderate inhibition of HCV infection by B-CD or SMase treatment of host cells. It has recently been reported that cholesterol depletion or SM hydrolysis from the host cell membrane decreases HCV infection, in part by decreasing the level of CD81 on the cell surface (19, 53). The involvement of the lipid environment of the host cell plasma membrane in HCV infection was investigated in our HCVcc infection system. Prior to infection, Huh-7 cells were treated with B-CD or SMase and then washed with the medium five times. Cholesterol depletion from Huh-7 cells by B-CD at 1 or 5 mg/ml inhibited HCV core levels by 20 and 75%, respectively, compared to levels in untreated cells (Fig. 5A). We also found that hydrolysis of SM by SMase at 1 or 10 U/ml on the cells, respectively, led to moderate reduction of the viral infection, by 20 or 55% of the infection level of the untreated control (Fig. 5B). There was no influence on cell viability under the conditions of these treatments (data not shown). These findings, compared with the results in Fig. 1A and 2A, suggest that the raft-like environment on the plasma membrane likely serves as a portal for HCV entry, but HCV virion-associated cholesterol and sphingolipid more readily play more critical roles in viral infection.

Inhibitors of the sphingolipid biosynthetic pathway suppress the production of HCVcc, but not RNA replication, for a JFH-1-derived replicon. In the course of studying the involvement of lipid metabolism in the HCV life cycle, we observed that inhibitors of the sphingolipid biosynthetic pathway, including ISP-1 and HPA-12, which specifically inhibit serine palmitoyltransferase (31) and ceramide trafficking from the ER to the Golgi apparatus (55), influenced subgenomic replicons derived from the HCV-N isolate (genotype 1b), but not those derived from JFH-1. A dose-dependent decrease in HCV RNA copy numbers among HCV-N replicon cells was observed upon exposure to ISP-1 or HPA-12, as previously reported (43, 52). In contrast, these compounds had little or no effect on viral RNA accumulation in JFH-1 replicon cells (Fig. 6A). Furthermore, these compounds did not affect luciferase activity in the lysates of Huh-7 cells transfected with an in vitro-transcribed JFH-1 replicon RNA containing a luciferase reporter gene (22) (data not shown). Figure 6B shows the effects of ISP-1 and HPA-12 on de novo sphingolipid biosynthesis by replicon cells. No differences in the inhibitory effects of each compound were observed in replicon cells derived from HCV-N versus JFH-1. When de novo synthesis of sphingolipids was examined by metabolic labeling with [^{14}C]serine, ISP-1 almost completely inhibited the production of both ceramide and SM, while HPA-12 greatly inhibited the synthesis of SM but not ceramide. Levels of phosphatidylethanolamine and phosphatidylserine, into which serine is incorporated by a pathway distinct from that of sphingolipid biosynthesis, were not influenced by these drugs. These results suggest that suppression of HCV RNA replication by inhibitors of sphingolipid biosynthesis might be dependent on the viral genotype or isolate.

This observation prompted us to investigate whether inhibitors of the sphingolipid biosynthetic pathway might have the ability to prevent HCV virion production. Interestingly, when Huh-7 cells producing JFH-1 HCVcc were treated with ISP-1 or HPA-12 under conditions similar to those the replicon cells, viral core levels in the culture supernatants were greatly reduced in a dose-dependent manner. For example, exposure to 10 μM ISP-1 or 1 μM HPA-12 reduced viral core protein levels more than 85% from those for control cells (Fig. 6C). The 50% inhibitory concentrations of both drugs were less than 0.1 μM, 50-fold less than those obtained for the RNA replication of the HCV-N-replicon. Together, these results suggest that the sphingolipid biosynthetic pathway plays an important role in the production of HCV particles, but not in genome replication, in JFH-1-based HCVcc.

DISCUSSION

In this study, we demonstrated the role of HCV virion-associated cholesterol and sphingolipid in viral infectivity. Although dependence on virion-associated cholesterol for virus entry has been shown for a number of viruses (4, 6, 28, 49), this is the first study to demonstrate the importance of envelope cholesterol in a virus belonging to the family *Flaviviridae*. Furthermore, to our knowledge, the functional role of virion membrane-associated SM has not been examined in viruses. Our previous studies using Chinese hamster ovary cell mutants deficient in SM synthesis have demonstrated that reduction of cellular SM levels enhances cellular cholesterol efflux in the presence of B-CD (9, 12). Thus, it may be possible that SM plays a role in the retention of cholesterol on HCV particles due to interaction between cholesterol and SM. The finding that B-CD or SMase treatment of HCVcc markedly inhibited virus internalization but not cell attachment (Fig. 3) suggests that HCV membrane-associated cholesterol and sphingolipid are crucial for the interaction of viral glycoproteins with the virus-receptor/coreceptor required for cell entry. Cholesterol depletion or sphingolipid hydrolysis might induce a conformational change in the viral envelope, resulting in instability of the virion structure. Since the cholesterol/phospholipid ratios of membranes affect bilayer fluidity, the maturation of viral envelopes with high cholesterol/phospholipid ratios via association with rafts may be important for the stability of HCV

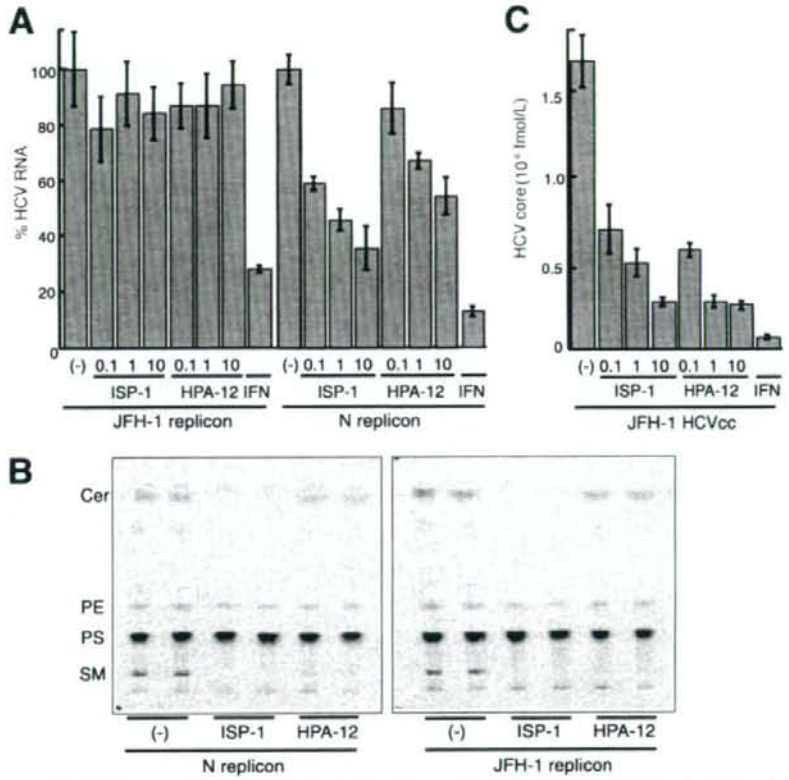

FIG. 6. Anti-HCV effects of inhibitors of the sphingolipid biosynthetic pathway. Subgenomic replicon cells derived from HCV isolate N or JFH-1, as well as HCVcc-producing cells, were treated with ISP-1 (0.1, 1, or 10 μM), HPA-12 (0.1, 1, or 10 μM) or alpha interferon (IFN) (100 U/ml) for 72 h. HCV RNA titers in the replicon cells (A) and the HCV core protein content of the culture medium of infected cells (C) were determined. Data are means from four independent experiments. Error bars, standard deviations. (B) De novo synthesis of sphingolipid in the absence or presence of ISP-1 (10 μM) and HPA-12 (10 μM) was monitored in duplicate by metabolic labeling with [^{14}C]serine for 2 h at 37°C. Cer, ceramide; PE, phosphatidylethanolamine; PS, phosphatidylserine.

particles. Replenishing the viral membrane with cholesterol following treatment with 5 mg/ml B-CD successfully restored viral infectivity to the same level as that of untreated virus (Fig. 1), suggesting that reversible B-CD-induced changes in HCV structure might critically influence viral infectivity. However, we were unable to restore viral infectivity by replenishing cholesterol after pretreatment of the virion with concentrations of B-CD exceeding 10 mg/ml (data not shown). Under these conditions, it is likely that large holes in the viral membrane destroy the virus, a result that cannot be reversed by supplying exogenous cholesterol.

How are cholesterol and sphingolipid involved in the HCV virion during the process of virus maturation? Like most positive-stranded RNA viruses, HCV is thought to assemble at the ER membrane. However, Miyanari et al. (32) reported that lipid droplets are important for HCVcc formation. These authors have shown that the characteristics of lipid-droplet-associated membranes in Huh-7 cells differ from those of ER membranes. In the case of flaviviruses, for which the mechanism of viral assembly and budding remains unclear (15), a few studies have demonstrated budding at the plasma membrane (13, 36, 37, 41), and it has been proposed that the site of budding may be virus and cell type dependent (27). We demonstrate here that subpopulations of HCV structural proteins partition into cellular detergent-resistant, lipid-raft-like membrane fractions in HCVcc-producing cells (Fig. 4) and that inhibitors of the sphingolipid biosynthetic pathway block HCV virion production (Fig. 6). Furthermore, a large proportion of HCV E2 protein incorporated into HCVcc is endoglycosidase H resistant (data not shown). Thus, membrane compartments containing cholesterol- and sphingolipid-rich microdomains may be involved in HCV virion maturation. Another explanation for the recruitment of these lipids to the HCV membrane may be an association between the virus and very-low-density lipoprotein (VLDL) or low-density lipoprotein. Recently, Huang et al. (16) demonstrated a close link between HCV production and VLDL assembly, suggesting that an HCV-VLDL complex is generated and secreted from cells.

Recent reports have demonstrated that CD81-mediated HCV infection is partly dependent on cell membrane choles-

terol (19) and SM (53). We further characterized the role of lipid on the plasma membrane in viral infectivity and found that cholesterol depletion by B-CD, as well as hydrolysis of SM by SMase, moderately inhibits HCV infectivity (Fig. 5). These results suggest that cholesterol and sphingolipid in the plasma membrane environment may assist HCV entry, while HCV virion-associated cholesterol and sphingolipid appear to play critical roles in viral infection.

We previously demonstrated that HCV RNA and nonstructural proteins are present in DRM structures, likely in the context of a lipid-raft structure, and that viral RNA is likely synthesized at a raft membrane structure in cells containing the genotype 1b HCV replicon (2, 10, 46). Here we observed that ISP-1 and HPA-12 suppress HCVcc production, but not viral RNA replication, by the JFH-1 replicon (Fig. 6). Impairment of particle assembly and maturation, rather than suppression of genome replication, by these drugs may account for the inhibition of HCV production in the JFH-1 system. Viral RNA replication of the HCV-N replicon, however, was efficiently inhibited by these compounds, as found in previous reports (43). The virus strain specificity of the anti-HCV activity of cyclosporine has recently been demonstrated: JFH-1 replication is less sensitive to cyclosporine than replication of genotype 1b strains. Furthermore, the requirement for interaction with a cellular replication cofactor, cyclophilin B, differs among HCV strains (18). It appears that ISP-1 and HPA-12 are further examples of diverse effects on HCV strain replication.

In summary, our data here demonstrate important roles of cholesterol and sphingolipid in HCV infection and virion maturation. Specifically, mature HCV particles are rich in cholesterol. Depletion from HCV or hydrolysis of virion-associated SM results in a loss of infectivity. Moreover, the addition of exogenous cholesterol restores infectivity. In addition, cholesterol and sphingolipid on the HCV membrane play key roles in virus internalization, and portions of structural proteins are localized at lipid-raft-like membrane structures within cells. Finally, inhibitors of the sphingolipid biosynthetic pathway efficiently block virion production. These observations suggest that agents capable of modifying virion-associated lipid content might function as antivirals by preventing and/or blocking HCV infection and production.

ACKNOWLEDGMENTS

We thank M. Matsuda, M. Sasaki, S. Yoshizaki, T. Shimoji, M. Kaga, and T. Date for technical assistance and T. Mizoguchi for secretarial work.

This work was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labor, and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science, and Technology, as well as by a Research on Health Science Focusing on Drug Innovation grant from the Japan Health Sciences Foundation.

REFERENCES

1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology **27:** 621–627.
2. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology **324:**450–461.
3. **Akazawa, D., T. Date, K. Morikawa, A. Murayama, M. Miyamoto, M. Kaga, H. Barth, T. F. Baumert, J. Dubuisson, and T. Wakita.** 2007. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J. Virol. **81:**5036–5045.
4. **Bender, F. C., J. C. Whitbeck, H. Lou, G. H. Cohen, and R. J. Eisenberg.** 2005. Herpes simplex virus glycoprotein B binds to cell surfaces independently of heparan sulfate and blocks virus entry. J. Virol. **79:**11588–11597.
5. **Blanchard, E., D. Brand, S. Trassard, A. Goudeau, and P. Roingeard.** 2002. Hepatitis C virus-like particle morphogenesis. J. Virol. **76:**4073–4079.
6. **Chazal, N., and D. Gerlier.** 2003. Virus entry, assembly, budding, and membrane rafts. Microbiol. Mol. Biol. Rev. **67:**226–237.
7. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature **446:**801–805.
8. **Ezelle, H. J., D. Markovic, and G. N. Barber.** 2002. Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. J. Virol. **76:**12325–12334.
9. **Fukasawa, M., M. Nishijima, H. Itabe, T. Takano, and K. Hanada.** 2000. Reduction of sphingomyelin level without accumulation of ceramide in Chinese hamster ovary cells affects detergent-resistant membrane domains and enhances cellular cholesterol efflux to methyl-β-cyclodextrin. J. Biol. Chem. **275:**34028–34034.
10. **Gao, L., H. Aizaki, J. W. He, and M. M. Lai.** 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J. Virol. **78:**3480–3488.
11. **Guo, J. T., V. V. Bichko, and C. Seeger.** 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. **75:**8516–8523.
12. **Hanada, K., T. Hara, M. Fukasawa, A. Yamaji, M. Umeda, and M. Nishijima.** 1998. Mammalian cell mutants resistant to a sphingomyelin-directed cytolysin. Genetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. J. Biol. Chem. **273:**33787–33794.
13. **Hase, T., P. L. Summers, K. H. Eckels, and W. B. Baze.** 1987. An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito cells: maturation events. Arch. Virol. **92:**273–291.
14. **Heider, J. G., and R. L. Boyett.** 1978. The picomole determination of free and total cholesterol in cells in culture. J. Lipid Res. **19:**514–518.
15. **Heinz, F. X., and S. L. Allison.** 2003. Flavivirus structure and membrane fusion. Adv. Virus Res. **59:**63–97.
16. **Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, and J. Ye.** 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc. Natl. Acad. Sci. USA **104:**5848–5853.
17. **Ikeda, M., M. Yi, K. Li, and S. M. Lemon.** 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. **76:**2997–3006.
18. **Ishii, N., K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, and K. Shimotohno.** 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. **80:**4510–4520.
19. **Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari.** 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J. Virol. **81:**374–383.
20. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. **64:**334–339.
21. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125:**1808–1817.
22. **Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T. Wakita.** 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J. Clin. Microbiol. **43:**5679–5684.
23. **Kato, T., T. Date, A. Murayama, K. Morikawa, D. Akazawa, and T. Wakita.** 2006. Cell culture and infection system for hepatitis C virus. Nat. Protoc. **1:**2334–2339.
24. **Kobayashi, S., K. Kakumoto, and M. Sugiura.** 2002. Transition metal salts-catalyzed aza-Michael reactions of enones with carbamates. Org. Lett. **18:** 1319–1322.
25. **Koutsoudakis, G., E. Herrmann, S. Kallis, R. Bartenschlager, and T. Pietschmann.** 2007. The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. J. Virol. **81:**588–598.
26. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113.
27. **Mackenzie, J. M., and E. G. Westaway.** 2001. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J. Virol. **75:**10787–10799.
28. **Manes, S., G. del Real, R. A. Lacalle, P. Lucas, C. Gomez-Mouton, S. Sanchez-Palomino, R. Delgado, J. Alcami, E. Mira, and A. C. Martinez.**

2000. Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. EMBO 1:190–196.
29. **Matsuo, E., H. Tani, C. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S. Yagi, A. H. Patel, T. Miyamura, and Y. Matsuura.** 2006. Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. Biochem. Biophys. Res. Commun. **340**:200–208.
30. **Matto, M., C. M. Rice, B. Aroeti, and J. S. Glenn.** 2004. Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. J. Virol. **78**:12047–12053.
31. **Miyake, Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki.** 1995. Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. Biochem. Biophys. Res. Commun. **211**: 396–403.
32. **Miyanari, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno.** 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. **9**:1089–1097.
33. **Morikawa, K., Z. Zhao, T. Date, M. Miyamoto, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, and T. Wakita.** 2007. The roles of CD81 and glycosaminoglycans in the adsorption and uptake of infectious HCV particles. J. Med. Virol. **79**:714–723.
34. **Murakami, K., K. Ishii, Y. Ishihara, S. Yoshizaki, K. Tanaka, Y. Gotoh, H. Aizaki, M. Kohara, H. Yoshioka, Y. Mori, N. Manabe, I. Shoji, T. Sata, R. Bartenschlager, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology **351**:381–392.
35. **Nakai, K., T. Okamoto, T. Kimura-Someya, K. Ishii, C. K. Lim, H. Tani, E. Matsuo, T. Abe, Y. Mori, T. Suzuki, T. Miyamura, J. H. Nunberg, K. Moriishi, and Y. Matsuura.** 2006. Oligomerization of hepatitis C virus core protein is crucial for interaction with the cytoplasmic domain of E1 envelope protein. J. Virol. **80**:11265–11273.
36. **Ng, M. L., J. Howe, V. Sreenivasan, and J. J. Mulders.** 1994. Flavivirus West Nile (Sarafend) egress at the plasma membrane. Arch. Virol. **137**:303–313.
37. **Ng, M. L., S. H. Tan, and J. J. Chu.** 2001. Transport and budding at two distinct sites of visible nucleocapsids of West Nile (Sarafend) virus. J. Med. Virol. **65**:758–764.
38. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108**:193–199.
39. **Pessin, J. E., and M. Glaser.** 1980. Budding of Rous sarcoma virus and vesicular stomatitis virus from localized lipid regions in the plasma membrane of chicken embryo fibroblasts. J. Biol. Chem. **255**:9044–9050.
40. **Pitha, J., T. Irie, P. B. Sklar, and J. S. Nye.** 1988. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. Life Sci. **43**:493–502.
41. **Rahman, S., T. Matsumura, K. Masuda, K. Kanemura, and T. Fukunaga.** 1998. Maturation site of dengue type 2 virus in cultured mosquito C6/36 cells and Vero cells. Kobe J. Med. Sci. **44**:65–79.
42. **Rouser, G., G. Galli, and G. Kritchevsky.** 1967. Lipid composition of the normal human brain and its variations during various diseases. Pathol. Biol. **15**:195–200.
43. **Sakamoto, H., K. Okamoto, M. Aoki, H. Kato, A. Katsume, A. Ohta, T. Tsukuda, N. Shimma, Y. Aoki, M. Arisawa, M. Kohara, and M. Sudoh.** 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat. Chem. Biol. **1**:333–337.
44. **Sato, K., H. Okamoto, S. Aihara, Y. Hoshi, T. Tanaka, and S. Mishiro.** 1993. Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. Virology **196**:354–357.
45. **Serafino, A., M. B. Valli, F. Andreola, A. Crema, G. Ravagnan, L. Bertolini, and G. Carloni.** 2003. Suggested role of the Golgi apparatus and endoplasmic reticulum for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected in vitro. J. Med. Virol. **70**:31–41.
46. **Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai.** 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. **77**:4160–4168.
47. **Shinitzky, M., and M. Inbar.** 1976. Microviscosity parameters and protein mobility in biological membranes. Biochim. Biophys. Acta **433**:133–149.
48. **Shirakura, M., K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K. Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Moriishi, Y. Matsuura, T. Wakita, T. Suzuki, P. M. Howley, T. Miyamura, and I. Shoji.** 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. **81**:1174–1185.
49. **Stuart, A. D., H. E. Eustace, T. A. McKee, and T. D. Brown.** 2002. A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. J. Virol. **76**:9307–9322.
50. **Takikawa, S., K. Ishii, H. Aizaki, T. Suzuki, H. Asakura, Y. Matsuura, and T. Miyamura.** 2000. Cell fusion activity of hepatitis C virus envelope proteins. J. Virol. **74**:5066–5074.
51. **Tani, H., Y. Komoda, E. Matsuo, K. Suzuki, I. Hamamoto, T. Yamashita, K. Moriishi, K. Fujiyama, T. Kanto, N. Hayashi, A. Owsianka, A. H. Patel, M. A. Whitt, and Y. Matsuura.** 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. J. Virol. **81**:8601–8612.
52. **Umehara, T., M. Sudoh, F. Yasui, C. Matsuda, Y. Hayashi, K. Chayama, and M. Kohara.** 2006. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. Biochem. Biophys. Res. Commun. **346**:67–73.
53. **Voisset, C., M. Lavie, F. Helle, A. Op De Beeck, A. Bilheu, J. Bertrand-Michel, F. Tercé, L. Cocquerel, C. Wychowski, N. Vu-Dac, and J. Dubuisson.** 2008. Ceramide enrichment of the plasma membrane induces CD81 internalization and inhibits hepatitis C virus entry. Cell. Microbiol. **10**:606–617.
54. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
55. **Yasuda, S., H. Kitagawa, M. Ueno, H. Ishitani, M. Fukasawa, M. Nishijima, S. Kobayashi, and K. Hanada.** 2001. A novel inhibitor of ceramide trafficking from the endoplasmic reticulum to the site of sphingomyelin synthesis. J. Biol. Chem. **276**:43994–44002.
56. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102**:9294–9299.

Journal of General Virology (2008), **89**, 1587–1592 DOI 10.1099/vir.0.83618-0

Short Communication

Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines

Kyoko Murakami,[1] Toshiro Kimura,[1] Motonao Osaki,[1] Koji Ishii,[1] Tatsuo Miyamura,[1] Tetsuro Suzuki,[1] Takaji Wakita[1] and Ikuo Shoji[1,2]

Correspondence
Ikuo Shoji
ishoji@med.kobe-u.ac.jp

[1]Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

[2]Division of Microbiology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

While hepatocytes are the major site of hepatitis C virus (HCV) infection, a number of studies have suggested that HCV can replicate in lymphocytes. However, *in vitro* culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

Received 25 November 2007
Accepted 18 March 2008

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo *et al.*, 1989; Saito *et al.*, 1990). Infection with HCV is frequently associated with B-cell-related diseases, such as mixed cryoglobulinaemia and non-Hodgkin's lymphoma (Hausfater *et al.*, 2000). A number of studies have suggested that HCV can replicate not only in hepatocytes, but also in lymphocytes (Ducoulombier *et al.*, 2004; Karavattathayyil *et al.*, 2000, Lerat *et al.*, 1998), whereas the determinants of HCV tropism are still unknown. The development of HCV strain JFH-1, which generates infectious HCV in culture, has made an important contribution to the study of the HCV life cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The HCV life cycle is divided into several steps. After entry into the cell and uncoating, the HCV life cycle leads to translation, polyprotein processing, RNA replication, virion assembly, transport and release. The JFH-1 subgenomic replicon can replicate in non-hepatic cell lines, such as HeLa cells and 293 cells, suggesting that the host factors required for HCV replication are not hepatocyte-specific (Kato *et al.*, 2005b). The SB strain of HCV (genotype 2b strain) was isolated from an HCV-infected non-Hodgkin's B-cell lymphoma and has been reported to infect B and T cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). The virus titres of the SB strain in lymphocytes were, however, lower than those of JFH-1 in Huh-7 cells and the expression of HCV proteins was not confirmed (Kondo *et al.*, 2007). It is unknown whether HCV JFH-1 can infect and replicate in lymphocytes. To gain more insight into the tissue tropism of HCV infection, we investigated the infection, replication, IRES-dependent translation and polyprotein processing of the JFH-1 strain in nine lymphocytic cell lines.

We first sought to determine whether HCV JFH-1 can infect lymphocytic cell lines. We chose nine lymphocytic cell lines derived from Burkitt's lymphoma, the EBV-immortalized human B cell line, lymphoblasts and acute T-cell leukaemia. C1R, IB4, Namalwa, P3HR1 and Raji cells were Epstein–Barr virus (EBV)-positive (Table 1). Infectious HCV was generated from HCV JFH-1 RNA in Huh-7 cells (Shirakura *et al.*, 2007; Wakita *et al.*, 2005) and the calculation of the 50 % tissue culture infectious dose ($TCID_{50}$) was based on methods described previously (Lindenbach *et al.*, 2005). These cell lines (1×10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5×10^3 or 5×10^4 $TCID_{50}$ ml^{-1}) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics). As shown in Fig. 1(a), increasing the HCV titre of the inoculum resulted in a 7.2-fold increase in the levels of HCV core protein in Huh-7 cells at 3 h p.i. Increasing the HCV titre of the inoculum resulted in a 1.5- to 3.2-fold increase in the levels of the core protein in C1R, BL41,

Table 1. Summary of the virological characterization of HCV JFH-1 in lymphocytes

Name	Source	EBV	Transfection			Concentration of G418 for selection (μg ml^{-1})	HCVcc infection	HCV-RNA replication	Translation*		Polyprotein processing†
			Buffer	Program	Efficiency				HCV-IRES	EMCV-IRES	
Bjab	Burkitt's lymphoma	−	T	T−16	>90 %	600–800	−	−	+	++	+
BL41	Burkitt's lymphoma	−	V	I−10	60–70 %	1000	−	−	+	++	ND
C1R	B lymphoblast	+	V	T−20	70–80 %	100	−	−	++++	++++	+
IB4	Lymphoblastoid	+	V	T−20	80–90 %	1000	−	−	+++	++++	+
Jurkat	Acute T cell leukaemia	−	V	I−10	60–70 %	600	−	−	++	+	ND
Namalwa	Burkitt's lymphoma	+	V	M−13	60–70 %	600–800	−	−	+++	++++	+
P3HR1	Burkitt's lymphoma	+	V	A−23	60–70 %	800	−	−	+++	++++	ND
Raji	Burkitt's lymphoma	+	V	T−27	70–80 %	800	−	−	++	++++	+
Ramos	Burkitt's lymphoma	−	V	M−13	40–60 %	400	−	−	+	+	ND
Huh7	Hepatoma		−	T	T−14	70–80 %	500	+	+	+++	+++	+

*+, <0.25 fold IRES activity of Huh-7; ++, 0.25–0.75 fold; +++, 0.75–1.5-fold; ++++, >1.5-fold.
†ND, Not determined.

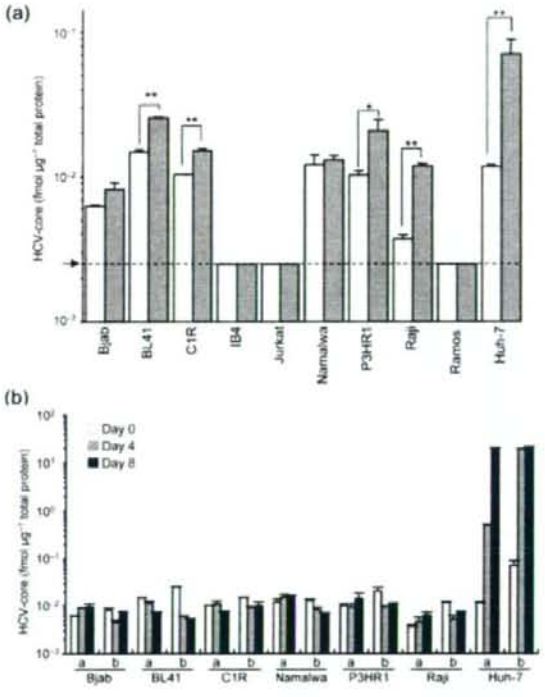

Fig. 1. HCV infection assay. (a) HCV core protein levels 3 h after infection. A total of 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [white bars] or 5×10^4 [grey bars] $TCID_{50}$ ml^{-1}) for 3 h at 37 °C and harvested at 3 h p.i. HCV core protein in cell lysate was quantified by ELISA. The average values with standard deviations from triplicate samples are shown. The cut-off value of the immunoassay is indicated by an arrow and a dotted line. The difference between low m.o.i. (white bars) and high m.o.i. (grey bars) was significant (*, $P<0.05$; **, $P<0.01$, Student's *t*-test). (b) Time-course of HCV core protein levels after infection. In total, 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [a] or 5×10^4 [b] $TCID_{50}$ ml^{-1}) for 3 h and harvested at 0, 4 and 8 days p.i. HCV core protein in cell lysate was quantified by ELISA. Average values ± SD from triplicate samples are shown.

P3HR1 and Raji cells, suggesting that HCV can bind to these cell lines (Fig. 1a). In contrast, the levels of HCV core protein in IB4, Jurkat and Ramos cells at 3 h p.i. were below the detection limits and there were no significant differences in the levels of the core protein in Bjab cells and Namalwa cells, suggesting that HCV binding to these cells was very inefficient (Fig. 1a). Moreover, the levels of HCV core protein increased in Huh-7 cells but, in the case of all lymphocytic cell lines, including Raji cells, the core titre did not increase at day 4 and 8 p.i., suggesting that HCV JFH-1 does not infect and/or replicate efficiently in these lymphocytic cell lines (Fig. 1b).

To assess the replication of JFH-1 in our lymphocytic cell lines, we utilized the HCV replicon system. To visualize the

replicating cells, a reporter replicon plasmid was constructed as follows. The gene encoding green fluorescence protein (GFP) was fused to the neomycin resistance gene using an overlap PCR amplification technique and the fusion product was inserted into pSGR-JFH1. The resultant plasmid was pSGR-GFPneo-JFH1. This plasmid was linearized with *Xba*I and used as a template for *in vitro* transcription using an AmpliScribe T7 High Yield Transcription kit (Epicentre Biotechnologies). RNA was transfected with high transfection efficiency and low cytotoxicity using the Nucleofector system (Amaxa Biosystems) (Coughlin *et al.*, 2004; Miyahara *et al.*, 2005; Van De Parre *et al.*, 2005). The transfection efficiencies ranged from 60 to 80 % after optimization of transfection conditions (Table 1). GFP expression was monitored periodically during the selection of HCV-replicon cells by G418 (Table 1). The GFP-expressing cells were detected at day 3 post-transfection (p.t.) in Huh-7, P3HR1, Raji, C1R and Namalwa cells. The rate of GFP expression in Huh-7 cells was more than 50 %. The rate of GFP-expression in lymphocytic cell lines was less than 1 %, despite the high transfection efficiencies. After 3 weeks of G418 selection, SGR-GFPneo-JFH1 replicon cells were established in Huh-7 cells, but not in lymphocytic cells. These data suggest that JFH-1 subgenomic replicon RNA cannot replicate in the lymphocytic cell lines.

To facilitate quantification of replication, we performed luciferase assays using subgenomic replicon RNA (SGR-JFH1/Luc) carrying firefly luciferase as a reporter. SGR-JFH1/Luc RNA was *in vitro*-transcribed using the linearized pSGR-JFH1/Luc (Kato *et al.*, 2005a) as template DNA. Cells were harvested at 4, 24, 48 and 72 h p.t. and luciferase activities were assayed with luciferase assay reagent (Promega). Assays were performed at least in triplicate. There were significant differences in luciferase activities at 4 h p.t. among the cell lines, probably because there were differences in transfection efficiencies and the doubling time of the cell lines. Thus, the replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1 (Fig. 2a). HCV subgenomic replicon RNA efficiently replicated in Huh-7 cells (Fig. 2a). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B served as a negative control in Huh-7 cells. The luciferase activities of replication-deficient subgenomic replicon RNA in lymphocytic cell lines also decreased rapidly (data not shown). As shown in Fig. 2(a), the luciferase activities of HCV subgenomic replicon RNA in lymphocytic cell lines decreased rapidly, suggesting that HCV subgenomic replicon RNA did not replicate efficiently in lymphocytic cell lines. Thus, these two different replicon assays demonstrated that the HCV JFH-1 subgenomic replicon failed to replicate in our lymphocytic cell lines.

To determine which steps of the HCV life cycle are impaired, we further examined translation and polyprotein processing. At first, we assessed HCV IRES-dependent translational efficiencies in the lymphocytic cell lines. Cells were co-transfected with the subgenomic replicon RNA (SGR-JFH1/Luc) and a capped RNA encoding *Renilla* luciferase (cap-luc). Cap-luc RNA was *in vitro*-transcribed using a T7 mMessage mMachine kit (Ambion). The HCV IRES activities in IB4, Namalwa and P3HR1 cells were as high as in Huh-7 cells. The HCV IRES activities in Jurkat and Raji cells were about 50 % of those in Huh-7 cells, and the HCV IRES activities in Bjab, BL41 and Ramos cells were less than 25 % of those in Huh-7 cells. On the other hand, the HCV IRES activity in C1R cells was about twofold higher than in Huh-7 cells (Fig. 2b). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B showed a luciferase activity level similar to that of the wild-type, suggesting that the luciferase activity at 4 h after transfection reflected translational levels but not replication levels (data not shown). Our data indicate high HCV IRES activities in all cell lines, except in Bjab, BL41 and Ramos.

The HCV polyprotein is translated in subgenomic replicon cells in an encephalomyocarditis virus (EMCV) IRES-dependent manner. To rule out the possibility that the EMCV IRES-dependent translation is impaired in lymphocytic cell lines, we assessed the EMCV IRES-dependent translational efficiencies. We assayed EMCV IRES activity using EMCV IRES-driven luciferase RNA (EMC-luc) and Cap-luc RNA. The EMCV IRES activity was five- to tenfold higher in C1R, Namalwa, IB4 and P3HR1 than in Huh-7 cells (Fig. 2c). From these results, HCV IRES and EMCV IRES exhibited sufficient translational activity in C1R, Namalwa, P3HR1 and Raji cells, suggesting that IRES-dependent translation was not impaired in these lymphocytic cell lines.

To determine whether HCV polyprotein is properly processed in lymphocytes, we examined the processing of HCV non-structural (NS) proteins. The construct pSGR-JFH1/Luc expresses the polyprotein NS3-NS4A-NS4B-NS5A-NS5B. The HCV NS3/4A protease is responsible for proteolytic processing at each cleavage site. We used the eukaryotic transient-expression system based on a recombinant vaccinia virus carrying bacteriophage T7 RNA polymerase (T7vac) (Fuerst *et al.*, 1989). To express the SGR-JFH1/Luc encoding HCV NS proteins, 5×10^6 cells were transfected with 5 μg pSGR-JFH1/Luc and infected with 2.5×10^9 p.f.u. T7vac, harvested at 24 h p.i., and analysed by Western blotting. Completely processed NS3, NS5A and NS5B proteins were detected in Bjab, Raji, IB4 and Namalwa cells as well as in pSGR-JFH1/Luc-transfected Huh-7 cells and HCV-JFH1-infected Huh-7 cells (Fig. 2c). The unprocessed polyprotein was not detected by immunoblotting in these lymphocytic cell lines (data not shown). These results suggest that the HCV polyprotein is efficiently processed in these lymphocytic cells.

In this study, we demonstrated that HCV JFH-1 failed to infect and replicate in nine lymphocytic cell lines. In contrast, HCV IRES-dependent translation and polyprotein processing by NS3/NS4A protease functioned properly

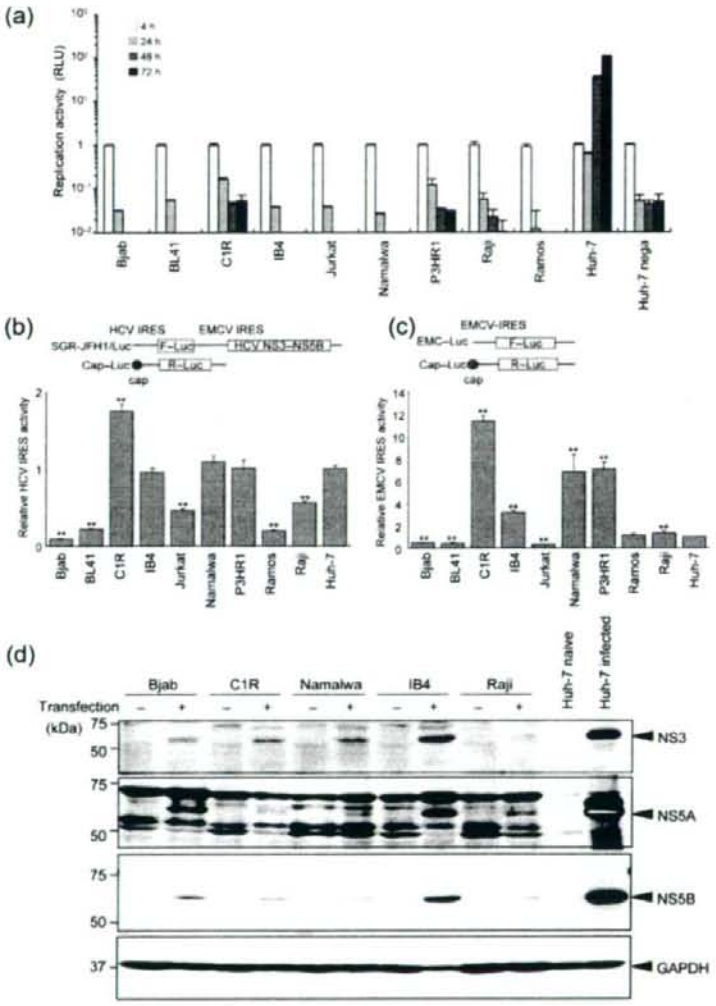

Fig. 2. Replication, HCV IRES-dependent translational efficiencies and polyprotein processing. (a) Subgenomic replicon assay. JFH-1 subgenomic replicon RNA was transfected into several cell lines and harvested at 4, 24, 48 and 72 h p.t. The replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1. RLU, Relative luciferase units; Huh-7 nega, Huh-7 cells transfected with SGR-JFH1/Luc GND, served as a negative control. (b) HCV IRES-dependent translational efficiency. To determine the HCV IRES activities, we co-transfected cells with SGR-JFH1/Luc RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of HCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in HCV IRES activity between Huh-7 cells and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (c) EMCV IRES-dependent translational efficiency. To determine the EMCV IRES activities, we co-transfected cells with EMCV-firefly luciferase RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of EMCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in EMCV IRES activity between Huh-7 cell and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (d) Polyprotein processing by NS3/4A protease in lymphocytic cell lines. pSGR-JFH1/Luc-transfected cells were infected with T7vac and harvested at 24 h p.i. HCV NS proteins, NS3, NS5A and NS5B were detected by using anti-NS3 rabbit polyclonal antibody (PAb), anti-NS5A rabbit PAb and anti-NS5B rabbit PAb. Arrowheads indicate the processed NS3, NS5A and NS5B proteins, respectively.

in these cells. Moreover, subgenomic replicon RNA failed to replicate in these cell lines. Our data suggest that lymphocytic cell lines may lack some host factors required for infection and replication of HCV-JFH1.

Viral entry often requires sequential interactions between viral proteins and several cellular factors. Several molecules (CD81, Claudin-1, Scavenger receptor class B member IR, LDL-receptor and glycosaminoglycans) have been reported to be involved in HCV binding and entry (Barth *et al.*, 2003; Evans *et al.*, 2007; Pileri *et al.*, 1998; Scarselli *et al.*, 2002). Further investigation will be required to clarify HCV binding and entry into lymphocytic cell lines.

HCV IRES and EMCV IRES exhibited sufficient translational activities in C1R, IB4, P3HR1, Namalwa and Raji cells. All these cell lines are EBV-positive. EBV-encoded nuclear antigen (EBNA1) has been reported to support HCV replication (Sugawara *et al.*, 1999). Two small EBV-encoded RNA species (EBERs) bind to the HCV IRES region (Wood *et al.*, 2001). These findings raise the possibility that HCV IRES activities may be modified by the EBV genome.

HCV JFH-1 subgenomic replicon RNA could not replicate in all lymphocytes tested in this study. The HCV SB strain, however, has been reported to infect Raji, Daudi, Molt-4

and Jurkat cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). Still unknown is how hepatotropism and lymphotropism of HCV are determined. The GB virus B (GBV-B) is most closely related to HCV and the GBV-B infection of tamarins has been proposed as a good surrogate model for chronic hepatitis C (Bukh *et al.*, 2001; Jacob *et al.*, 2004; Lanford *et al.*, 2003; Martin *et al.*, 2003). A recent report has shown that GBV can disseminate to not only liver but also a variety of extrahepatic tissues such as haematolymphoid and genital tissues in tamarins (Ishii *et al.*, 2007). Viral RNA cloned from plasma and liver from the tamarins showed no sequence heterogeneity, suggesting that host factors determine the pleiotropism (Ishii *et al.*, 2007). It remains unclear how host factors and/or viral factors determine the tissue tropism of HCV. Further studies will be required to clarify the molecular mechanisms of HCV tissue tropism.

Acknowledgements

The authors gratefully acknowledge Drs Sanae Machida (Saitama Medical School, Saitama, Japan), Shizuko Harada (NIID, Tokyo, Japan) and Isao Hamaguchi (NIID, Tokyo, Japan) for the cell lines, and Dr Hideki Aizaki (NIID, Tokyo, Japan) for helpful discussion. This work was supported in part by grants-in-aid from the Ministry of Health, Labour and Welfare, by a grant for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, and by grant-in aid for young scientists (B).

References

Barth, H., Schafer, C., Adah, M. I., Zhang, F., Linhardt, R. J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T. H. & other authors (2003). Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* **278**, 41003–41012.

Bukh, J., Apgar, C. L., Govindarajan, S. & Purcell, R. H. (2001). Host range studies of GB virus-B hepatitis agent, the closest relative of hepatitis C virus, in New World monkeys and chimpanzees. *J Med Virol* **65**, 694–697.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.

Coughlin, C. M., Vance, B. A., Grupp, S. A. & Vonderheide, R. H. (2004). RNA-transfected CD40-activated B cells induce functional T-cell responses against viral and tumor antigen targets: implications for pediatric immunotherapy. *Blood* **103**, 2046–2054.

Ducoulombier, D., Roque-Afonso, A. M., Di Liberto, G., Penin, F., Kara, R., Richard, Y., Dussaix, E. & Feray, C. (2004). Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. *Hepatology* **39**, 817–825.

Evans, M. J., von Hahn, T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J. A., Bieniasz, P. D. & Rice, C. M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801–805.

Fuerst, T. R., Fernandez, M. P. & Moss, B. (1989). Transfer of the inducible *lac* repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector. *Proc Natl Acad Sci U S A* **86**, 2549–2553.

Hausfater, P., Rosenthal, E. & Cacoub, P. (2000). Lymphoproliferative diseases and hepatitis C virus infection. *Ann Med Interne (Paris)* **151**, 53–57.

Ishii, K., Iijima, S., Kimura, N., Lee, Y. J., Ageyama, N., Yagi, S., Yamaguchi, K., Maki, N., Mori, K. & other authors (2007). GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues *in vivo*. *Microbes Infect* **9**, 515–521.

Jacob, J. R., Lin, K. C., Tennant, B. C. & Mansfield, K. G. (2004). GB virus B infection of the common marmoset (*Callithrix jacchus*) and associated liver pathology. *J Gen Virol* **85**, 2525–2533.

Karavattathayyil, S. J., Kalkeri, G., Liu, H. J., Gaglio, P., Garry, R. F., Krause, J. R. & Dash, S. (2000). Detection of hepatitis C virus RNA sequences in B-cell non-Hodgkin lymphoma. *Am J Clin Pathol* **113**, 391–398.

Kato, T., Date, T., Miyamoto, M., Sugiyama, M., Tanaka, Y., Orito, E., Ohno, T., Sugihara, K., Hasegawa, I. & other authors (2005a). Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. *J Clin Microbiol* **43**, 5679–5684.

Kato, T., Date, T., Miyamoto, M., Zhao, Z., Mizokami, M. & Wakita, T. (2005b). Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J Virol* **79**, 592–596.

Kondo, Y., Sung, V. M., Machida, K., Liu, M. & Lai, M. M. (2007). Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. *Virology* **361**, 161–173.

Lanford, R. E., Chavez, D., Notvall, L. & Brasky, K. M. (2003). Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia. *Virology* **311**, 72–80.

Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M. A., Trepo, C. & Inchauspe, G. (1998). In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* **91**, 3841–3849.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R. & other authors (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626.

Martin, A., Bodola, F., Sangar, D. V., Goettge, K., Popov, V., Rijnbrand, R., Lanford, R. E. & Lemon, S. M. (2003). Chronic hepatitis associated with GB virus B persistence in a tamarin after intrahepatic inoculation of synthetic viral RNA. *Proc Natl Acad Sci U S A* **100**, 9962–9967.

Miyahara, Y., Naota, H., Wang, L., Hiasa, A., Goto, M., Watanabe, M., Kitano, S., Okumura, S., Takemitsu, T. & other authors (2005). Determination of cellularly processed HLA-A2402-restricted novel CTL epitopes derived from two cancer germ line genes, MAGE-A4 and SAGE. *Clin Cancer Res* **11**, 5581–5589.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D. & other authors (1998). Binding of hepatitis C virus to CD81. *Science* **282**, 938–941.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M. & other authors (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* **87**, 6547–6549.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* **21**, 5017–5025.

Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shimoji, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M. & other authors (2007).

E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. *J Virol* **81**, 1174–1185.

Sugawara, Y., Makuuchi, M., Kato, N., Shimotohno, K. & Takada, K. (1999). Enhancement of hepatitis C virus replication by Epstein–Barr virus-encoded nuclear antigen 1. *EMBO J* **18**, 5755–5760.

Sung, V. M., Shimodaira, S., Doughty, A. L., Picchio, G. R., Can, H., Yen, T. S., Lindsay, K. L., Levine, A. M. & Lai, M. M. (2003). Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. *J Virol* **77**, 2134–2146.

Van De Parre, T. J., Martinet, W., Schrijvers, D. M., Herman, A. G. & De Meyer, G. R. (2005). mRNA but not plasmid DNA is efficiently transfected in murine J774A.1 macrophages. *Biochem Biophys Res Commun* **327**, 356–360.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Wood, J., Frederickson, R. M., Fields, S. & Patel, A. H. (2001). Hepatitis C virus 3′X region interacts with human ribosomal proteins. *J Virol* **75**, 1348–1358.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* **102**, 9294–9299.