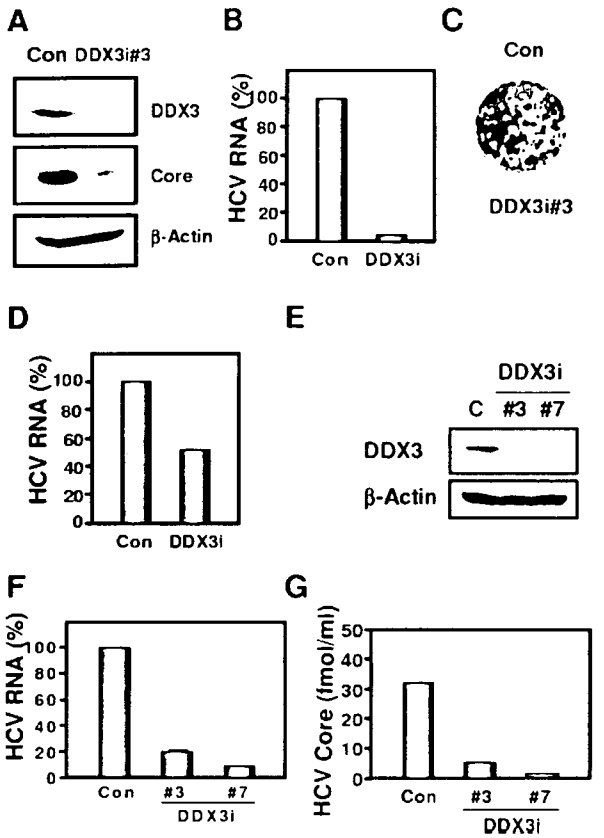

FIG. 1. Requirement of DDX3 for HCV replication. (A to D) Effect of DDX3 knockdown on HCV RNA replication. (A) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 (ProSci), anti-HCV core (CP-9; Institute of Immunology), or an anti-β-actin antibody (Sigma) in O cells expressing shRNA against DDX3 (DDX3i#3) as well as in O cells transduced with a control lentivirus vector (Con) are shown. (B) The level of genome-length HCV RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (C) Efficiency of colony formation in DDX3 knockdown cells. In vitro-transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the DDX3 knockdown Oc cells (DDX3i#3) or the Oc cells transduced with a control lentivirus vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and representative results are shown. (D) The level of subgenomic replicon RNA was monitored by real-time LightCycler PCR. Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (E to G) Effect of DDX3 knockdown on HCV infection. (E) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 or an anti-β-actin antibody for RSc cells expressing the shRNA DDX3i#3 or DDX3i#7 and for RSc cells transduced with a control lentivirus vector (Con) are shown. (F) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the cell culture-generated HCVcc. Results from three independent experiments are shown. (G) The levels of the HCV core in the culture supernatants were determined by an enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in duplicate, and bars represent the mean HCV core protein levels.

tion system (23). We found 80 to 90% reductions in the accumulation of JFH1 RNA and 82 to 94% reductions in the release of the core into the culture supernatants in DDX3 knockdown HuH-7-derived RSc cured cells at 4 days after inoculation of HCVcc (Fig. 1E to G). Thus, DDX3 seems to be required for HCV RNA replication.

Previously, DDX3 was identified as an HCV core-interacting protein by yeast two-hybrid screening. This interaction required the N-terminal domain of the core (aa 1 to 59) and the C-terminal domain of DDX3 (aa 553 to 622) (17, 19, 25). To determine whether the core can interact with DDX3 regardless of the HCV genotype, we used the HCV-O core (genotype 1b) and the JFH1 core (genotype 2a) (Table 1). We first examined their subcellular localization by confocal laser scanning microscopy as previously described (3). Consistent with previous reports (17, 19, 25), both the HCV-O core and JFH1 core mostly colocalized with DDX3 in the perinuclear region (Fig. 2A). Then we immunoprecipitated lysates from 293FT cells in which hemagglutinin (HA)-tagged DDX3 and HCV-O core, JFH1 core, or their 40-aa N-truncated mutants were overexpressed with an anti-HA antibody. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Bio-Sciences). Precleared supernatants were incubated with 1 μg of anti-HA antibody (3F10; Roche) at 4°C for 1 h. Following absorption of the precipitates

TABLE 1. Primers used for construction of the HCV core-expressing plasmids[a]

Plasmid name	Direction	Primer sequence
pCXbsr/core(HCV-O)	Forward	5′-GGAATTCCCACCATGAG CACGAATCCTAAACCTC-3
	Reverse	5′-ATAAGAATGCGGCCGCC TATCAAGCGGAAGCTGG GATGGT-3′
pcDNA3/core(HCV-O)	Forward	5′-CGGGATCCAAGATGAGC ACGAATCCTAAACCTCAA AGA-3′
pcDNA3/FLAG-core(HCV-O)	Reverse	5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′
pcDNA3/Δcore(HCV-O)	Forward	5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′
pcDNA3/FLAG-Δcore(HCV-O)	Reverse	5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′
pcDNA3/core(JFH1)	Forward	5′-CGGGATCCAAGATGAGC ACAAATCCTAAACCTCAA AGA-3′
pcDNA3/FLAG-core(JFH1)	Reverse	5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′
pcDNA3/Δcore(JFH1)	Forward	5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′
pcDNA3/FLAG-Δcore(JFH1)	Reverse	5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′

[a] To construct pCXbsr/core(HCV-O), a DNA fragment encoding the core was amplified by PCR from pON/C-5B (13) with the indicated primers. The PCR product was digested with EcoRI-NotI and subcloned into the same site of pCX4bsr (1). To construct pcDNA3/core(HCV-O), pcDNA3/FLAG-core(HCV-O), pcDNA3/Δcore(HCV-O), and pcDNA3/FLAG-Δcore(HCV-O), DNA fragments encoding the core were amplified by PCR from pON/C-5B (13) with the indicated primer sets. To construct pcDNA3/core(JFH1), pcDNA3/FLAG-core(JFH1), pcDNA3/Δcore(JFH1), and pcDNA3/FLAG-Δcore(JFH1), DNA fragments encoding the core were amplified by PCR from pJFH1 (23) with the indicated primer sets. The PCR products were digested with BamHI and XhoI and then subcloned into the same site of pcDNA3 (Invitrogen) or pcDNA3-FLAG (2).

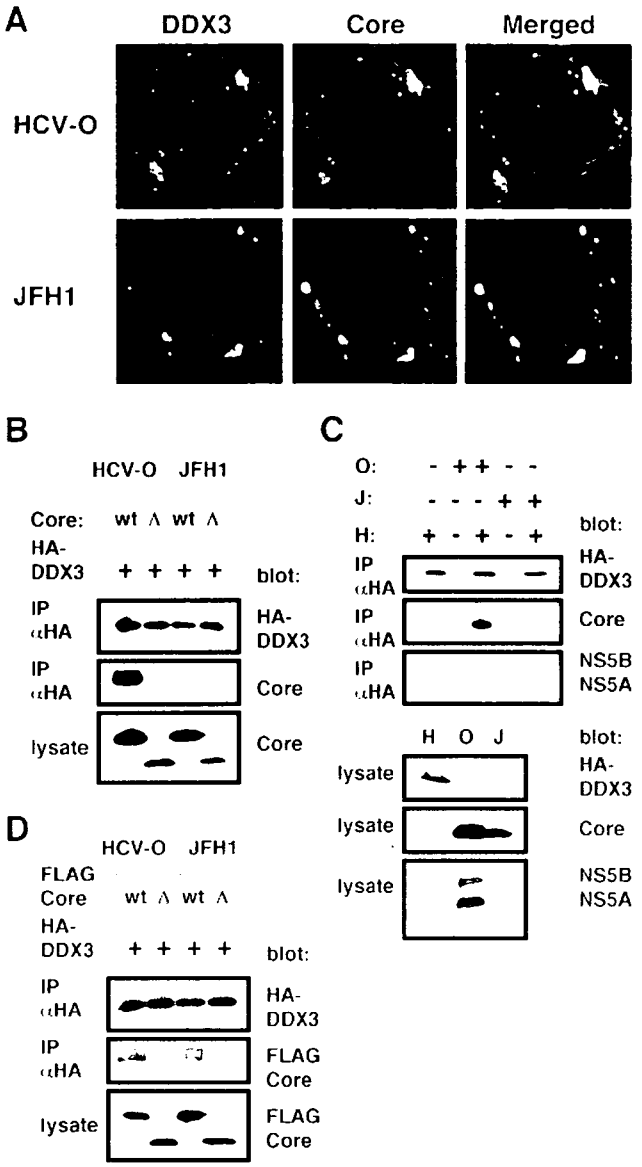

FIG. 2. Interaction of the HCV core with DDX3. (A) The HCV core colocalizes with DDX3. 293FT cells cotransfected with 100 ng of pCXbsr/core(HCV-O) or pcDNA3/core(JFH1) and 200 ng of pHA-DDX3 were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV core (CP-9 and CP-11 mixture) and anti-DDX3 antibodies and were then visualized with fluorescein isothiocyanate (DDX3) or Cy3 (core). Images were visualized using confocal laser scanning microscopy (LSM510; Carl Zeiss). The right panels exhibit the two-color overlay images (Merged). Colocalization is shown in yellow. (B) The core binds to DDX3. 293FT cells were cotransfected with 4 μg of pHA-DDX3 and 4 μg of pCXbsr/core(HCV-O) (wt), pcDNA3/Δcore(HCV-O) (Δ), pcDNA3/core(JFH1) (wt), or pcDNA3/Δcore(JFH1) (Δ). The cell lysates were immunoprecipitated with an anti-HA antibody (3F10; Roche), followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). (C) 293FT cells transfected with 4 μg of pHA-DDX3 (H), O cells (O), or RSc cells 3 days after inoculation of HCVcc (JFH1) (J) were lysed and immunoprecipitated (IP) with 1 μg of anti-HA antibody (3F10), followed by immunoblotting with anti-HA (HA-7), anti-core (CP-9 and CP-11 mixture), or anti-HCV NS5A (no. 8926) and anti-HCV NS5B. (D) 293FT cells transfected with 4 μg of pHA-DDX3 and 4 μg of pcDNA3/FLAG-core(HCV-O) (wt), pcDNA3/FLAG-Δcore(HCV-O) (Δ), pcDNA3/FLAG-core(JFH1) (wt), or pcDNA3/FLAG-Δcore(JFH1) (Δ) were lysed and immunoprecipitated with 1 μg of an anti-HA antibody (3F10), followed by immunoblotting with an anti-HA (HA-7) or anti-core (CP-9 and CP-11 mixture) antibody.

on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). We observed that the HCV-O core but not its N-truncated mutant could strongly bind to HA-tagged DDX3 (Fig. 2B). In contrast, the binding activity of the JFH1 core to HA-tagged DDX3 seemed to be fairly weak (Fig. 2B). As well, immunoprecipitation of lysates of 293FT cells expressing HA-tagged DDX3, O cells, or JFH1-infected RSc cells, or mixtures of these lysates, with an anti-HA antibody revealed that HCV-O core but not JFH1 core could bind strongly to DDX3 (Fig. 2C). The anti-HCV core antibody we used could detect both HCV-O core and JFH1 core (Fig. 2), while both anti-HCV NS5A and anti-NS5B antibodies failed to detect JFH1 NS5A and NS5B (Fig. 2C). At least, we failed to detect an interaction between DDX3 and either HCV-O NS5A or NS5B under experimental conditions that permitted the core to interact with DDX3 by immunoprecipitation (Fig. 2C). In contrast, the FLAG-tagged JFH1 core could bind to HA-tagged DDX3 just as efficiently as the FLAG-tagged HCV-O core could (Fig. 2D). Thus, the binding affinity or stability of the complex formed between the JFH1 core and DDX3 might be weaker than that between the HCV-O core and DDX3.

Since DDX3 is required for HIV-1 and HCV replication, we hypothesized that the HCV core might affect the function of HIV-1 Rev when both proteins were coexpressed. To test this hypothesis, we used the Rev-dependent luciferase-based reporter plasmid pDM628, harboring a single intron that includes both the Rev-responsive element (RRE) and the luciferase coding sequences (Fig. 3A) (10). In this system, Rev binds to RRE on the unspliced reporter mRNA, allowing its export from the nucleus for luciferase reporter gene expression, while the intron containing the luciferase gene is excised during RNA splicing when cells are transiently transfected with pDM628 alone. As previously reported (10), the luciferase activity in 293FT cells transfected with this reporter plasmid was stimulated by Rev, which induced a fourfold increase in the reporter signal (Fig. 3B). Luciferase activity was increased eightfold by the combination of Rev and DDX3, whereas neither the HCV-O core nor the JFH1 core had any effect on this Rev function (Fig. 3B). Since the Rev-binding domain (the N-terminal domain) and the core-binding domain (the C-terminal domain) do not overlap in DDX3, the HCV core might not compete with HIV-1 Rev for binding with DDX3. However, the development of a novel DDX3 inhibitor might provide a powerful antiviral agent against both HIV-1 and HCV (15).

Taking these results together, this study has shown for the first time that DDX3 is required for HCV RNA replication.

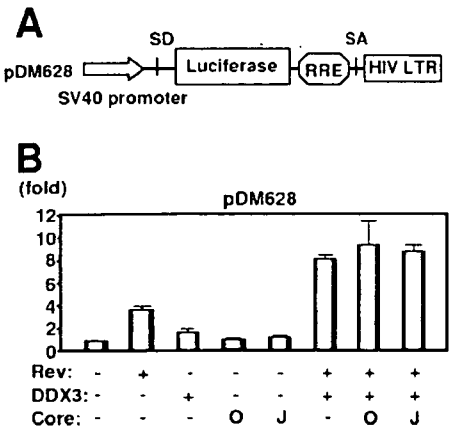

FIG. 3. HCV core does not affect the DDX3-mediated synergistic activation of Rev function. (A) Schematic representation of HIV-1 Rev-dependent luciferase-based reporter plasmid pDM628 harboring a splicing donor (SD), splicing acceptor (SA), and RRE. (B) 293FT cells were cotransfected with 100 ng of pDM628, 200 ng of pcRev, 200 ng of pHA-DDX3, and/or 100 ng of either pcDNA3/core(HCV-O) (O) or pcDNA3/core(JFH1) (J). A luciferase assay was performed 24 h later. All transfections utilized equal total amounts of plasmid DNA owing to the addition of the empty vector pcDNA3 to the transfection mixture. The relative stimulation of luciferase activity (*n*-fold) is shown. The results shown are means from three independent experiments.

Since helicases are motor enzymes that use energy derived from nucleoside triphosphate hydrolysis to unwind double-stranded nucleic acids, the DDX3–core complex might unwind the HCV double-stranded RNA and separate the RNA strands or might contribute to the function of HCV NS3 helicase. Since the replication of subgenomic replicon RNA was also partially suppressed in DDX3 knockdown cells (Fig. 1D), DDX3 might be associated with an HCV nonstructural protein(s) or HCV RNA itself. Indeed, Tingting et al. recently reported that DDX1 bound to both the HCV 3′ untranslated region (3′ UTR) and the HCV 5′ UTR and that short interfering RNA-mediated knockdown of DDX1 caused a marked reduction in the replication of subgenomic replicon RNA (22). Furthermore, Goh et al. demonstrated that DDX5/p68 associated with HCV NS5B and that depletion of endogenous DDX5 correlated with a reduction in the transcription of negative-strand HCV RNA (11). However, we failed to observe an interaction between DDX3 and NS5A or NS5B by immunoprecipitation under our experimental conditions in which the core could interact with DDX3 (Fig. 2C). Importantly, our DDX3 knockdown study demonstrated a more significant reduction in the accumulation of genome-length HCV RNA (95% reduction) than in the accumulation of subgenomic replicon RNA (52% reduction) (Fig. 1B and D). To date, it has been demonstrated that the 5′ UTR, the 3′ UTR, and the NS3-to-NS5B coding region are sufficient for HCV RNA replication (16); however, the core might be partly involved in the replication of genome-length HCV RNA. Importantly, DDX1 and DDX3 were specifically detected in the lipid droplets of core-expressing Hep39 cells by proteomic analysis (21), suggesting that DDX3 might be associated with HCV assembly or might incorporate into the HCV virion through interaction with the core to act as an RNA chaperone.

Recent studies have suggested a potential role of DDX3 and DDX5 in the pathogenesis of HCV-related liver diseases. DDX3 expression is deregulated in HCV-associated HCC (7, 8), and Huang et al. identified single-nucleotide polymorphisms in the DDX5 gene that were significantly associated with an increased risk of advanced fibrosis in patients with chronic hepatitis C (12). Interestingly, DDX3 might be a candidate tumor suppressor. DDX3 inhibits colony formation in various cell lines, including HuH-7, and up-regulates the $p21^{waf1/cip1}$ promoter (8). If DDX3 could in fact suppress tumor growth, then the core might overcome DDX3-mediated cell growth arrest and down-regulate $p21^{waf1/cip1}$ through interaction with DDX3, and it might also be involved in HCC development.

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Comprehensive Analysis of the Effects of Ordinary Nutrients on Hepatitis C Virus RNA Replication in Cell Culture▽

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To date, only a limited number of studies have reported finding an influence of ordinary nutrients on hepatitis C virus (HCV) RNA replication. However, the effects of other nutrients on HCV RNA replication remain largely unknown. We recently developed a reporter assay system for genome-length HCV RNA replication in hepatoma-derived HuH-7 cells (OR6). Here, using this OR6 assay system, we comprehensively examined 46 nutrients from four nutrient groups: vitamins, amino acids, fatty acids, and salts. We found that three nutrients—β-carotene, vitamin D_2, and linoleic acid—inhibited HCV RNA replication and that their combination caused additive and/or synergistic effects on HCV RNA replication. In addition, combined treatment with each of the three nutrients and interferon alpha or beta or fluvastatin inhibited HCV RNA replication in an additive manner, while combined treatment with cyclosporine synergistically inhibited HCV RNA replication. In contrast, we found that vitamin E enhanced HCV RNA replication and negated the effects of the three anti-HCV nutrients and cyclosporine but not those of interferon or fluvastatin. These results will provide useful information for the treatment of chronic hepatitis C patients who also take anti-HCV nutrients as an adjunctive therapy in combination with interferon. In conclusion, among the ordinary nutrients tested, β-carotene, vitamin D_2, and linoleic acid possessed anti-HCV activity in a cell culture system, and these nutrients are therefore considered to be potential candidates for enhancing the effects of interferon therapy.

Hepatitis C virus (HCV) is a major pathogen of chronic hepatitis (CH) and leads to fatal liver diseases, such as liver cirrhosis and hepatocellular carcinoma (1, 23, 38). Approximately 170 million people worldwide are infected with HCV (40). Therefore, HCV infection is a global health problem. The combination of pegylated interferon (IFN) with ribavirin is currently the most effective therapy for CH C (11, 28), and long-term treatment has been shown to improve the sustained virological response (SVR) rate (37). However, the SVR rate still remains at approximately 55% (11). Combination therapy occasionally also causes adverse effects, such as severe anemia (10) and cerebral vascular lesions (7), and reduction of the dosage leads to insufficient treatment. These adverse effects are more serious in older patients. Therefore, it remains necessary to identify alternative agents that have fewer side effects to couple with IFN. In developing countries, it is difficult to administer expensive IFN therapy. Hence, in such countries, inexpensive anti-HCV reagents are especially desirable (36).

The lack of a small-animal model and a cell culture system to support efficient HCV RNA replication hampered the development of anti-HCV reagents. Since an HCV subgenomic replicon system was developed in 1999 (25), the mechanism of HCV RNA replication has been gradually elucidated by a number of groups (2). However, this subgenomic replicon system may not necessarily reflect actual HCV RNA replication in hepatocyte cell lines, due to the absence of structural proteins. To overcome this problem, a cell culture system for genome-length HCV RNA replication was developed by several groups (4, 15, 33). We also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter, which facilitated the prompt and precise monitoring of HCV RNA replication in hepatocyte cell lines (13, 30). In the OR6 assay system, the luciferase activity was well correlated with HCV RNA levels when cells were treated with IFN-α (13). Therefore, we quantified the luciferase activity instead of HCV RNA for the indirect evaluation of HCV RNA replication, although the OR6 assay system doesn't strictly quantify the HCV RNA replication. More recently, several groups have developed cell culture systems that produce infectious viral particles (genotype 2a), which can be used to reconstruct the life cycle of HCV infection in hepatocyte cell lines (24, 39, 45).

Using this OR6 assay system, we demonstrated that mizoribine (30), as an immunosuppressant, and fluvastatin (FLV) (14), as the reagent for hypercholesterolemia, inhibited HCV RNA replication in hepatocyte cell lines. Another immunosuppressant, cyclosporine (CsA), was also identified as an anti-HCV reagent in a subgenomic replicon system (41). These results suggested the expansion of the primary application of the existing therapeutic drugs to new anti-HCV therapy.

Other studies have also revealed that certain ordinary nutrients contained in common foods can influence HCV RNA replication (5, 8, 9, 17, 21, 26). However, in these studies, only a limited number of nutrients were tested. To date, there has

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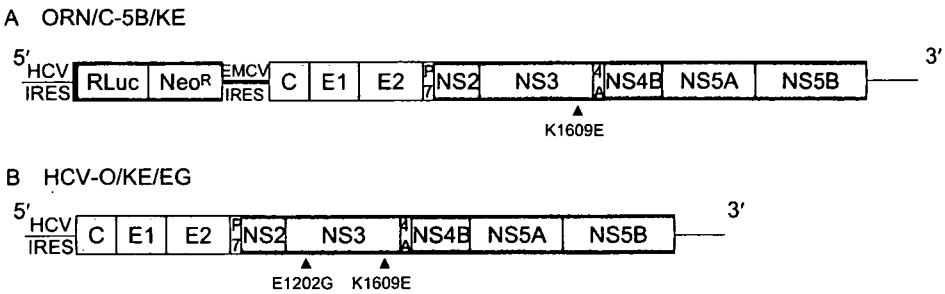


FIG. 1. Schematic gene organization of genome-length HCV RNA. (A) ORN/C-5B/KE RNA replicated in OR6 cells. RL (RLuc) is expressed as a fusion protein with neomycin phosphotransferase (NeoR). The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) Authentic HCV RNA, HCV-O/KE/EG, was introduced into the OR6c cells by electroporation as previously described (13). The positions of two adaptive mutations, E1202G and K1609E, are indicated by black triangles. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus.

been no comprehensive analysis of the effects of nutrients on HCV RNA replication. Thus, we inclusively investigated the effects of nutrients on genome-length HCV RNA replication using the OR6 assay system. Here, we report finding that three nutrients, namely, β-carotene (BC), vitamin D_2 (VD2), and linoleic acid (LA), inhibited HCV RNA replication and that combination treatments including each of these nutrients and CsA synergistically inhibited HCV RNA replication. Furthermore, we found that vitamin E (VE) enhanced HCV RNA replication, and that the anti-HCV activities of each of the three nutrients and CsA were abrogated by VE.

MATERIALS AND METHODS

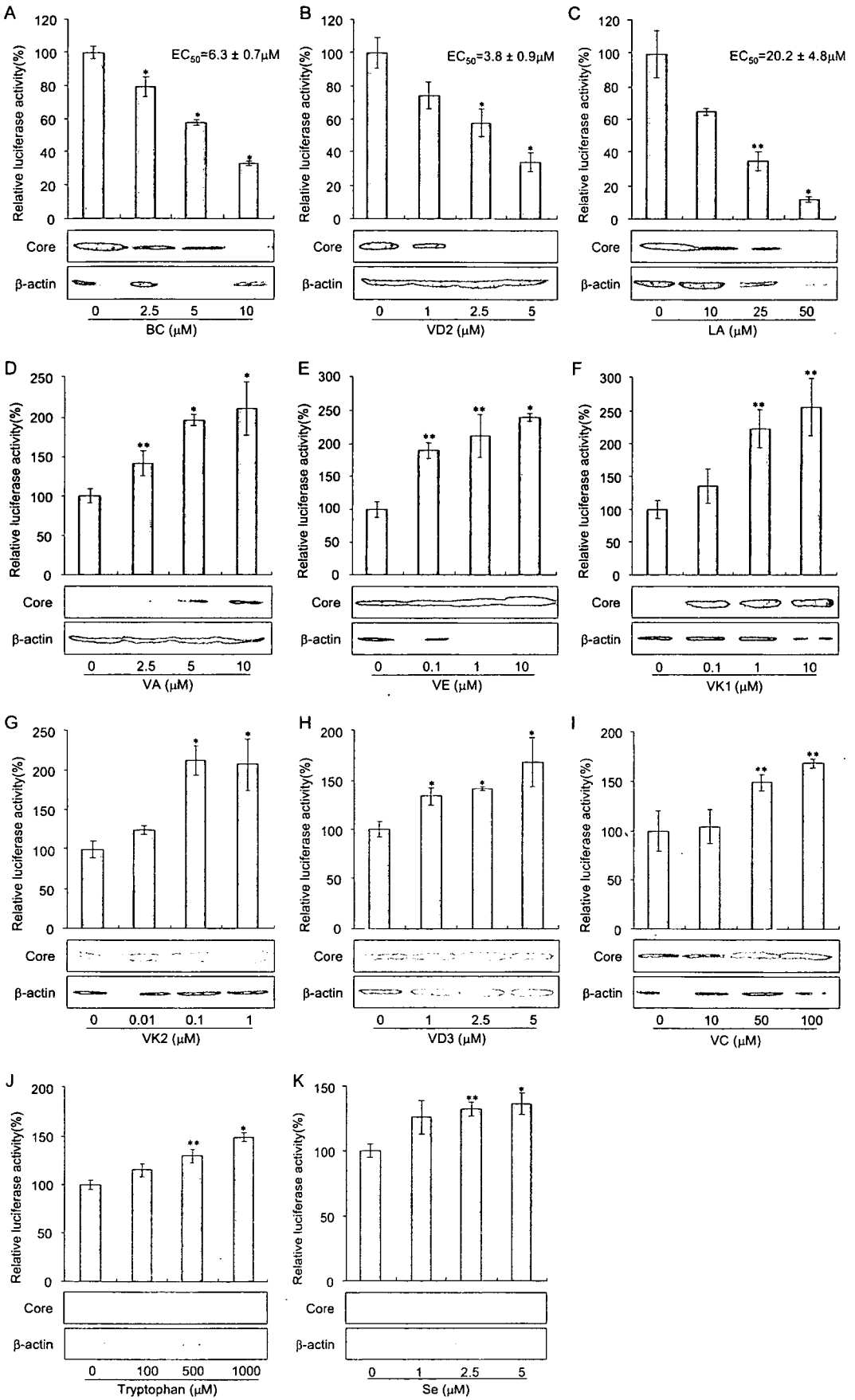
Reagents. Vitamin B_{12}, vitamin K_1 (VK1), vitamin K_3, elaidic acid, and vaccenic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BC, vitamin A (VA), vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin C (VC), VD2, vitamin D_3 (VD3), VE, vitamin K_2 (VK2), pantothenic acid, biotin, folic acid, inositol, leucine, isoleucine, valine, tryptophan, phenylalanine, tyrosine, lauric acid, palmitic acid, stearic acid, behenic acid, oleic acid, elaidic acid, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), $Fe(II)SO_4$, Na_2SeO_4, $Fe(III)(NO_3)_3$, $ZnCl_2$, NaCl, KCl, $CaCl_2$, PCl_3, $MgCl_2$, $CuCl_2$, $MnCl_2$, and IFN-α were purchased from Sigma (St. Louis, MO), and CsA and FLV were purchased from Calbiochem (Los Angeles, CA). IFN-β was kindly provided by Toray Industries, Inc. Fatty acids, except for LA-albumin and oleic acid-albumin compounds (Sigma), were compounded with fatty acid-free bovine serum albumin (Sigma) as described in a previous report (6). We treated the HCV RNA-harboring cells at various concentrations of salts [$Fe(II)SO_4$ at 5, 25, and 50 μM, $Fe(III)(NO_3)_3$ at 10, 100, and 200 μM, $ZnCl_2$ at 20, 50, and 100 μM, Na_2SeO_4 at 1, 2.5, and 5 μM, NaCl at 100, 150, and 300 μM, KCl at 5, 10, and 20 μM, $CaCl_2$ at 2, 4, and 8 μM, PCl_3 at 1, 2.5, and 5 μM, $MgCl_2$ at 0.5, 2.5, and 5 μM, $CuCl_2$ at 20, 50, and 100 μM, and $MnCl_2$ at 30, 60, and 120 μM]. Dimethyl sulfoxide or ethanol was used for the dissolution of the liposoluble nutrients, and the final concentrations (0.1%) of each were equal for the cells with nutrients and those without.

Cell cultures. OR6 cells, polyclonal genome-length HCV RNA (ORN/C-5B/KE)-replicating cells, and subgenomic replicon (ORN/3-5B/KE) cells, which were derived from hepatoma cell line, HuH-7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin (designated as the control medium in this study), and G418 (300 μg/ml) (Geneticin; Invitrogen, Carlsbad, CA), and the cells were passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ORN/3-5B/KE are derived from HCV-O (strain O of genotype 1b). OR6c cells are cured OR6 cells from which

TABLE 1. Summary of anti-HCV activities of various nutrients

Nutrient type	Nutrient(s) with the indicated characteristic for HCV[a]		
	Inhibitory	Enhancing	Ineffective
Vitamins			
Liposoluble	BC, VD2	VA (retinol), VE, VK1, VK2, VK3	VD3
Water soluble		VC	*VB1*, *VB2*, *VB3* (*niacin*), *VB6*, VB12, *pantothenic acid*, biotin, *folic acid*, *inositol*
Amino acids			
Branched-chain			*Leucine*, *isoleucine*, *valine*
Aromatic		*Tryptophan*	*Phenylalanine*, *tyrosine*
Fatty acids			
Saturated			Lauric acid (C_{12}), palmitic acid (C_{16}), stearic acid (C_{18}), behenic acid (C_{22})
Mono-unsaturated			Oleic acid (C_{18}; 9-unsaturated), elaidic acid (C_{18}; *trans*-form of oleic acid), vaccenic acid (C_{18}; 11-unsaturated)
Polyunsaturated	LA ($C_{18:2}$, n-6), AA ($C_{20:4}$, n-6), EPA ($C_{20:5}$, n-3), DHA ($C_{22:6}$, n-3)		
Salts	$Fe(II)SO_4$, *$Fe(III)(NO_3)_3$*, $ZnCl_2$	Na_2SeO_4	*NaCl*, *KCl*, *$CaCl_2$*, *PCl_3*, *$MgCl_2$*, $CuCl_2$, $MnCl_2$

[a] Nutrients already contained in the medium are indicated in italics. VB1, vitamin B_1; VB2, vitamin B_2; VB3, vitamin B_3; VB6, vitamin B_6; VB12, vitamin B_{12}; VK3, vitamin K_3.



genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described (13).

Luciferase reporter assay. For the *Renilla* luciferase (RL) assay, approximately 1.0×10^4 to 1.5×10^4 OR6 cells (72-hour treatment) or 0.5×10^4 OR6 cells (120-hour treatment) were plated onto 24-well plates in triplicate and cultured for 24 h. The cells were treated with each nutrient or compound for 72 or 120 h. Then, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to the RL assay according to the manufacturer's protocol.

Western blot analysis. For Western blot analysis, 4×10^4 to 4.5×10^4 OR6c cells harboring HCV-O/KE/EG (strain O of genotype 1b) (K. Abe, M. Ikeda, and N. Kato, unpublished data) were plated onto six-well plates and cultured for 24 h and then were treated with each nutrient or compound for 72 h. Preparation of the cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were then performed as previously described (18). The antibodies used in this study were those specific to HCV core antigen (CP11; Institute of Immunology, Tokyo) and β-actin (Sigma). The epitope of CP11 was located within amino acid positions 21 to 40 of the core antigen. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

Cell viability. To examine the suppressive effects of nutrients on OR6 cell viability, approximately 4.5×10^4 to 5×10^4 OR6 cells (72-hour viability assay) or approximately 1×10^4 to 1.5×10^4 cells (120-hour viability assay) were plated onto six-well plates in triplicate and were cultured for 24 h. The cells were treated without nutrients or with each nutrient for 72 or 120 h, and then the number of viable cells was counted after trypan blue dye treatment as previously described (30).

Statistical analysis and synergistic statistics. Differences between the anti-HCV activities of the nutrients at each concentration and controls were tested using Student's *t* test. *P* values of less than 0.05 were considered statistically significant. Then, an isobologram method was used to evaluate the effects of a combination of nutrients or compounds on HCV RNA replication (21). OR6 cells were treated with each combination of nutrients or compounds at various concentrations for 72 h. The 50% effective concentration (EC_{50}) against HCV RNA replication in each combination treatment was analyzed by sigmoid regression, and isoboles of EC_{50} were plotted using the resulting data.

RESULTS

Effects of ordinary nutrients on HCV RNA replication. To date, information about the anti-HCV effects of ordinary nutrients has been limited to only a few studies, and in those studies, a plasmid (26), a subgenomic replicon (21), and recombinant HCV proteins (5, 8, 9) were used in the assays. We recently developed OR6 assay system by the selection after introducing genome-length ORN/C-5B/KE RNA (Fig. 1A) into HuH-7 cells. Our OR6 assay system renders it possible to carry out the prompt and precise evaluation of genome-length HCV RNA replication (13, 30). Therefore, we comprehensively analyzed 46 ordinary nutrients to determine their effects on HCV RNA replication using our novel OR6 assay system (Table 1). The effects of the preexistent nutrients in the medium on HCV RNA replication were under a significant level, because the concentrations of the nutrients already contained in the medium were less than a one-thousandth part of the minimum concentration in the treatment.

We first examined 8 liposoluble vitamins and 10 water-soluble vitamins to investigate their effects on HCV RNA replication. Among the liposoluble vitamins, VA (Fig. 2D), VE (Fig. 2E), and VK (Fig. 2F and G) significantly enhanced HCV RNA replication. However, BC and VD2 significantly inhibited HCV RNA replication in a dose-dependent manner (the mean EC_{50}s ± standard deviations [SDs] were 6.3 ± 0.7 μM and 3.8 ± 0.9 μM, respectively) (Fig. 2A and B). In contrast with VD2, VD3 apparently enhanced relative luciferase activity, but this promotive effect was thought to result from cell proliferation, since the amount of β-actin increased in a manner similar to that of HCV core antigen (Fig. 2H). Most of the water-soluble vitamins exerted no effect on HCV RNA replication (data not shown), while only VC moderately enhanced HCV RNA replication (Fig. 2I).

We next examined three branched-chain amino acids and three aromatic amino acids for their effects on HCV RNA replication. We tested the six amino acids at concentrations of 0, 100, 500, and 1,000 μM, and only tryptophan exerted moderate promotive effects on HCV RNA replication (Fig. 2J).

We further examined four saturated fatty acids, three monounsaturated fatty acids, and four polyunsaturated fatty acids (PUFAs) to assess their effects on HCV RNA replication. As has been noted in previous reports (17, 21), all of the PUFAs, i.e., LA, AA, EPA, and DHA, inhibited HCV RNA replication in OR6 cells in a dose-dependent manner (the mean EC_{50}s ± SDs were 20.2 ± 4.8 μM, 22.1 ± 1.7 μM, 36.2 ± 2.5 μM, and 37.0 ± 3.6 μM, respectively). However, we found that with the exception of LA, treatment with 50 μM of PUFA resulted in

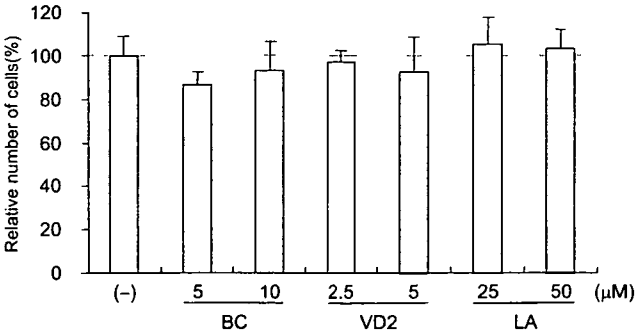


FIG. 3. The anti-HCV activities of three nutrients were not due to the suppression of cell growth. Cell viabilities after treatment with BC, VD2, and LA are shown. OR6 cells were cultured in control medium [(−)] and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), and LA (25 and 50 μM) for 72 h, and then the number of viable cells was counted after trypan blue dye treatment. Shown here are the percent relative cell numbers calculated when the relative cell number of untreated cells was assigned as 100%. The data indicate means ± SDs of triplicates from at least two independent experiments.

FIG. 2. Effects of ordinary nutrients on HCV RNA-replicating cells. (A through K) Reporter assay and Western blot analysis of nutrient sensitivity of HCV RNA replication. OR6 cells were treated with each nutrient at a four-grade-modulated concentration in the medium. After 72 h of treatment, the RL assay was performed as described in Materials and Methods. Shown here are the percent relative luciferase activities calculated when the RL activity of untreated cells was assigned the value of 100%. The data indicate means ± SDs of triplicate samples from at least three independent experiments. Subsequently, OR6c cells, into which authentic HCV RNA was introduced, were treated with nutrients exhibiting either inhibitory effects, i.e., BC (A), VD2 (B), and LA (C), or promotive effects, i.e., VA (D), VE (E), VK1 (F), VK2 (G), VD3 (H), VC (I), tryptophan (J), and Se (K) at the same concentrations as those used in the OR6 assay (bar graphs). After 72 h of treatment, the production of HCV core antigen was analyzed by immunoblotting using antibody specific to HCV core antigen (upper lanes). β-Actin was used as a control for the amount of protein loaded per lane (lower lanes). *, $P < 0.01$; **, $P < 0.05$.

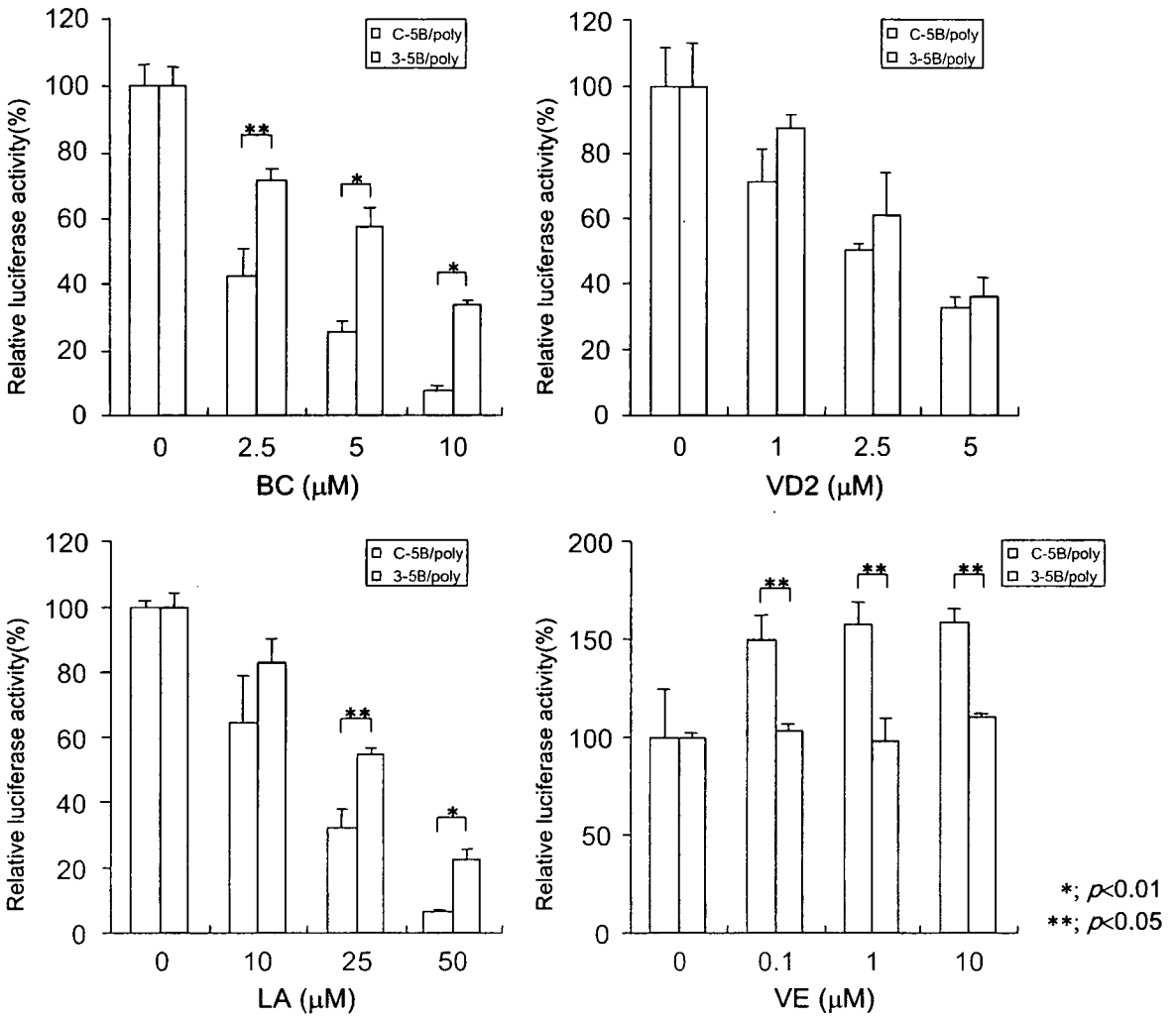


FIG. 4. The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. Both polyclonal genome-length HCV RNA-replicating cells (ORN/C-5B/KE/poly) and subgenomic replicon cells (ORN/3-5B/KE/poly) were treated with BC, VD2, LA, or VE according to the same protocol as that used for the OR6 assay. The RL assay was performed at 72 h postapplication, and then RL activity was calculated as described in the legend to Fig. 2.

the suppression of cell growth due to cytotoxicity (data not shown). These data indicate that among PUFAs, only LA exhibited a significant inhibitory effect on HCV RNA replication without concomitant cytotoxicity (Fig. 2C and 3).

Finally, we examined 11 salts in order to assess their effects on HCV RNA replication. Iron [Fe(II) in the form of $FeSO_4$ and Fe(III) in the form of $Fe(NO_3)_3$] and zinc (in the form of $ZnCl_2$) exhibited anti-HCV effects without cytotoxicity at concentrations of up to 50% inhibition, but beyond 50% inhibition, cell growth was dose-dependently affected by the cytotoxicity of these minerals. Selenium (in the form of Na_2SeO_4), a typical antioxidant, slightly enhanced HCV RNA replication (Fig. 2K). We also confirmed these results using authentic HCV RNA-replicating cells (Fig. 1B and 2A through K).

These results suggest that the ordinary nutrients tested here have different profiles in terms of their effects on HCV RNA replication. The results are summarized in Table 1. Most of the nutrients were found to have no effect on HCV RNA replication. Eight nutrients enhanced HCV RNA replication, and the antioxidant nutrients VA, VC, VE, and Se were included in this group. Among the 46 nutrients tested with the OR6 assay system, we found that BC, VD2, and LA exerted anti-HCV effects without cytotoxicity. To the best of our knowledge, this is the first study to demonstrate the anti-HCV effects of BC and VD2. Therefore, we focused on the anti-HCV effects of BC, VD2, and LA in the following study.

The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. OR6 cells are among the cloned cell lines selected by G418. Therefore, we examined polyclonal cells harboring genome-length HCV RNA (ORN/C-5B/KE/poly) to exclude the possibility that the anti-HCV effects of BC, VD2, and LA were an OR6 clone-specific phenomenon. Furthermore, polyclonal cells harboring subgenomic HCV RNA (ORN/3-5B/KE/poly) were also used to examine the effects of the anti-HCV nutrients on RNA replication in the absence of the structural HCV proteins. The

results revealed that all of these three nutrients exhibited a dose-dependent suppression of HCV RNA replication in both cell systems, although the three nutrients had stronger anti-HCV effects in the polyclonal genome-length HCV RNA-replicating cells than they did in the subgenomic HCV RNA-replicating cells (Fig. 4). These results indicated that the anti-HCV activities of these nutrients were not due to cell clonality, and the sensitivities of the reagents were found to differ between subgenomic and genome-length HCV RNA-replicating cells. One possible explanation of this difference is that the different genome sizes of subgenomic (9-kb) and genome-length (12-kb) HCV RNA might affect the replication efficiencies and lead to the difference in the sensitivities of antiviral reagents. These differences were significant, especially in BC- and LA-treated cells. A subgenomic replicon system may underestimate the effects of anti-HCV reagents and therefore might fail to identify potentially effective anti-HCV reagents. Therefore, our genome-length HCV RNA replication system (OR6) is advantageous for evaluating anti-HCV candidates.

We also tested VE's effect on subgenomic and genome-length HCV RNA-replicating cells. VE enhanced the replication of genome-length HCV RNA. However, interestingly, VE did not affect subgenomic HCV RNA replication. These results suggest that the subgenomic HCV RNA replication system may not be able to evaluate the reagent-enhancing HCV RNA replication.

Anti-HCV activities of three nutrients were not due to inhibition of cell growth. Since it has been reported that HCV RNA replication is dependent on cell growth (34), we examined whether the anti-HCV activities of three nutrients were due to their respective cytotoxicities. OR6 cells were treated with each nutrient (BC, 5 and 10 μM; VD2, 2.5 and 5 μM; LA, 25 and 50 μM) for 72 h. These results suggest that the anti-HCV effects of BC, VD2, and LA are not due to their cytotoxicities.

Time course assay of inhibitory effects of three nutrients on HCV RNA replication. A kinetics analysis of the anti-HCV effects of reagents provides information about inhibitory mechanisms and optimized drug administration. Therefore, we conducted a time course assay (24, 72, and 120 h after treatment) of the anti-HCV effects of three nutrients, BC, VD2, and LA, using our OR6 assay system. The results revealed that BC and VD2 exhibited stronger inhibition of HCV RNA replication than did LA at 24 h after treatment. However, the anti-HCV activities of BC and VD2 only slightly increased at 72 or 120 h after treatment (Fig. 5A). On the other hand, LA inhibited HCV RNA replication in dose- and time-dependent manners. It is noteworthy that about 90% inhibition of RL activity was observed at 120 h after LA (50 μM) treatment of OR6 cells (Fig. 5A).

We examined whether these reductions in relative RL activity induced by all three nutrients at 120 h were due to the suppression of cell growth. Compared to the number of untreated cells, at 120 h after treatment with each nutrient (BC, 5 and 10 μM; VD2, 2.5 and 5 μM; LA, 25 and 50 μM), no significant reduction in the number of treated cells was observed (Fig. 5B). These results indicate that the anti-HCV effects of these three nutrients were not due to their respective cytotoxicities.

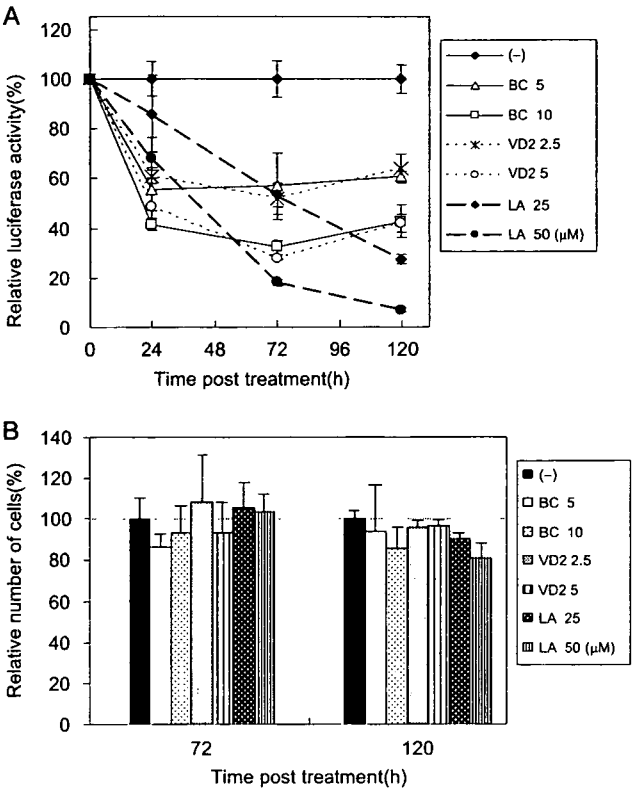


FIG. 5. Time course assay of the anti-HCV activities of three nutrients. (A) Time course of the inhibitory effects of three nutrients on HCV RNA replication. OR6 cells were treated with control medium [(−)], BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and the RL assay was performed at 24, 72, and 120 h postapplication. Relative RL activity was calculated as described in the legend to Fig. 2. (B) Time course of cell viability after the application of three nutrients. OR6 cells were cultured in the control medium and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and at 72 and 120 h postapplication, the number of viable cells was counted after trypan blue dye treatment. Shown here are the percent relative cell numbers calculated as described in the legend to Fig. 3.

HCV RNA replication was additively inhibited by each combination of three nutrients and was synergistically inhibited by all three. As described above, we found that BC, VD2, and LA possessed anti-HCV activities. However, these nutrients appeared to be insufficient for eliminating HCV by mono-treatment. Therefore, we examined the anti-HCV effects of two or three nutrients in combination.

To evaluate the effects of each combination treatment, OR6 cells were cotreated with two nutrients at the listed concentrations for 72 h (BC, approximately 0 to 5 μM; VD2, approximately 0 to 3 μM; LA, approximately 0 to 20 μM). Isoboles of 50% inhibition of HCV RNA replication were obtained for each data point. An analysis of the 50% isoboles of each combination treatment graphed nearly a straight line in each case. These results indicate that the inhibitory effects of all combinations on HCV RNA replication were additive (Fig. 6A).

Treatment with all three nutrients at various concentrations resulted in stronger suppression of HCV RNA replication in OR6 cells than we had predicted as an additive effect (Fig. 6B).

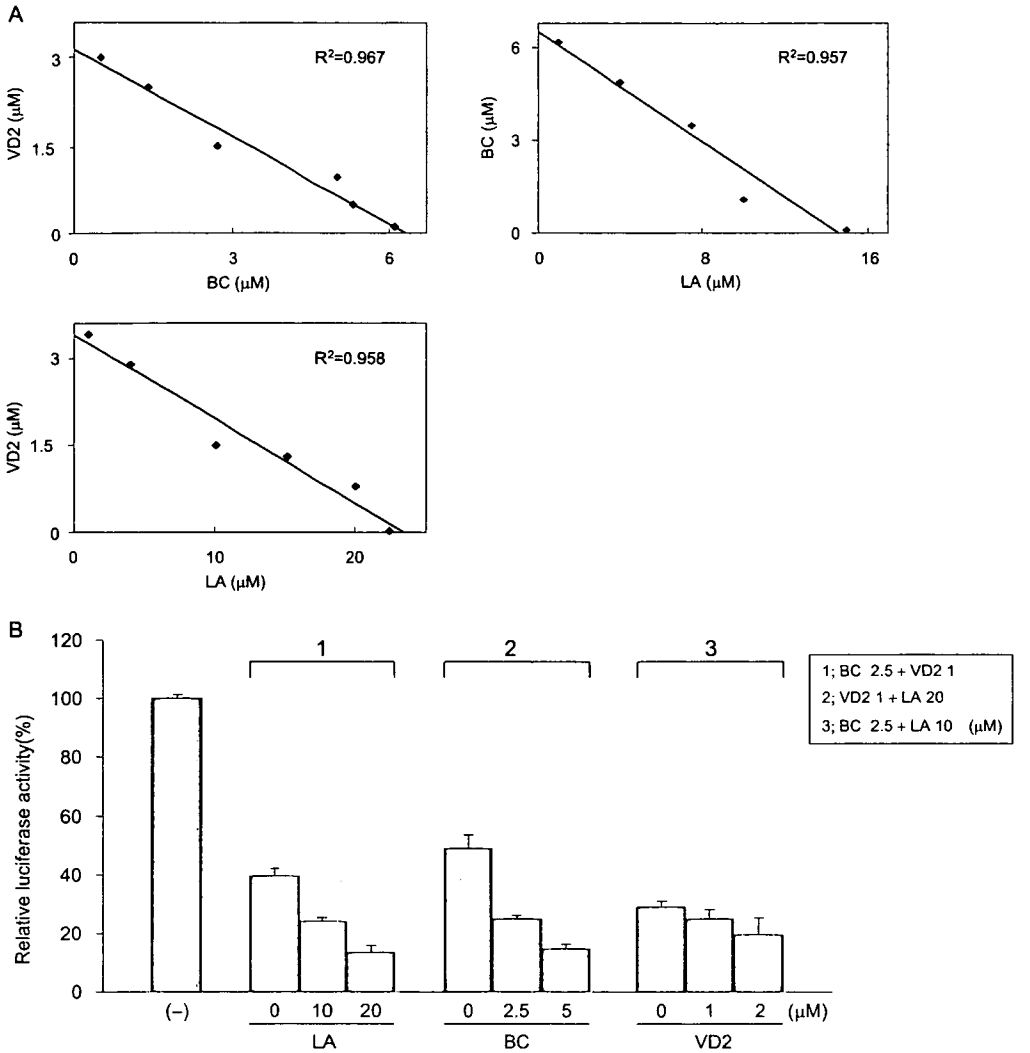


FIG. 6. Effects of treatment with each combination or all of the three nutrients on HCV RNA replication. (A) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with each combination of three nutrients, BC (0, 0.5, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.1, 0.5, 1, 1.5, 2, and 3 μM), and LA (0, 1, 5, 10, 15, and 20 μM), for 72 h, and RL assay was performed as described in the legend to Fig. 2 to obtain 50% isoboles. The R^2 value indicates the coefficient of determination. (B) The effect of treatment with all three nutrients on HCV RNA replication was synergistic. OR6 cells were treated with LA (0, 10, and 20 μM) in addition to 2.5 μM BC plus 1 μM VD2, BC (0, 2.5, and 5 μM) in addition to 1 μM VD2 plus 20 μM LA, or VD2 (0, 1, and 2 μM) in addition to 2.5 μM BC plus 10 μM LA. After 72 h of treatment, the RL assay was performed, and then relative RL activity was calculated as described in the legend to Fig. 2.

For instance, in the sample cotreated with 2.5 μM BC ($\approx EC_{20}$) in addition to 1 μM VD2 ($\approx EC_{30}$) plus 20 μM LA ($\approx EC_{50}$) (Fig. 2A through C), the actual effect on HCV RNA replication was 90% inhibition, which was 20% greater than we had originally estimated (i.e., approximately 70%; $1 - 0.8 \times 0.7 \times 0.5 = 0.72$) (Fig. 6B). In addition, no suppression of cell growth was observed during these cotreatments (data not shown). These results suggest that treatment with a mixture of these three nutrients may exert synergistic inhibitory effects on HCV RNA replication.

Treatment with each of three nutrients in combination with IFN or FLV additively inhibited HCV RNA replication, and CsA synergistically inhibited HCV RNA replication. Recently, CsA was proposed as a novel candidate to be paired with IFN in similar studies using a cell culture system (41). We have also reported findings obtained with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, exerted diverse anti-HCV effects, and FLV was found to exert the strongest inhibitory effect on HCV RNA among the statins tested (14).

Therefore, we examined the anti-HCV effects of each of three nutrients in combination with IFN, FLV, or CsA by using OR6 cells. OR6 cells were treated for 72 h with IFN-α (0, 0.2, 0.5, and 1 IU/ml) in combination with each of the nutrients at various concentrations (BC, approximately 0 to 5 μM; VD2, approximately 0 to 4 μM; LA, approximately 0 to 20 μM) (Fig. 7A). FLV (approximately 0 to 2 μM) or CsA (approximately 0 to 1 μg/ml) was also used for treatment in combination with BC, VD2, or LA at the concentration mentioned above (Fig. 7B and C). Isoboles of 50% inhibition of HCV RNA replication were generated from each sample. An analysis of 50%

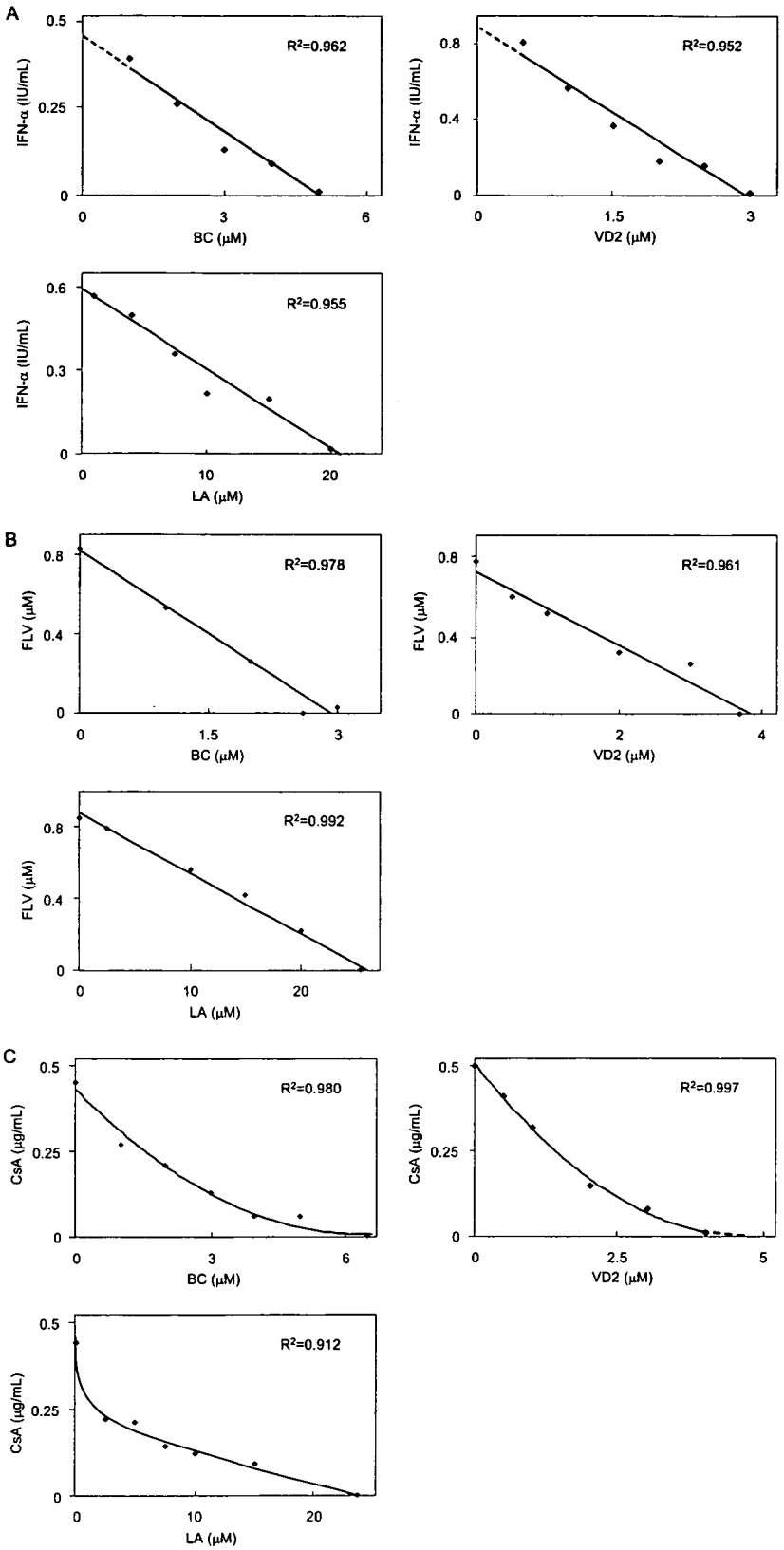
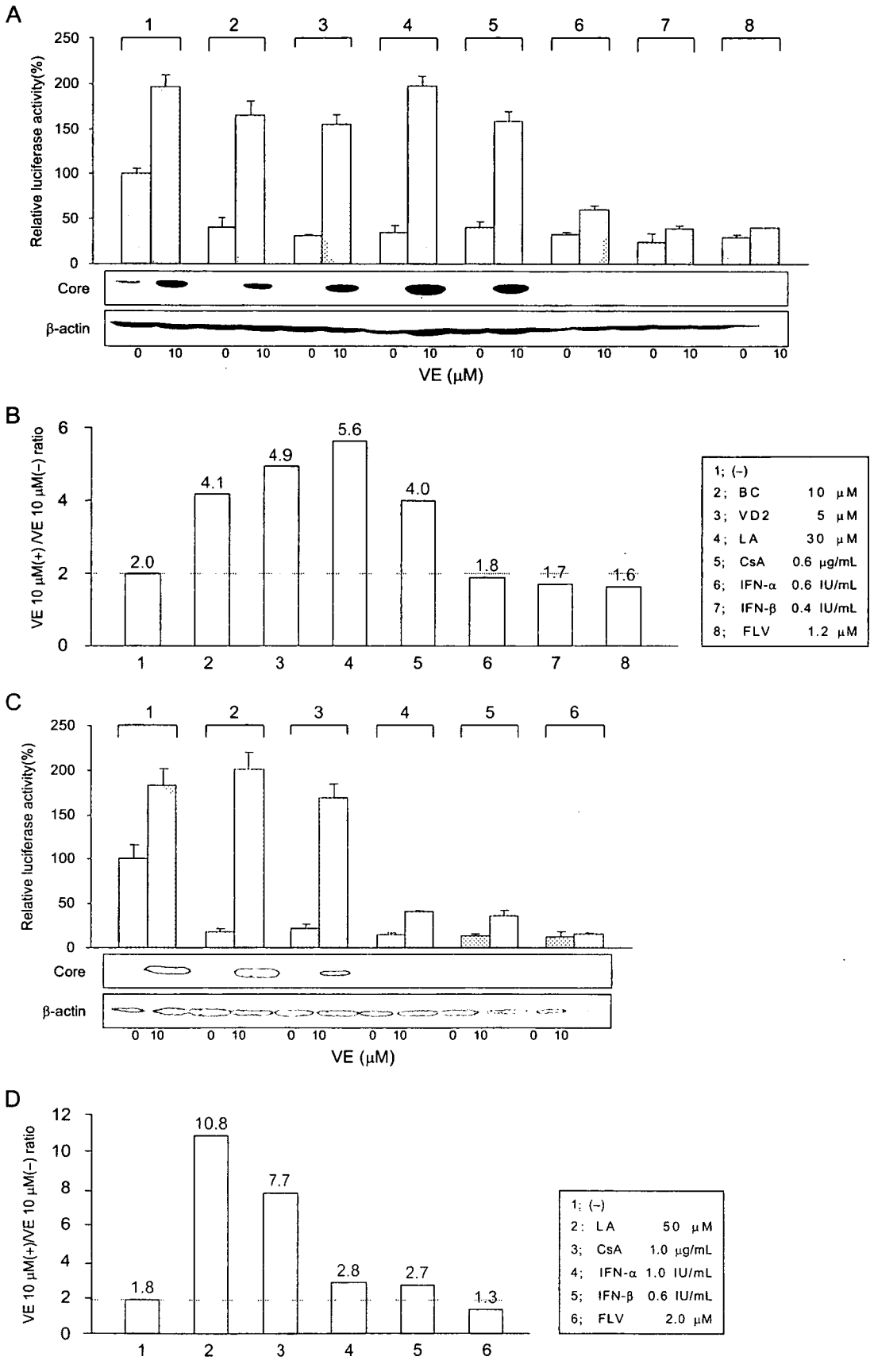


FIG. 7. Additive inhibitory effects of each of three nutrients in combination with IFN-α or FLV on HCV RNA replication, and synergistic effects observed with Cs. (A to C) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with BC (0, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.5, 1, 2, 3, and 4 μM), and LA (0, 2.5, 5, 10, 15, and 20 μM) in combination with IFN-α (0, 0.2, 0.5, and 1 IU/ml) (A), FLV (0, 0.5, 1, and 2 μM) (B), or CsA (0, 0.2, 0.5, and 1 μg/ml) (C) for 72 h, and the RL assay was performed as described in the legend to Fig. 2 to obtain 50% isoboles. The R^2 value indicates the coefficient of determination.



isoboles in combinations using each nutrient and IFN-α or FLV graphed nearly straight lines in each case, indicating that the suppressive effects of these cotreatments on HCV RNA replication were additive (Fig. 7A and B). Similar additive effects were obtained in combination with IFN-β (data not shown). It was noteworthy that all cotreatments with each nutrient and CsA resulted in curved, concave plots of 50% isoboles, thus suggesting that these combinations with CsA exerted synergistic inhibitory effects on HCV RNA replication (Fig. 7C). These results indicate that these three nutrients, administered as a supportive nutritional therapy, could potentially improve the SVR rate associated with IFN therapy alone.

The anti-HCV activities of BC, VD2, and LA, as well as that of CsA but not those of IFN and FLV, were completely canceled by VE. Among the 46 nutrients tested, BC and VD2 exhibited inhibitory effects on HCV RNA replication up to 70%, and LA exhibited inhibitory effects up to 90%, without exhibiting any cytotoxicity (Fig. 5A). In contrast, most of the liposoluble vitamins enhanced HCV RNA replication in OR6 cells. We used VE in the following studies because VE is one of the most common vitamins in the daily diet and it exerts a strong enhancing effect on HCV RNA replication. To clarify the mechanism of these opposing effects, we investigated whether the anti-HCV effects of BC, VD2, and LA were canceled by the addition of VE. We also tested representative anti-HCV compounds (i.e., CsA, IFN-α, IFN-β, and FLV) in combination with VE. We first examined the influence of 10 μM VE on the nutrients and compounds at the 70% inhibitory concentration level (Fig. 8A and B). The inhibitory effects of IFN-α, IFN-β, and FLV were hardly influenced by cotreatment with VE, whereas the anti-HCV effects of BC, VD2, LA, and CsA were canceled to a significant level by VE in the OR6 cells (Fig. 8A, upper panel). These results were also confirmed using authentic HCV RNA-replicating cells (Fig. 8A, lower panel). To normalize these results, we divided the luciferase value observed in the presence of VE by that in the absence of VE, and we considered this value to represent the effects of VE. When this value was larger than the value obtained in the absence of anti-HCV reagent (2.0; column 1 in Fig. 8B), we interpreted it as indicative of a reagent whose anti-HCV effects were canceled by VE. According to this criterion, BC (4.1), VD2 (4.9), LA (5.6), and CsA (4.0) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (columns 2, 3, 4, and 5 in Fig. 8B). The anti-HCV effects of IFN-α, IFN-β, and FLV were not affected by VE (columns 6, 7, and 8 in Fig. 8B). We next examined the influence of 10 μM VE on the anti-HCV nutrients and compounds at the 90% inhibitory concentration level (Fig. 8C and D). BC and VD2 were not assessed in this experiment, because the maximum inhibitory effect was 70% in the case of these nutrients (Fig. 5A). Similar results were obtained in this experiment. LA (10.8) and CsA (7.7) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (compare columns 2 and 3 to column 1 in Fig. 8D), although IFN-α (2.8) and IFN-β (2.7) were slightly affected by VE at this concentration (Fig. 8D, compare columns 4 and 5 to column 1). Judging by these results, it appears that BC, VD2, LA, and CsA may share some mechanism by which VE negated their anti-HCV activities.

DISCUSSION

The differential effects of BC and VA, as well as those of VD2 and VD3, which belong to the same categories as VA and VD, respectively, are of interest. We observed that whereas BC and VD2 inhibited HCV RNA replication, VA enhanced it, and VD3 exhibited basically no effect. The mechanism governing how these vitamins from the same category exert different effects on HCV RNA remains to be elucidated. However, liposoluble vitamins have been reported to exhibit various physiological activities with each nuclear receptor, consequently acting as hormone-like substances (19, 20, 27, 35). Differences in the gene products induced by each of these vitamins may lead to differences in the effects on HCV RNA replication. Another explanation might be considered in the light of findings suggesting that VA is an antioxidant, and yet recently, BC has been reported to induce oxidative stress (32, 43). This diversity of activities among vitamins in the same category, VA, might result in a variety of influences on HCV RNA replication. Further studies are still needed to account for why these different consequences are generated.

Previous studies have demonstrated that PUFAs such as AA, EPA, and DHA inhibit HCV RNA replication in cell culture systems (17, 21). However, saturated and mono-unsaturated fatty acids have been shown to enhance HCV RNA replication (17). In the prior studies, the cells tolerated the presence of PUFAs at concentrations of up to 50 μM. In contrast, in our study, 50 μM PUFAs were toxic, with the exception of LA. Furthermore, saturated and mono-unsaturated fatty acids hardly exhibited any effects on HCV RNA replication in our OR6 cell culture system. These discrepancies might be due to differences in both the clonalities of the cells and the HCV strains used in each experiment.

FIG. 8. VE canceled the anti-HCV activities of BC, VD2, LA, and CsA. (A and B) Effects of VE on the nutrients and compounds at the 70% inhibitory concentration. Both OR6 cells and OR6c cells, into which authentic HCV RNA was introduced, were treated with control medium [(−)], 10 μM BC, 5 μM VD2, 30 μM LA, 0.6 μg/ml of CsA, 0.6 IU/ml of IFN-α, 0.4 IU/ml of IFN-β, or 1.2 μM FLV in either the absence or presence of 10 μM VE for 72 h. After treatment, an RL assay of harvested OR6 cell samples was performed, and then the relative RL activity was calculated as described in the legend to Fig. 2. Subsequently, the production of HCV core antigen in OR6c cells was analyzed by immunoblotting using antibody specific to HCV core antigen. β-Actin was used as a control for the amount of protein loaded per lane (A). Then, the ratio of RL activity in the presence of 10 μM VE (+) to the RL activity in the absence of VE (−) was calculated. The horizontal line indicates the promotive effect of 10 μM VE alone on HCV RNA replication as a baseline (B). (C and D) Effects of VE on the nutrients and compounds at the 90% inhibitory concentration. Both OR6 cells and OR6c cells were treated with control medium, 50 μM LA, 1 μg/ml of CsA, 1 IU/ml of IFN-α, 0.6 IU/ml of IFN-β, and 2 μM FLV in either the absence (−) or presence (+) of 10 μM VE for 72 h. After treatment, the RL assay and Western blot analysis were performed (C), and then the ratio of RL activity in the presence of 10 μM VE to the RL activity in the absence of VE was calculated in the same manner as that described above (D).

Here, we demonstrated that three nutrients, BC, VD2, and LA, exhibited anti-HCV effects in polyclonal genome-length and subgenomic HCV RNA (strain O of genotype 1b)-replicating cells. These results indicated that the inhibitory activities of at least three anti-HCV nutrients are not limited to a specific cell clone (OR6).

Moreover, IFN or FLV exhibited additive anti-HCV effects when the cells were cotreated with each of the three anti-HCV nutrients. However, CsA showed synergistic anti-HCV effects in combination with each of these three nutrients. Interestingly, these results coincided with the experiment using VE, as VE canceled the anti-HCV effects of CsA but not those of IFN or FLV. It was recently demonstrated that the anti-HCV effects of CsA are related to the inhibition of cyclophilin (31, 42). CsA is also known as an oxidant that can cause renal or vascular dysfunction, and interestingly, antioxidants, including VE, attenuate these CsA-induced side effects (16, 22). Furthermore, we confirmed that another antioxidant, Se, also weakened the anti-HCV effects of BC, VD2, and LA (data not shown). Therefore, BC, VD2, and LA may possess an anti-HCV mechanism similar to that of CsA, and oxidative stress may be involved in these anti-HCV effects to some extent. Among the nutrients tested, VA, VC, VE, and Se enhanced HCV RNA replication, and these nutrients functioned as antioxidants. In contrast, four PUFAs inhibited HCV RNA replication, and they served as oxidants (29, 44). These results are further evidence of the involvement of oxidative stress in HCV RNA replication.

CH C patients may take excessive doses of VE during the course of interferon therapy, because as an antioxidant, VE has been expected to prevent injury to hepatocytes caused by oxidative stress. However, our results suggest that the potentially negative effects of VE on therapy for CH C patients should be carefully considered. To date, the significance of the role played by ordinary nutrients in viral infections has not been well characterized and has even been underestimated. We believe that our findings will shed light on the field of viral infection from the perspective of the nutritional sciences.

It is difficult to determine the blood concentrations of the nutrients tested in this study because the administration conditions may affect the concentrations in the blood. Rühl et al. (35) reported that the concentrations of BC in human serum are between 0.34 to 0.54 μM and that the average concentration in the human liver is 4.4 μM. Hagenlocher et al. (12) reported that the concentration of LA in human serum is 0.8 to 11.9 μg/100 μl. Armas et al. (3) reported that the concentration of VD2 in human serum at 24 h after a 50,000-IU administration is about 50 nM. The concentration of the nutrient in this study is higher than that in those reports. Therefore, monotreatment of the nutrient may not eliminate HCV. However, these nutrients may boost the effect of IFN treatment in combination like ribavirin does.

It is worth trying to examine the effects of BC, VD2, and LA on the recently developed JFH1 infectious virus production system in a future study. Here, it remains unclear whether these three nutrients affect the production of the virus. Furthermore, the comparison of the effects of these three nutrients between HCV genotypes 1 and 2 will provide useful information for the HCV therapy, as HCV genotypes 1 and 2 respond differentially to IFN treatment.

The precise mechanism underlying the anti-HCV activities of the nutrients has not been clarified in this study. The nutrients may inhibit viral RNAs and proteins, including the internal ribosome entry site, NS3-4A serine protease, and NS5B polymerase. Further in vitro study will be needed to clarify the targets of the nutrients responsible for their anti-HCV activities. Another possibility is that the nutrients inhibit the cellular proteins required for HCV RNA replication. We are now planning a study to clarify the mechanism underlying the nutrients' anti-HCV activities.

In conclusion, we found that three nutrients, BC, VD2, and LA, inhibited HCV RNA replication in a cell culture system and that Se, tryptophan, and various vitamins (A, C, E, and K) enhanced HCV RNA replication. The anti-HCV effects of BC, VD2, and LA were reversed by VE. These results are expected to provide useful information for the improvement of the SVR rates of patients receiving the currently standard IFN therapy. In addition, these findings may contribute to the development of a nutritional supplement specific to the treatment of people with CH C.

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Serum-free cell culture system supplemented with lipid-rich albumin for hepatitis C virus (strain O of genotype 1b) replication

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Abstract

HuH-7 is a highly differentiated hepatoma cell line and the only cell line that supports robust RNA replication of the hepatitis C virus (HCV). HuH-7 cells cause cell death in serum-free culture condition. However, the effect is reversed by supplementation with selenium. Serum-free cell cultures are advantageous for vaccine development and experimental reproducibility. However, HCV RNA replication in HuH-7 cells in serum-free medium had not yet been achieved. Therefore, we tried to develop a system for robust HCV RNA replication in a serum-free cell culture. Although HuH-7 cells grew in serum-free medium in the presence of selenium, HuH-7 cells under these conditions did not support HCV RNA replication in long-term culture. Among the supplements tested, serum-free medium with lipid-rich albumin (LRA) was found to yield robust HCV RNA replication. HCV proteins were detected for more than 9 months in serum-free medium supplemented with LRA. This is the first report to demonstrate a long-term, serum-free cell culture that successfully maintained robust HCV RNA replication. This cell culture system is expected to be a useful tool for vaccine development, as well as for further investigation of cellular factors that are essential for HCV RNA replication.

Keywords: HCV; Serum-free cell culture; Selenium; Lipid-rich albumin; Vaccine

1. Introduction

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (Feld and Hoofnagle, 2005). HCV, a member of the *Flaviviridae* family, is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1996). This polyprotein is processed by a combination of host and viral proteases into at least 10 proteins: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993).

The discovery of the HCV subgenomic replicon in 1999 was a turning point for HCV RNA replication in cultured cells (Lohmann et al., 1999). Furthermore, genome-length HCV RNA replication systems were developed using N, Con1, and H strains (Blight et al., 2002; Ikeda et al., 2002; Pietschmann et al., 2002). We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6) as an effective screening system (Ikeda et al., 2005). The development of infectious virus-producing cells has been a remarkable breakthrough in the fields of virology (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

HuH-7 is a highly differentiated hepatoma cell line that is commonly used for replication and infection studies. However, these cells have been reported by a number of groups to be heterogeneous, and the replication efficiency of this cell line differed among subclonal HuH-7 cells. Parental HuH-7 cells showed low capacity for HCV RNA replication and low susceptibility for HCV infection, but Huh7.5, Huh-Lunet, and our recently developed the RSc cells efficiently support HCV RNA replication and infection (Blight et al., 2002; Pietschmann et al., 2006; Ikeda et al., in preparation). In addition to these sub-

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clonal HuH-7 cell features, fetal bovine serum (FBS) may be another factor that affects HCV RNA replication and infection, as FBS is a pooled material containing unknown factors from different origins. Therefore, different FBS lots may affect the reproducibility of experiments conducted by different research groups. Furthermore, pathogens contained in FBS may introduce additional problems during the development of an HCV vaccine.

To resolve these issues, in this study, we investigated whether or not HCV RNA could replicate in serum-free cell culture. As HuH-7 cells produce an autocrine growth factor, hepatoma-derived growth factor, additional supplementation with growth factor seemed to be unnecessary (Nakamura et al., 1989, 1994). HuH-7 cells cause cell death in serum-free culture condition. However, when HuH-7 cells were cultured in serum-free medium supplemented with selenium, they produce a number of plasma proteins and liver-specific enzymes essential for their survival (Nakabayashi et al., 1982, 1984). Therefore, the serum-free culture of HuH-7 cells can be maintained by the addition of selenium alone. However, HCV RNA replication was not yet maintainable under these conditions.

In the present study, we found that HCV RNA replicates robustly for more than 9 months in serum-free medium supplemented with selenium and lipid-rich albumin (LRA). These results indicate the requirement of the lipid for HCV RNA replication. This cell culture system is expected to be a useful tool for the development of an HCV vaccine, and will also enhance the reproducibility of experiments, including those that evaluate anti-HCV reagents.

2. Materials and methods

2.1. Reagents

Sodium selenite (Na_2SeO_3), insulin, linoleic acid, oleic acid, IFN-α, and cyclosporine A (CsA) were purchased from Sigma–Aldrich (St. Louis, MO). Fluvastatin (FLV) and low-density lipoprotein (LDL) were purchased from Calbiochem (San Diego, CA). Lipid-rich albumin (ALBUMAX I™) was purchased from Invitrogen and is referred to as LRA in this study.

2.2. Cell cultures

The OR6 cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin–streptomycin (referred to as 10% FBS medium in this study), and G418 (300 μg per ml; Geneticin, Invitrogen) in a 5% CO_2 atmosphere at 37 °C. The cells were supplied with fresh medium twice a week at a 5:1 split ratio. The serum-free medium was DMEM containing 100 nM sodium selenite (Na_2SeO_3, Sigma–Aldrich) with LRA. The cells were cultured on six-well plates in 10% FBS medium or the serum-free medium. The cells cultured in the serum-free medium were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis.

2.3. Cell count

To examine cell growth in selenium-containing medium with 10% FBS, 2 mg per ml of LRA, or no supplementation, OR6 cells were seeded at a density of 1×10^5 cells per well onto six-well plates in the absence of G418. Then, the number of the cells was counted in an improved Neubauer-type hemotocytometer after trypan blue dye (Invitrogen) treatment.

2.4. Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblot analysis with a polyvinylidene difluoride membrane were performed as described previously (Kato et al., 2003). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, UK) and β-actin (AC-15; Sigma–Aldrich). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

2.5. Luciferase reporter assay

A luciferase reporter assay was performed as described previously (Ikeda et al., 2006). Briefly, 2×10^4 cells were plated onto 24-well plates and cultured in 10% FBS or the serum-free medium, at least in triplicate for each assay, and the cells were cultured for 24 h. Then, the cells were treated with human IFN-α, CsA, or FLV at several concentrations for 72 h. The cells were then harvested and subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). The cells were washed twice with phosphate-buffered saline and were then extracted with 100 μl of *Renilla* lysis reagent. The relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

3. Results

3.1. Efficiency of HCV RNA replication in HuH-7 cells with supplements in serum-free medium

At the early stage of the establishment of the HuH-7 cells, the serum-free cell culture was examined; the HuH-7 cells were found to replicate continuously for more than 9 months in a chemically defined medium containing selenium (Nakabayashi et al., 1982). Furthermore, the HuH-7 cells were maintained for a period of more than 3 years in improved serum-free medium containing additional supplements, i.e., oleic acid, linoleic acid, and insulin (Nakabayashi et al., 1984). We first investigated whether these serum-free conditions would support HCV RNA replication using the OR6 reporter system. The OR6 cells supported the replication of genome-length HCV-O RNA, into which the luciferase gene had been introduced (Ikeda et al., 2005). Using this OR6 system, we were able to monitor the level of HCV RNA

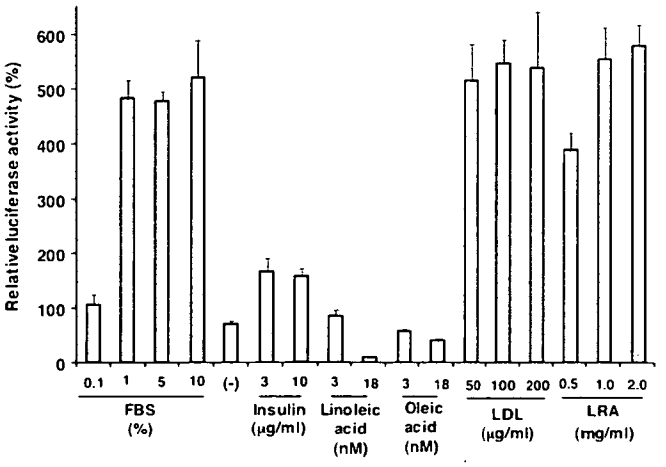

Fig. 1. HCV RNA replication in OR6 cells under different cell culture conditions. The OR6 cells were cultured in DMEM containing 100 nM sodium selenium with FBS (0.1, 1, 5, and 10%), insulin (3 and 10 μg per ml), linoleic acid (3 and 18 nM), oleic acid (3 and 18 nM), LDL (50, 100, and 200 μg per ml), or LRA (0.5, 1.0, and 2.0 mg per ml). The cells were harvested at 24 and 96 h and were subjected to luciferase assay as described in Section 2. Relative luciferase activities (%) were obtained from the value at 96 h, when the value at 24 h was assigned as 100%. The data indicate means ± standard deviations (S.D.s) from three independent experiments. (−) indicates culture in DMEM containing 100 nM sodium selenium.

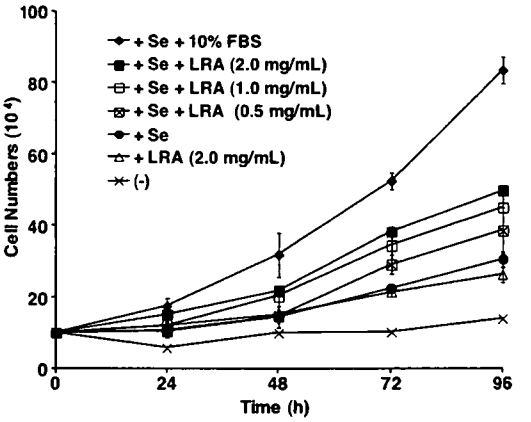

Fig. 2. Cell growth of HuH-7 cells in serum-free medium with LRA or in 10% FBS medium. The OR6 cells were plated at 1×10^5 cells per well onto six-well plates in triplicate. The cells were cultured in DMEM containing 100 nM sodium selenium with 10% FBS or 0.5, 1.0 and 2.0 mg per ml LRA or no supplement and were harvested at 24, 48, 72, and 96 h. The cells were cultured in DMEM or DMEM with LRA in the absence of selenium and were harvested at indicated time points.

replication by measuring the activity of luciferase. Luciferase activity at 96 h was five times higher than that at 24 h in 10% FBS medium (Fig. 1). However, HCV RNA replication was reduced when the OR6 cells were cultured in serum-free medium containing only selenium (Fig. 1). Serum-free medium supplemented with insulin reduced HCV RNA replication to about one-third of that observed in cultures maintained in 10% FBS medium supplemented with selenium. When used in combination with linoleic acid or oleic acid, the serum-free medium with selenium remarkably reduced HCV RNA replication (Fig. 1). However, in the serum-free medium with selenium in combination with LDL (50, 100, or 200 μg per ml) or LRA (1 or 2 mg per ml), HCV RNA replication was supported at the same level as that in 10% FBS medium, although the replication of HCV RNA was slightly low level in serum-free medium with selenium and LRA supplement at 0.5 mg per ml. These results suggest that chemically conditioned serum-free medium supplemented with selenium is not sufficient to support HCV RNA replication, but the addition of either LDL or LRA restored HCV RNA replication to almost the same level as that observed in 10% FBS medium. Thus, some of the elements essential for HCV RNA replication may be contained in LDL and LRA.

3.2. Cell growth of HuH-7 cells in selenium-containing medium supplemented with FBS or LRA

As HCV RNA replication depends on cell growth (Guo et al., 2001; Pietschmann et al., 2001), we next determined the number of cells at 24, 48, 72, and 96 h of culture. The doubling time of the OR6 cells was estimated to be approximately 29, 43, and 64 h in selenium-containing medium with 10% FBS, or 0.5, 1.0, and 2 mg per ml of LRA, or no supplementation, respectively (Fig. 2). Selenium or LRA containing medium enhanced the growth of OR6 cells and the combination of the selenium with LRA further enhanced the cell growth in a LRA dose-dependent manner (Fig. 2). Interestingly, the cell culture in serum-free medium supplemented with 2 mg per ml of LRA and selenium supported HCV RNA replication as efficiently as did that supplemented with 10% FBS and selenium (Fig. 1), although the cell growth of the culture in the medium with LRA and selenium was slower than that in the medium supplemented with 10% FBS and selenium. These results indicate that LRA may contain factors that enhance HCV RNA replication, and these LRA-derived factors appear to function in a manner that is not dependent on the cell growth factor.

3.3. Expression of HCV proteins in HuH-7 cells at 1 month of cell culture under various medium conditions

We continued to maintain the culture of OR6 cells for 1 month in different types of conditioned media. NS3 and Core HCV proteins were detected in the OR6 cell culture for 1 month in medium containing selenium with 10% or 5% FBS, but not with 1% or 0.5% FBS (Fig. 3A). The protein expression levels were higher in the cell culture with 10% FBS medium than in that with the 5% FBS medium. HCV proteins were not detected in the OR6 cells cultured in serum-free medium containing selenium alone (Fig. 3A). In contrast, HCV proteins were detected in LRA- and selenium-containing cell cultures. The levels of expression of HCV proteins were almost equal to those in the cell culture with selenium and 5% FBS (Fig. 3A). To further confirm the results, we performed luciferase assay for the OR6 cells cultured for 1 month after RNA transfection (Fig. 3B). These results indicated that HCV RNA replication was not maintained for more than 1 month in low concentrations (less than 1%) of FBS with selenium. However, the cell culture in serum-free medium with selenium and LRA at concentrations of 0.5, 1, or 2 mg per ml did efficiently support HCV RNA replication for more than

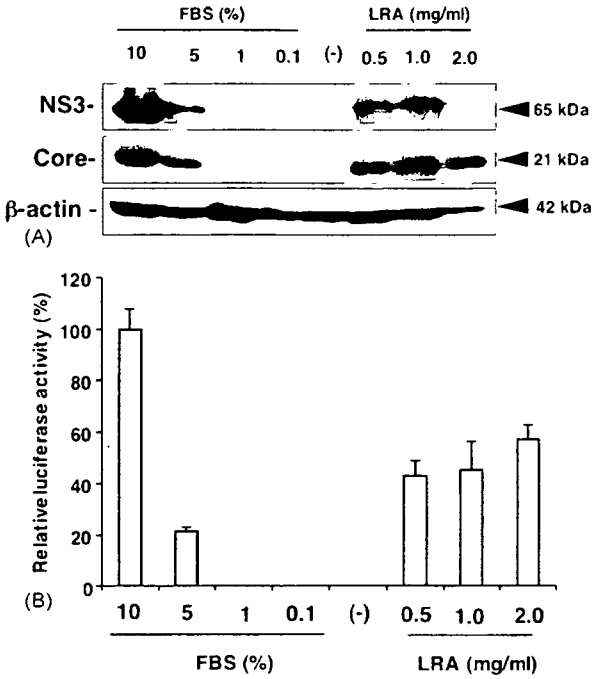

Fig. 3. Expression of HCV proteins in HuH-7 cells cultured in serum-free medium with LRA or in 10% FBS medium. OR6 cells were cultured for 30 days in DMEM containing 100 nM sodium selenium with LRA (0.5, 1.0, and 2.0 mg per ml) or FBS (0.1, 1, 5, and 10%). (−) indicates the culture in DMEM with sodium selenium. (A) The production of Core and NS3 in the OR6 cells was analyzed by immunoblotting using anti-Core and anti-NS3 antibodies. β-actin was used as a control for the amount of protein loaded per lane. (B) Relative luciferase activity was determined for the cells from 24-well plates in triplicate.

1 month. Therefore, we concluded that LRA could serve as an alternative supplement to FBS, when used in combination with selenium for HCV RNA replication.

3.4. Core expression in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

Since OR6 cells cultured in serum-free medium with selenium and LRA could support HCV RNA replication at least for 1 month, we continued to culture the OR6 cells under the same conditions for 1 year. Core was detected for 9 months and for 6 months in serum-free medium containing selenium with LRA at 1.0 and 2.0 mg per ml, respectively (Fig. 4). These results suggest that serum-free cell culture supplemented with LRA supports HCV RNA replication at almost equal level to that in the culture supplemented with 10% FBS without G418 selection at least more than 9 months.

3.5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

IFN is currently used as a therapeutic treatment of patients with chronic hepatitis C. Subgenomic HCV replicon-harboring cells and genome-length HCV RNA-replicating cells have been used to evaluate IFN and other candidate anti-HCV reagents, as

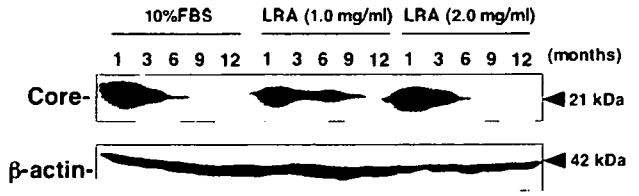

Fig. 4. Core expression in a long-term culture of OR6 cells in serum-free medium supplemented with LRA. The OR6 cells were cultured for 1 year in DMEM containing 100 nM sodium selenium with LRA at 1.0 or 2.0 mg per ml. The OR6 cells were also cultured in DMEM with 10% FBS and used as a positive control. Cells were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis for Core using anti-Core antibody. β-actin was used as a control for the amount of protein loaded per lane.

has recently been reported in the case of CsA and the statins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Watashi et al., 2003; Ye et al., 2003). However, one remaining problem with the evaluation of the anti-HCV activity of these reagents has been that FBS is derived from pooled blood materials and contains unknown cellular factors in various concentrations. These unknown factors can affect experimental results and reproducibility. Therefore, it is preferable to use a culture medium containing as few cellular factors as possible in order to ensure the reliability of the results. In this context, completely chemical conditioned medium is most ideal. Although our selenium and LRA culture system still contained animal proteins and lipids, it contained fewer unknown factors than medium containing FBS. We compared the anti-HCV activity of IFN-α, CsA, and FLV in serum-free medium with selenium and LRA, as well as in 10% FBS medium with selenium. OR6 cells were treated with these reagents for 72 h, and harvested cells were subjected to luciferase assay. When the OR6 cells were treated with IFN-α under either the 10% FBS medium condition or the selenium and LRA condition, HCV RNA replication was inhibited by IFN-α in a dose-dependent manner (range: 0.625–5 IU per ml; Fig. 5). However, the sensitivity of the cultures to IFN-α was greater in selenium and LRA medium than in 10% FBS medium (Fig. 5). When the OR6 cells were treated with CsA at low concentrations of 0.0625 and 0.125 μg per ml, sensitivity was greater in selenium and LRA medium than in 10% FBS medium, but almost identical sensitivity was observed under both medium conditions at concentrations of CsA of 0.25 and 0.5 μg per ml (Fig. 5). The most striking result was observed when the OR6 cells were treated with FLV in concentrations ranging from 1.25 to 10 μM. In contrast to the results obtained with CsA and IFN-α, the sensitivity associated with FLV was markedly reduced in the cultures treated with selenium and LRA medium, as compared to that of cultures in 10% FBS medium (Fig. 5).

4. Discussion

The development of serum-free cell culture systems will reduce the risk of contamination by infectious pathogens of animal origin in studies of vaccine development. Moreover, such systems will enhance the reproducibility of such experiments, because FBS contains unknown cellular factors that can affect the physiological state of cells. In this study, we developed a