Table 2 HCV NS3 protease quasispecies complexity, diversity and secondary structure features

	Duration of hepatitis C infection (years)*	d_N/d_S	Number of clones analysed	Number of different AA sequences	Number of different nt sequences	Genetic distance	Shannon entropy S_n (AA)	NS3 secondary structure (%)	
Cases									
K1	45	0.015	15	4	15	0.023	0.266	B1-1	100
K2	54	0.042	13	3	13	0.008	0.209	B1-2	92.3
								A2-2	7.7
K3	11	0.048	15	6	14	0.031	0.522	B1-1	66.7
								B1-2	26.7
								C-2	6.6
K4	43	0.057	15	8	15	0.048	0.631	B1-1	80
								B1-2	13.3
								C-2	6.7
K5	59	0.167	15	5	10	0.005	0.399	B1-1	93.3
								C-2	6.7
K6	25	0.067	15	7	13	0.011	0.513	B1-1	100
K7	42	0.094	13	6	13	0.013	0.591	B1-1	100
K8	48	0.093	15	9	13	0.032	0.701	B1-1	93.3
								C-2	6.7
K9	46	0.029	15	3	14	0.024	0.326	B1-1	100
K10	ND	0.053	15	4	12	0.007	0.317	B1-1	100
Median	45	0.041	15	5.5	13.5	0.096	0.46	B1-1	84.2†
Controls									
C11	38	0.070	14	9	13	0.046	0.745	B1-1	100
C12	ND	0.020	13	5	6	0.002	0.330	B1-1	92.3
								C-2	7.7
C13	22	0.333	15	2	13	0.013	0.214	B1-1	100
C14	23	0.026	15	2	13	0.006	0.09	B1-2	100
C15	ND	0.000	13	1	4	0.001	0	B1-2	100
C16	38	0.250	15	6	13	0.011	0.433	B1-2	86.6
								A1-2	6.7
								C-4	6.7
C17	17	0.097	14	5	12	0.014	0.377	B1-1	85.7
								A1-1	14.3
C18	27	0.143	15	7	14	0.04	0.620	B1-1	100
C19	ND	0.000	15	2	7	0.002	0.09	B1-2	100
C20	21	0.429	14	7	13	0.011	0.550	B1-1	85.7
								C-2	14.3
Median	23	0.05	14.5	5.1	13	0.139	0.35	B1-1	55.9†

ND, unknown; AA, amino acidic; nt, nucleotidic; Mean intrapatient genetic distance was estimated with the Kimura two-parameter method in MEGA3 software.
*Time between supposed date of contamination and intake of serum sample.
†Percentage of B1-1 clones in each group.

sequence that clustered with those of the other HCC cases. Bootstrap resampling values outside intrasample clusters were low, probably because of the weak phylogenetic signal of the NS3 protease region [31]. However, 20 clusters had a high level of reliability (most with bootstrap values exceeding 90%), corresponding to the quasispecies of the 20 patients. The topology of the tree is otherwise consistent with estimates of intrasample quasispecies diversity based on genetic distance.

Amino acid polymorphism

Amino acid consensus sequences were 100% identical in the two groups and were 100% and 95.58% similar to the

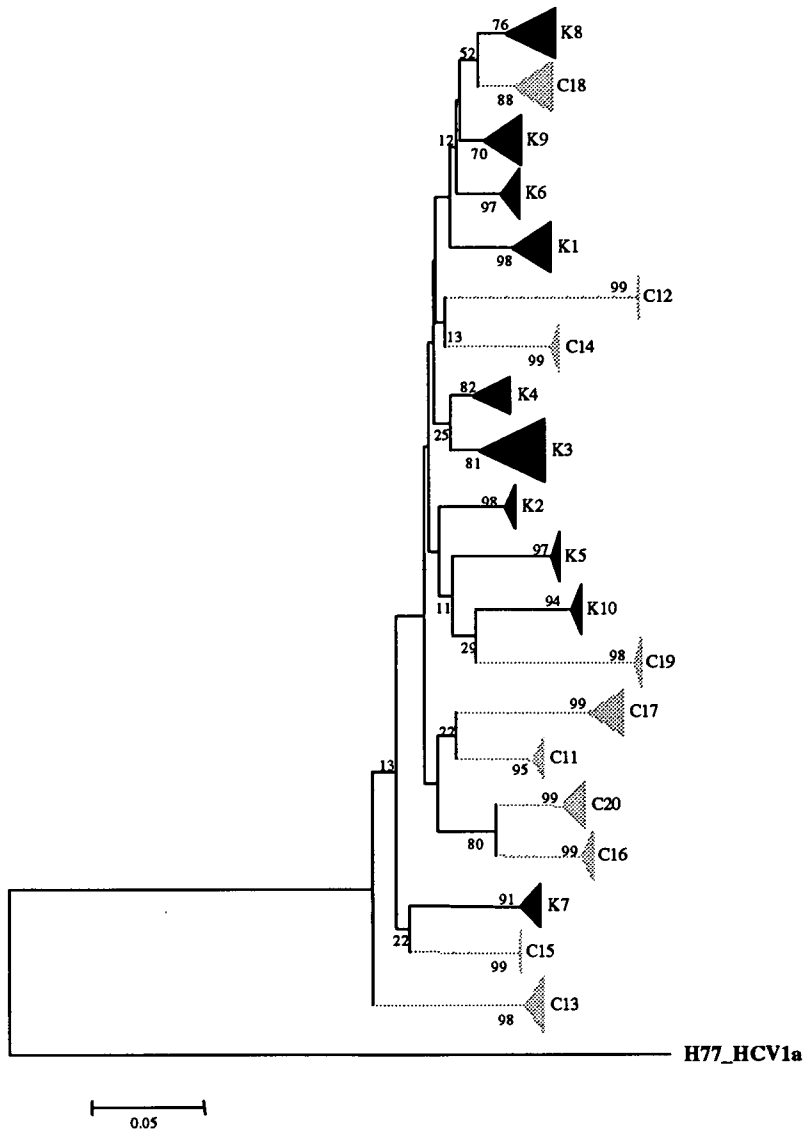

Fig. 1 Phylogenetic tree issued from nucleotide alignment of 289 NS3 protease clones analysed. Lineages of each 20 quasispecies are represented by a black (cases) or a grey (controls) solid elongated triangle whose thickness is proportional to the number of taxa condensed. HCV-1a H77 reference strain was included as outgroup. Tree was built with neighbour-joining method with a Kimura two-parameter and a gamma distribution ($\alpha = 0.29$). Bootstrap value higher than 10 are shown. Branch lengths are drawn to scale. The scale bar represents 5% of nucleotide sequence divergence.

sequences of the prototype HCV-BK [32] and HCV-J [33] strains, respectively (Fig. 2). Eight differences from the sequence of HCV-J were identified: substitutions E30D, V36L, Q86P, L94M, I114V, V132I, A150V and V170I.

Of the 181 amino acid residues in the HCV NS3 protease, 131 (72%) and 139 (77%) residues were conserved in cases and controls, respectively. Thirty and 17 sites, respectively, displayed substitution with respect to the consensus sequence in only one clone per group. NS3 protease substitutions occurring in at least two variants were detected at 20 (11%) and 25 (14%) sites in the case and control groups, respectively. Most of the mutations detected corresponded to conservative hydrophobic to hydrophobic substitutions (Fig. 2). The residues implicated in HCV resistance to NS3 protease inhibitors *in vitro* – D168, A156 and R155 [34,35] – were consistently conserved in the 289 variants analysed.

In none of the clones did polymorphism at a single NS3 position exceed 46.2% of control variants and 23.3% of case variants (Fig. 3). Neither in cases nor controls did we observe substitutions in the catalytic triad residues H57, D81 and S139 (Fig. 2), with the exception of one case, with a variant displaying a mutation in histidine 57 (H57R). Histidine 149, which holds the zinc ion on the top of the zinc finger, was substituted in one variant from several cases (H149Y). None of the major residues implicated in NS3 protease substrate binding (F154, L135, R161, K165) [36] were substituted in either of the groups, with the exception of alanine 157 from the S1 binding pocket (A157T/V), which was mutated in three and two proteases from the case and control groups, respectively.

Some identical mutations were found in the NS3 protease sequences of isolates from cases and controls, when these

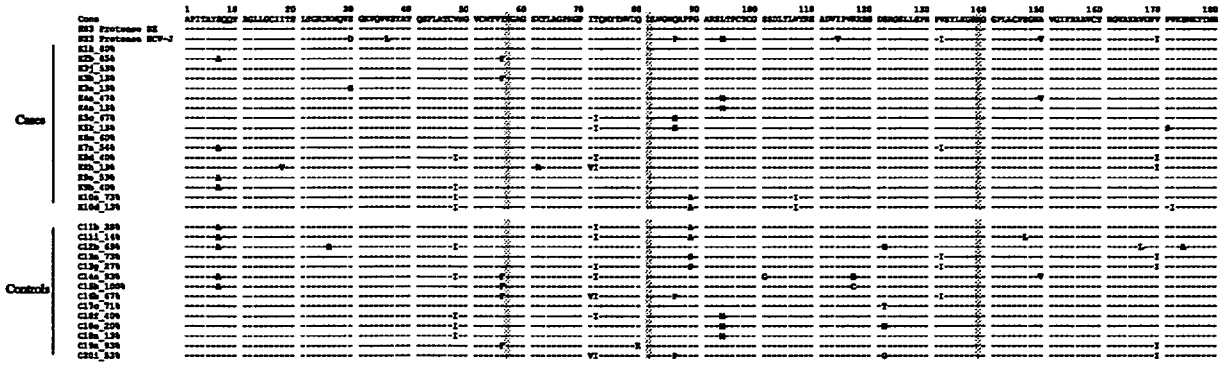

Fig. 2 NS3 protease sequence alignment for the predominant clones of each of the 20 quasispecies analysed. The consensus sequence 'cons' – obtained by aligning the sequences of every sequenced clone – the sequence of the BK strain [32] – used as the reference sequence for numbering residues – and the sequence of the HCV-J reference strain are shown for comparison. Only sequences corresponding to the predominant isolate sequences for a given quasispecies (frequency >13%) from cases and controls are aligned. The name and frequency of the predominant clone of each quasispecies is shown to the left of the sequences. The residues of the catalytic triad are shown in grey.

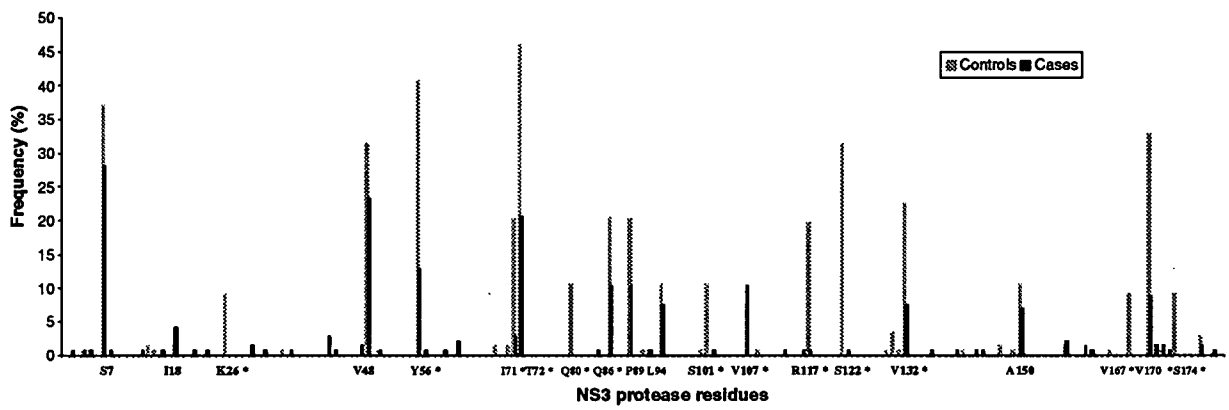

Fig. 3 Frequency of polymorphism mutations for each 181 NS3 protease residue as a function of subsequent progression from cirrhosis to HCC (cases, black bars) or absence of progression (controls, grey bars). The percentage polymorphism corresponds to differences in the amino acid sequence from the NS3 BK strain (accession number gi5542135). The proportion of substituted sequences for each 181 NS3 protease residue is shown along the *y*-axis. Asterisks indicate NS3 positions at which substitutions were significantly more prevalent in HCV isolates from one of the two groups.

sequences were compared with that of HCV-BK strain (Fig. 2). However, there were also several amino acid substitutions in control isolates that were not found in isolates from cases. Moreover, mutations affecting the consensus sequence were found exclusively in the control group at sites 26 (K26R), 80 (Q80K), 86 (Q86P), 89 (P89S), 101 (S101G), 117 (R117C), 122 (S122G/T/N), 167 (V167L) and 174 (V174A) (Table 3). Mutations at sites 26, 80, 101, 167 and 174 were restricted to all the quasispecies variants analysed from a single control patient. Polymorphism at site 122 was more widespread (31.5% of the control clones analysed), with three different amino acid substitutions (S122G/T/N). A Blast search of the GenBank™ database was conducted using the amino acid sequences of the HCV-1b NS3 protease domain from the consensus sequence for all 289 clones. A total of 250 HCV-1b isolates were identified, without taking into account hepatitis stage or clonal *vs* direct sequencing, and S122G/T/N polymorphism was also observed (G/T/N for 11.6%, 10% and 4.4% of sequences, respectively; data not shown). We compared HCV NS3 protease substitutions occurring in clones from at least two different samples between the two groups (Fig. 3). Differences, in terms of the percentage of clones with substitutions, were statistically significant for 10 NS3 sites. Thus, mutations Y56F, I71V, T72I, Q86P, P89S, S101G, R117H, S122G and V170I ($P < 0.001$), S122T ($P = 0.0013$) and V132I ($P = 0.003$) were more frequently observed in control than in case NS3 protease sequences.

Table 3 HCV NS3 protease substitutions occurring exclusively in cases or in controls group variants

HCV NS3 residues	Mutations	Patients		Mutated clones (%)*
		Cases	Controls	
26	K26R	–	C12	100
80	Q80K	–	C19	100
86	Q86H	K5	–	100
86	Q86P	–	C16	100
89	P89S	–	C13	100
101	S101G	–	C14	100
107	V107I	K10	–	100
117	R117H	–	C14	100
	R117C	–	C15	100
122	S122G	–	C12, C20	100, 100
	S122T	–	C17	100
	S122N	–	C18	27
167	V167L	–	C12	100
174	V174A	–	C12	100

*Mutation occurrence in each patient analysed quasispecies.

Only one residue, V107, was substituted in case NS3 sequences but not in control sequences (V107I, 100% of the 15 clones analysed from patient K10's serum sample) (Table 3). This site was substituted in only one of the 250 NS3 sequences from GenBank; this NS3 mutation was also reported in an HCC sample of a total of 146 (77 HCC and 69 non-HCC) [13]. Q86H substitution was observed in all clones from patient K5 sample and was not present in NS3 control clones.

Classification of NS3 protease clones based on the analysis of the secondary structure of an amino-terminal portion of the protease

The secondary structure of the amino-terminal 120 residues of NS3 was predicted for each of the 289 clones sequenced and for the sequence generated by direct sequencing of each of the 20 quasispecies. NS3 protease variants were assigned to three different groups – group A (4 clones), group B (277 clones) and an indeterminate group (8 clones) – according to their secondary structure (Table 2). Each group was further divided into a number of subgroups. The classification used corresponds to that previously defined by Ogata *et al.* [13,14].

We found that in 9 of the 10 cases and 6 of the 10 controls, 66.7–100% of the NS3 protease quasispecies variants had secondary structures of the B1-1 subgroup (Table 2). The difference between cases and controls was not significant ($P > 0.05$). The proportion of NS3 protease variants assigned to subgroup B1-1, which has been shown to be associated with HCC, differed significantly between cases and controls ($P < 0.001$). The number of quasispecies belonging to this secondary structure group was higher in cases (123 variants, 84.2%) than in controls (80 variants, 55.9%). No protease sequence was classified as belonging to subgroup B2-1, which has been shown to be associated with HCC [13]. Eight control and eight case amino acid sequences obtained by direct sequencing were 100% identical to the predominant sequence of the corresponding quasispecies and consequently displayed the same secondary structure (data not shown). Of the four discordant direct sequences, only that for the sample from patient C14 had a B1-1 secondary structure, despite the population analysed being relatively homogeneous and belonging to the B1-2 group.

DISCUSSION

The HCV NS3 protease is essential for viral replication and is also thought to be involved in hepatocarcinogenesis. As this enzyme is a key target for specific anti-HCV therapy, it is important to define its diversity at different stages of liver disease. We previously described its natural polymorphism, resulting in the existence of quasispecies in untreated patients with chronic hepatitis C [37]. We investigated the relationship between the progression of hepatic disease and features of the nucleotide and amino acid sequences corresponding to the HCV-1b NS3 protease region by analysing these sequences for viral quasispecies derived from patients with cirrhosis. One of the strong points of this study is the long interval (several years) between sample analysis and outcome determination (occurrence or absence of HCC). As no particular sequence motif was identified in the case group at the time of cirrhosis diagnosis, the NS3 protease population of cases presumably differentiated later, between the diagnosis of cirrhosis and HCC. It would therefore be of interest to analyse NS3 protease sequences at the HCC stage. Our comparison of these two viral populations is probably accurate, as the patients recruited to the two groups differed exclusively in terms of subsequent HCC occurrence. The patients recruited were matched for most criteria, without reported risk factors for HCC – women, with similar presumed modes of contamination (transfusion), devoid of toxic factors such as alcohol or drug abuse [38]. None of the 20 patients studied had HCC at the time of cirrhosis diagnosis or cirrhosis with extremely rapid progression to HCC. We chose to consider patients with HCV genotype 1 infections because this genotype has a high prevalence in European HCV patients, particularly those infected for long periods of time and because this genotype does not display a complete response to pegylated interferon–ribavirin bitherapy [39]. HCV genotype 1 infections are therefore more likely to progress to HCC than are infections with other HCV genotypes. Given the small numbers of patients in the two groups, we cannot exclude the possibility of a selection bias linked to the absence of a main NS3 sequence or NS3 secondary structure profile predictive of progression from cirrhosis to HCC in our study population.

NS3 protease polymorphism depended on the structural and functional constraints on the enzyme, with tolerance observed for substitutions occurring outside sites essential for catalytic function and three-dimensional structure. The polymorphic residues and types of substitution observed in the 289 NS3 variants from cirrhotic patients analysed are consistent with those previously identified in chronic hepatitis patients [37]. Substitutions that do not conserve the physicochemical properties of the original residue are exposed to solvent at the surface of the protease and are probably more sensitive to external pressures. NS3 protease gene complexity and quasispecies diversity varied considerably from patient to patient and was not related to the subsequent outcome of HCV cirrhosis. Quasispecies consisted of highly homogeneous populations or a 'cloud' of more divergent variants. Polymorphic sites 26, 80, 101, 167 and 174, which were restricted to variants from the control group, could not be considered as markers of subsequent nonprogression, as substitutions at these sites were observed exclusively in quasispecies from a single control patient. Moreover, mutations at positions 26, 80, 101 and 122 have previously been found in NS3 proteases from both HCC and non-HCC patients [13,14]. Two NS3 protease variants from the control group had a premature stop codon, at sites 85 and 86. These two variants probably correspond to defective viruses, as they were also detected in previous studies [40].

Although no amino acid signature patterns specific to cirrhotic patients displaying subsequent evolution to HCC could be identified, NS3 protease polymorphism was more frequent in quasispecies from patients not displaying subsequent progression to HCC. As patients were matched for most criteria, to minimize bias, we could not exclude the possibility of different levels of diversity related to the duration of infection, which was longer for cases (45 *vs* 23 years). This lower variability may be of clinical significance for disease progression, during which the major variant is selected.

It remains unclear whether there is an association between the evolution of genetic heterogeneity and the severity of HCV liver infection. Several studies have suggested that there is a correlation between the degree of HVR1 sequence diversity and the development of severe liver disease [41–43], but these findings have not been confirmed by other studies [44]. Diversity data have also been obtained for regions implicated in hepatocarcinogenesis other than NS3, with greater variability reported for patients with HCC than in patients without HCC for core protein [45] and the NS5A-PKR-binding domain [46,47]. Conversely, we show here that patients displaying progression to HCC had lower levels of NS3 protease polymorphism than patients who did not develop cancer. Comparative analyses of the core and NS3 helicase regions showed no specific amino acid differences between isolates from cirrhotic patients with and without HCC, suggesting that specific amino acid changes in these regions have no direct oncogenic role in patients with HCV-related cirrhosis [46]. Mutations in the HCV genome have been explored over a 13-year period in a single patient [48]; mutations clustering in the HVR-1 gene were found to have accumulated over time. Conversely, the carboxy-terminal NS3 and NS5B regions were found to be the most highly conserved. To date, no study has identified amino acid signatures associated with disease severity or disease outcome, in any region of the HCV genome.

An HCV NS3 protease secondary structure conformation, B1-1, has been identified as associated with progression to HCC [13,14]. We determined the secondary structures of our NS3 protease clones by the same method, to investigate whether this conformation was also observed in variants from cirrhotic patients who subsequently developed HCC. Surprisingly, the NS3 protease sequences from most of the cases and controls adopted the B1-1 configuration. This type of secondary structure may actually be more closely linked to fibrosis than to HCC. It would therefore be of interest to carry out analyses for a third paired group of patients without cirrhosis, over a similar period of follow-up for hepatitis C infection. Our study differed from the previous study in several respects: population studied (cirrhotic patients *vs* HCC and no HCC), sequence analysis (quasispecies *vs* direct sequencing). For a more accurate comparison, we also directly sequenced NS3 protease genes from each of the case and control samples. All but one of the secondary structures of the 20 quasispecies was consistent with that of the major variants. Among case and control NS3 protease variants, non-B1-1 secondary structures (B1-2) were found in the least complex quasispecies, mostly in cirrhotic patients without HCC (K2, C14, C15, C16 and C19) (Table 2). One substitution, Y56F was found exclusively in NS3 protease clones with the B1-2 secondary structure. This mutation, observed in 15% of the 250 GenBank NS3 protease sequences, and low quasispecies complexity may be two markers of non-HCC outcome. As reported by Ogata *et al.*, the presence of a phenylalanine in position 56 is associated with the absence of the turn structure at position 57 present in the B1-1 secondary structure. Interaction between the p53 tumour suppressor protein and the NS3 region above this turn structure (amino acids 29–174) would almost certainly be compromised by the structural modification [11].

In conclusion, we assessed and compared the variability of HCV NS3 protease in two groups of viruses in samples taken from cirrhotic patients at the time of cirrhosis diagnosis. Polymorphism differences have been shown, in favour of NS3 proteases from patients who did not subsequently develop HCC compared with those from patients displaying subsequent progression from cirrhosis to HCC. However, these differences did not allow the definition of a specific NS3 profile related to HCC occurrence. Our results suggest that there may be a relationship to fibrosis progression when diversity parameters are considered together with secondary structure profiles. If these findings could be confirmed, they would demonstrate the

potential utility of quasispecies analysis in attempts to interpret disease outcome. The mechanism of hepatocarcinogenesis in patients with hepatitis C infection remains to be determined. In addition to further NS3 protease sequence analysis, the cellular interactions of this enzyme require investigation. We are currently carrying out such studies, using a subgenomic replicon model.

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BRIEF REPORT

Risk of Hepatocellular Carcinoma and Secondary Structure of Hepatitis C Virus (HCV) NS3 Protein Amino-Terminus, in Patients Infected with HCV Subtype 1b

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We conducted a retrospective study of 65 patients with chronic hepatitis C, to determine whether the secondary structure of the amino-terminal 120 residues of the hepatitis C virus (HCV) NS3 protein is associated with an increased risk of development of hepatocellular carcinoma (HCC). The cumulative incidence of HCC was highest among patients infected with group B HCV-1b, wherein the risk of HCC significantly increased compared with that among patients infected with group A (hazard ratio, 4.95 [95% CI, 1.43–17.11]) after adjustment for age and histological stage. This HCV-1b grouping may be a useful marker for detecting the risk of development of HCC.

Hepatocellular carcinoma (HCC) is a common malignancy, and the mortality associated with it is increasing in Japan [1]. The 3 major categories of risk factors that appear to influence the incidence of HCC are host, viral, and environmental factors. Epidemiologic and clinical studies have shown that chronic hepatitis C virus (HCV) infection is a major cause of HCC [2]. At present, HCV is classified into at least 6 genotypes and >60 subtypes [3, 4]. HCV subtype 1b (HCV-1b) is most common in Asian countries, including Japan [5]. Patients infected with HCV-1b have a greater risk of progression to HCC than do those infected with other subtypes [6]. However, it remains unclear whether all HCV strains are associated with HCC to an equal degree.

We have been interested in the possible involvement of the HCV NS3 region in hepatocarcinogenesis. It has been reported that an amino-terminal portion of NS3 (aa 1027–1295 and aa 1008–1246) has the potential to transform NIH 3T3 and rat fibroblast cells [7, 8]. Moreover, studies have reported (1) that an amino-terminal portion of NS3 (aa 1027–1459) renders NIH 3T3 cells more resistant to DNA damage–induced apoptosis [9, 10], which is thought to be a prerequisite for the malignant transformation of cells, and (2) that NS3 interacts differentially, in a sequence-dependent manner, with the p53 tumor suppressor [11].

Recently, we reported that HCV-1b strains can be classified into different groups based on the secondary structure of an amino-terminal portion of the NS3 protein and that group B strains are more prevalent among patients with HCC [12]. These results suggest the possibility that HCV-1b strains of group B cause HCC more frequently than do group A strains. Using a retrospective cohort study design, we precisely assessed the possible association between the HCV-1b NS3-protein group and the risk of development of HCC.

Patients and methods. This retrospective cohort study enrolled outpatients infected with HCV-1b, who were referred from general medical or hepatology clinics to the Yamagata University Hospital, to further investigate their liver status. They all had been diagnosed histologically as having chronic hepatitis or cirrhosis. Patients with alcohol-related liver injury or autoimmune hepatitis or who were positive for hepatitis B surface antigen were excluded. Furthermore, patients who were followed up for <12 months were excluded, to rule out the possibility that the cancer was present at the start of the study. Twenty patients were excluded because samples available for group analysis were inadequate. We consequently examined 65 patients. Observation began in October 1981 and ended in December 2005. The baseline condition was considered to be the initial histologic diagnosis, and the end points were considered to be either (1) development of HCC or (2) last ultrasonogram (US) or computed tomogram (CT) without a diagnosis of HCC. At baseline, none of the patients had a diagnosis of HCC, on the basis of screening tests using US, CT,

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Table 1. Baseline characteristics of patients infected with group A, B, and C strains of HCV.

Characteristic	Group A (n = 18)	Group B (n = 44)	Group C (n = 3)	P
Age				
≤55 years	11 (61)	18 (41)	1 (33)	.37[a]
>55 years	7 (39)	26 (59)	2 (67)	
Sex				
Male	13 (72)	21 (48)	2 (67)	.20[a]
Female	5 (28)	23 (52)	1 (33)	
Stage of liver fibrosis				
F1 or F2	10 (56)	22 (50)	3 (100)	.30[a]
F3 or F4	8 (44)	22 (50)	0 (0)	
Grade of inflammatory activity				
A1 or A2	13 (72)	28 (64)	3 (100)	.52[a]
A3	5 (28)	16 (36)	0 (0)	
Alanine aminotransferase level				
≤100 IU/L	9 (50)	27 (61)	0 (0)	.11[a]
>100 IU/L	9 (50)	17 (39)	3 (100)	
Time since diagnosis of chronic liver injury, years				
Median	1.3	0.7	1.5	.51[b]
Range	0.2–9.3	0.3–8.5	0.5–1.5	
Time since blood transfusion,[c] years				
Median	26.8	26.9	...	.67[d]
Range	5.7–35.6	5.5–36.0		

NOTE. Data are no. (%) of subjects, unless otherwise indicated.

[a] By Fisher's exact test.

[b] By Kruskal-Wallis test.

[c] Based on data from those patients who had a history of blood transfusion, including 10 infected with group A strains and 17 infected with group B strains.

[d] By Mann-Whitney test.

or serum α-fetoprotein (AFP) level. None of the patients without HCC died during the follow-up period. All of the patients underwent screening examination for HCC, by either US or CT, at least once every 6 months, as well as blood testing, including testing for AFP, every 1 or 2 months. None of the personnel involved in the decision to screen and in selection of participants in the study were aware of the group status. There was no evident difference in screening frequency among the groups. The cirrhotic patients at baseline were all in a state of Child class A, which means that the clinically evaluated severity of liver disease was mild. The HCV genotype was determined by reverse-transcription polymerase chain reaction (RT-PCR) using genotype-specific primers [13].

On the basis of the secondary structure of NS3 protein, the HCV-1b isolates from all patients were classified further—into group A, group B, and an indeterminate group, group C, as reported elsewhere [12]. After October 2001, stored frozen samples collected during the follow-up period were used to determine the group. To identify whether the group shifted to another group during the infection period, the group of 8 patients was examined by use of paired serum samples from each of them.

Histological findings were scored to determine the stage of liver fibrosis and the grade of inflammatory activity, according to the classification system of Desmet et al. [14], by institutional pathologists who were blinded to the subjects' respective subgroup classifications. The stage of fibrosis was assessed as ranging from stage F0 (no fibrosis) to F4 (cirrhosis), and the grade of inflammatory activity was scored from grade A0 (minimal) to grade A3 (severe).

Written informed consent was obtained from all the participants. The study was approved by the Ethics Committee of Yamagata University.

The characteristics of the groups of patients were compared by use of Fisher's exact test, Mann-Whitney test, or Kruskal-Wallis test. Cumulative incidence curves were estimated by use of the Kaplan-Meier method; differences between the groups were assessed by use of the log-rank test. The risk of development of HCC was evaluated by use of the hazard ratio (HR) and its 95% confidence interval (CI), which were estimated by

use of the Cox proportional hazard model. The following 5 variables at baseline and the average HCV RNA level during the follow-up period were analyzed to assess potential confounding for liver carcinogenesis: age (≤55 years or >55 years), sex (male or female), stage of liver fibrosis (F1/F2 or F3/F4), grade of inflammatory activity (A1/A2 or A3), alanine aminotransferase level (≤100 IU/L or >100 IU/L), and HCV RNA level (low or high). The serum HCV RNA level was designated as a high viral load when it was either $>10^6$ equivalents/mL, on the basis of branched DNA probe assay, or $>10^5$ copies/mL, on the basis of combined RT-PCR assay (Amplicor-HCV monitor assay). Also,patients who received interferon therapy, which is effective against HCV infection and is known to reduce the risk of development of HCC in patients with chronic hepatitis C, were compared with those who had not received it [15].

It was difficult to clarify the time when some of the patients in the present study became infected with HCV. Therefore, we evaluated 2 factors as a proxy of the infection period before enrollment: (1) time since diagnosis of chronic liver injury and (2) time since blood transfusion in 27 patients who had a history of transfusion. The proportional hazard assumption was checked for all covariates, and no relevant violations were found. We used SAS statistical software (version 8.2; SAS Institute, Inc.) for the analyses. $P < .05$ was considered to be statistically significant.

Results. Of the 65 HCV-1b isolates analyzed, 18 were classified as group A, 44 as group B, and 3 as group C. Baseline characteristics of the patients infected with group A, B, and C strains are summarized in table 1. For the factors listed in the table, there were no significant differences between the groups. The HCV RNA level also did not differ significantly between the 3 groups ($P = .158$): the proportions of patients with a high viral load were 67%, 86%, and 100% in those infected with group A, B, and C strains, respectively. The median follow-up period was 12.8 years (range, 1.0–19.5 years). The subgroups that had been determined on the basis of 8 paired serum samples did not shift to another group during a mean interval of 11 years. During the follow-up period, HCC developed in 3 (17%) of the patients infected with group A strains, in 28 (64%) of those infected with group B strains, and in 0 of those infected with group C strains. In all the cases, HCC was a solitary nodule or comprised double nodules with diameters ≤3.0 cm. Forty-one patients (63%) received interferon therapy at least once during the follow-up period. The proportion of patients infected with group A strains who received treatment was not significantly different from the proportion of patients infected with B strains who received treatment (72% and 57%, respectively; $P = .39$), and the patients who received treatment were all nonsustained virologic responders. Therefore, in the present study, the effect that treatment had on the development of HCC was considered to be small.

Figure 1 depicts the cumulative incidence of HCC (determined by the Kaplan-Meier method) in the 3 groups. The 10-year cumulative incidences for patients infected with group A strains and for those infected with group B strains were 0.16 (95% CI, 0.00–0.37) and 0.43 (95% CI, 0.28–0.58), respectively; the 15-year rates for these 2 groups were 0.16 (95% CI, 0.00–0.37) and 0.72 (95% CI, 0.54–0.90), respectively. Cumulative incidence differed significantly between patients infected with group A strains and those infected with group B strains ($P < .01$, by log-rank test).

For estimation of risk of development of HCC, we focused on group A strains and group B strains, because the number of group C strains was too small to allow accurate evaluation of this association. The crude HR for development of HCC among patients infected with group B strains (when the crude HR among patients infected with group A strains was considered to be the reference) was 4.92 (95% CI, 1.48–16.32), and group B strains were significantly associated with HCC after adjustment for each potential confounding factor: HR, 5.15 (95% CI, 1.52–17.38); HR, 5.79 (95% CI, 1.70–19.77); HR, 5.10 (95% CI, 1.53–17.04); HR, 4.05 (95% CI, 1.21–13.56); and HR, 5.77 (95% CI, 1.68–19.83) for age, sex, grade of inflammatory activity, stage of fibrosis, and HCV RNA level, respectively. On the basis of the results of 31 cases of development of HCC, we included 2 important factors—namely, age and

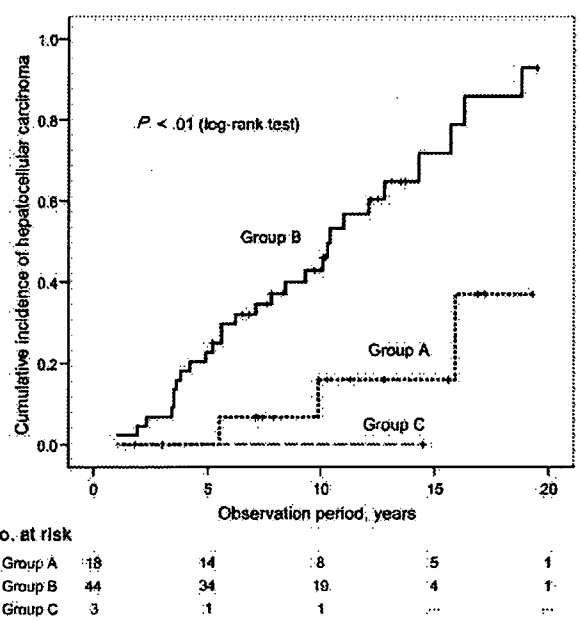

Figure 1. Cumulative incidence of hepatocellular carcinoma (HCC) among 65 patients infected with hepatitis C virus subtype 1b (HCV-1b), estimated by use of the Kaplan-Meier method. The graph shows Kaplan-Meier curves for patients in groups A ($n = 18$) *(dotted line)*, B ($n = 44$) *(solid line)*, and C ($n = 3$) *(dashed line)*. The cumulative incidence of HCC differed significantly between the 3 groups ($P < .01$, by log-rank test).

histologic stage—in the multivariate model; after adjustment for these 2 factors, the risk was significantly greater among the patients infected with group B strains (HR, 4.95 [95% CI, 1.43–17.11]).

Discussion. The findings of the present study suggest that the secondary structure of the amino-terminal 120 residues of the HCV NS3 protein may be independently associated with the risk of development of HCC. The mechanism underlying the relationship between hepatocarcinogenesis and sequence diversity remains unclear. It has been speculated that the difference in the secondary structure of NS3 causally associates with HCC, which reflects a conformational difference that might consequently affect interaction with the p53 tumor-suppressor gene [11]. However, there is another possibility—that the NS3-protein group B strains exert oncogenic function via a mechanism independent of interaction with p53. Moreover, we cannot exclude the possibility that the other biological functions of NS3, such as serine protease activity and antiapoptotic capacity, vary between different groups. In a previous study, which included 35 (54%) of the patients in the present study, we analyzed the amino acid sequences of the HCV isolate from patients with or without HCC and, among the majority of isolates from patients with HCC, did not find, at the primary-structure level, any particular residue that might be contributing to HCC [12]. Further experimental studies are necessary to elucidate these issues.

Long-term chronic inflammation is considered to be related to carcinogenesis. Unfortunately, it was difficult, in some patients in the present study, both to determine when they became infected with HCV and to evaluate the true duration of their infection. We chose, as the baseline, the time when liver biopsy was performed, because histologic characteristics are strong factors for development of HCC [15]. Although the presumed infection period before enrollment was not significantly different between the groups, it should be noted that there might have been unmeasured confounding, because of the limitation posed by the present study's lack of data with respect to true disease-inception status.

In conclusion, the present study shows that the long-term cumulative incidence of HCC in patients infected with HCV group A is significantly different from that in patients infected with HCV group B—and that, after adjustment for age and stage of fibrosis, the latter patients have a markedly increased HR. These findings suggest that HCV-1b grouping, based on the secondary structure of an amino-terminal portion of the NS3 protein, is a potential marker for a high risk of development of HCC in patients with chronic hepatitis C. It is important to further test and replicate the present study's results, in other populations and in a larger number of subjects who have a defined time point of infection, such as blood-transfusion recipients.

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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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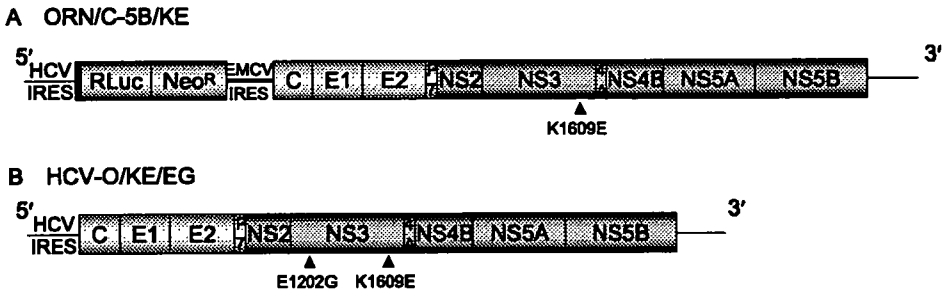


FIG. 1. Schematic gene organization of genome-length HCV RNA. (A) ORN/C-5B/KE RNA replicated in OR6 cells. RL (RLuc) is expressed as a fusion protein with neomycin phosphotransferase (NeoR). The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) Authentic HCV RNA, HCV-O/KE/EG, 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

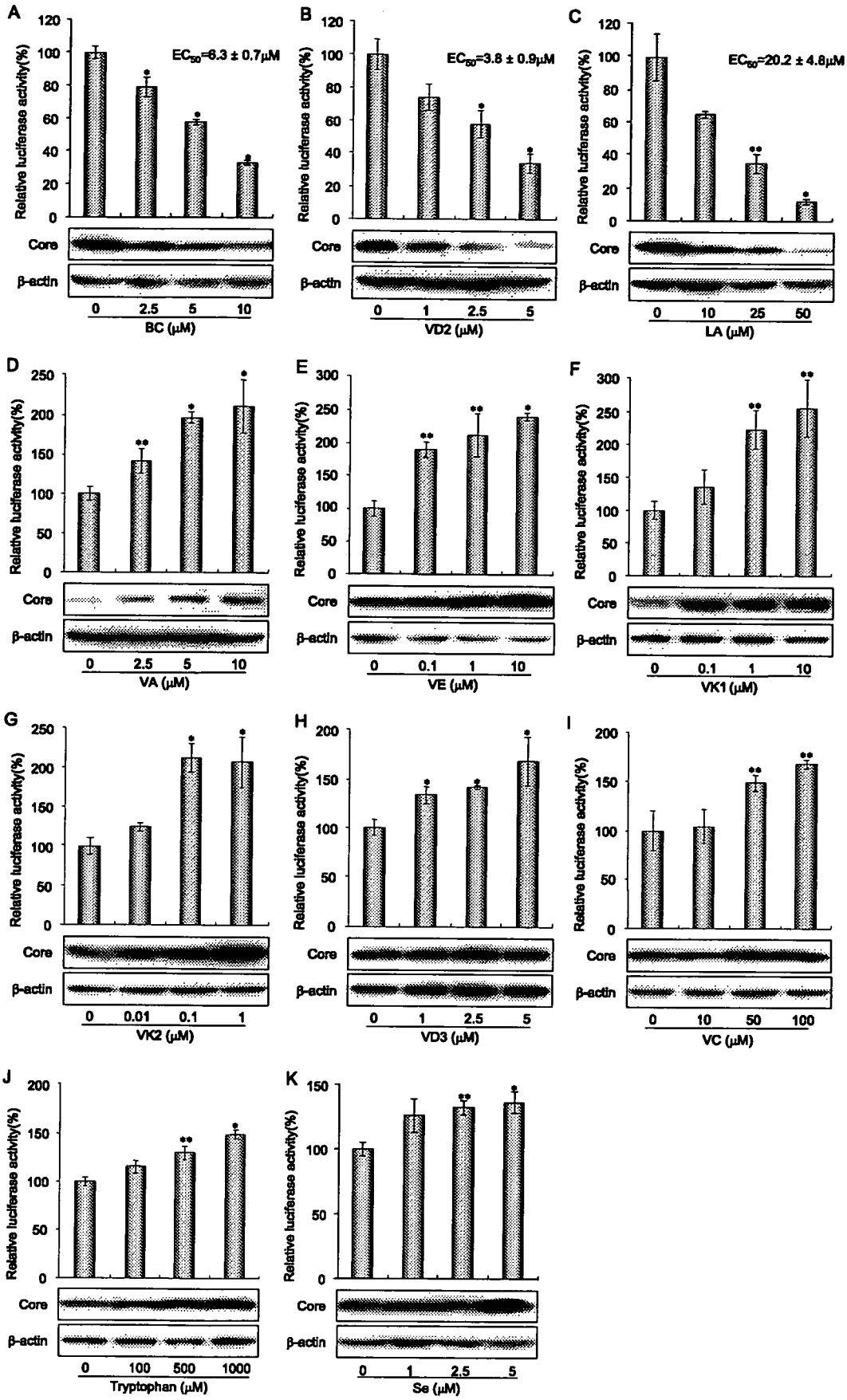
Reagents. Vitamin B_{12}, vitamin K_1 (VK1), vitamin K_3, elaidic acid, and vaccenic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BC, vitamin A (VA), vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin C (VC), VD2, vitamin D_3 (VD3), VE, vitamin K_2 (VK2), pantothenic 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

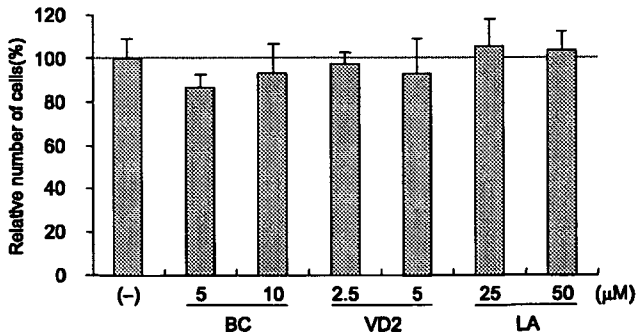


FIG. 3. The anti-HCV activities of three nutrients were not due to the suppression of cell growth. Cell viabilities after treatment with BC, VD2, and LA are shown. OR6 cells were cultured in control medium [(−)] and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), and LA (25 and 50 μM) for 72 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). *, $P < 0.01$; **, $P < 0.05$.

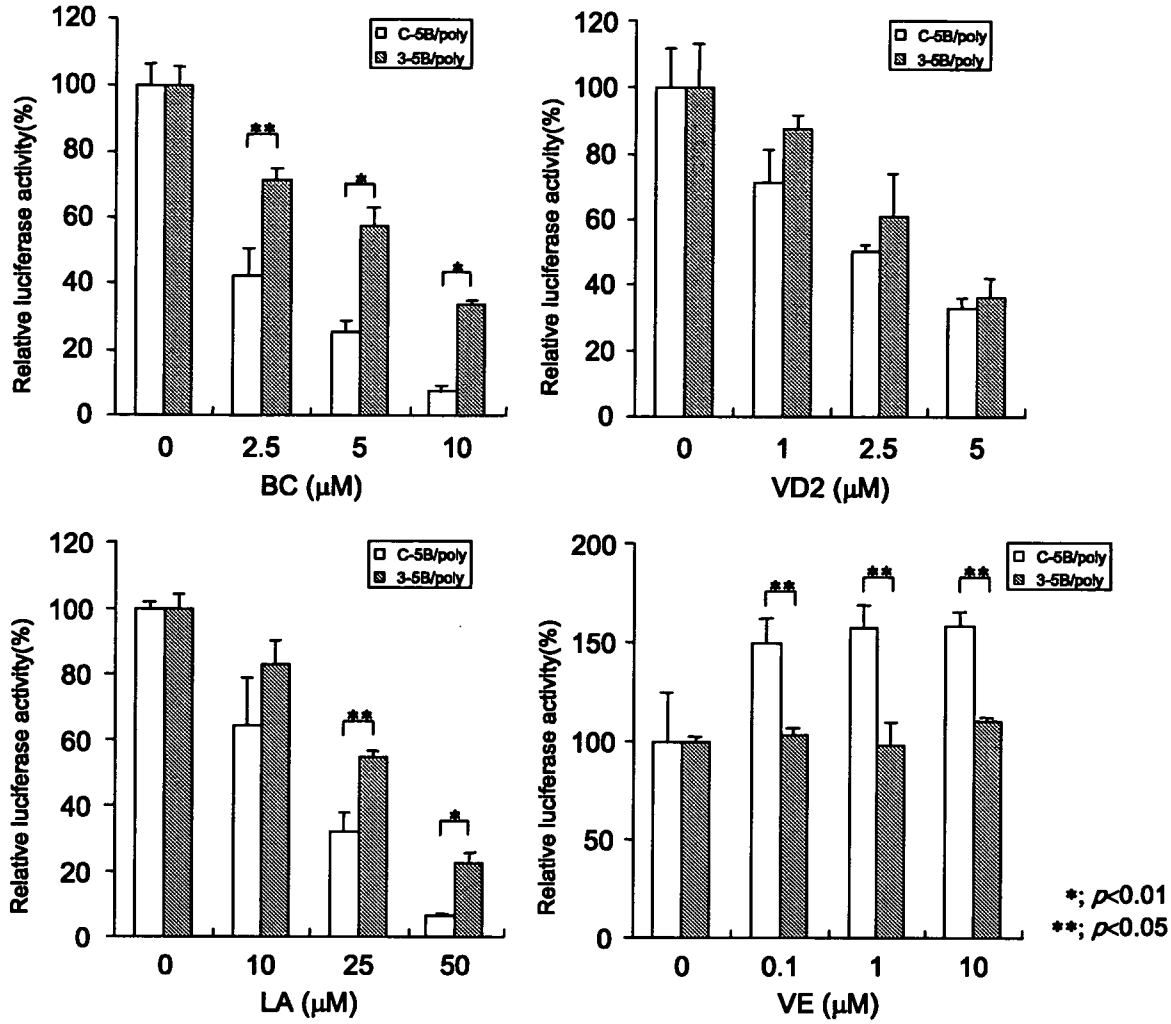


FIG. 4. The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. Both polyclonal genome-length HCV RNA-replicating cells (ORN/C-5B/KE/poly) and subgenomic replicon cells (ORN/3-5B/KE/poly) were treated with BC, VD2, LA, or VE according to the same protocol as 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.

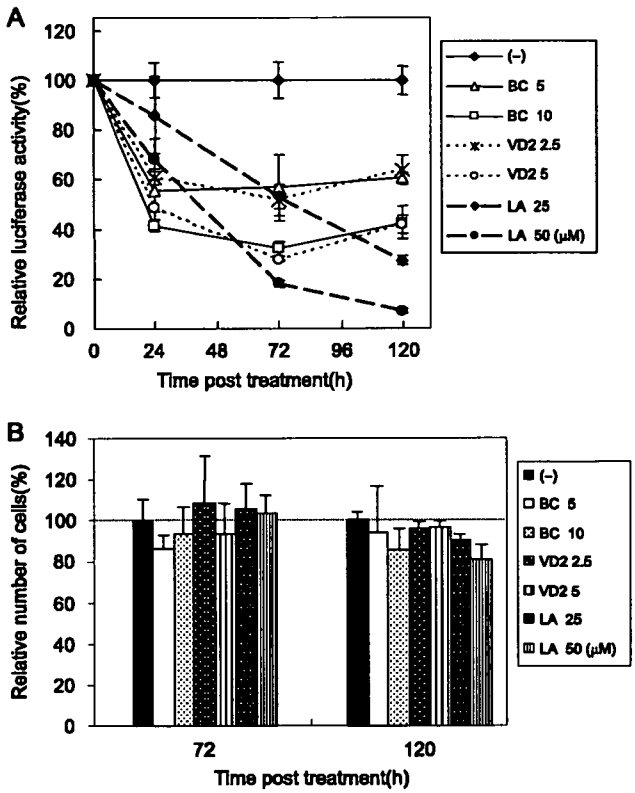


FIG. 5. Time course assay of the anti-HCV activities of three nutrients. (A) Time course of the inhibitory effects of three nutrients on HCV RNA replication. OR6 cells were treated with control medium [(−)], BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and the RL assay was performed at 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).

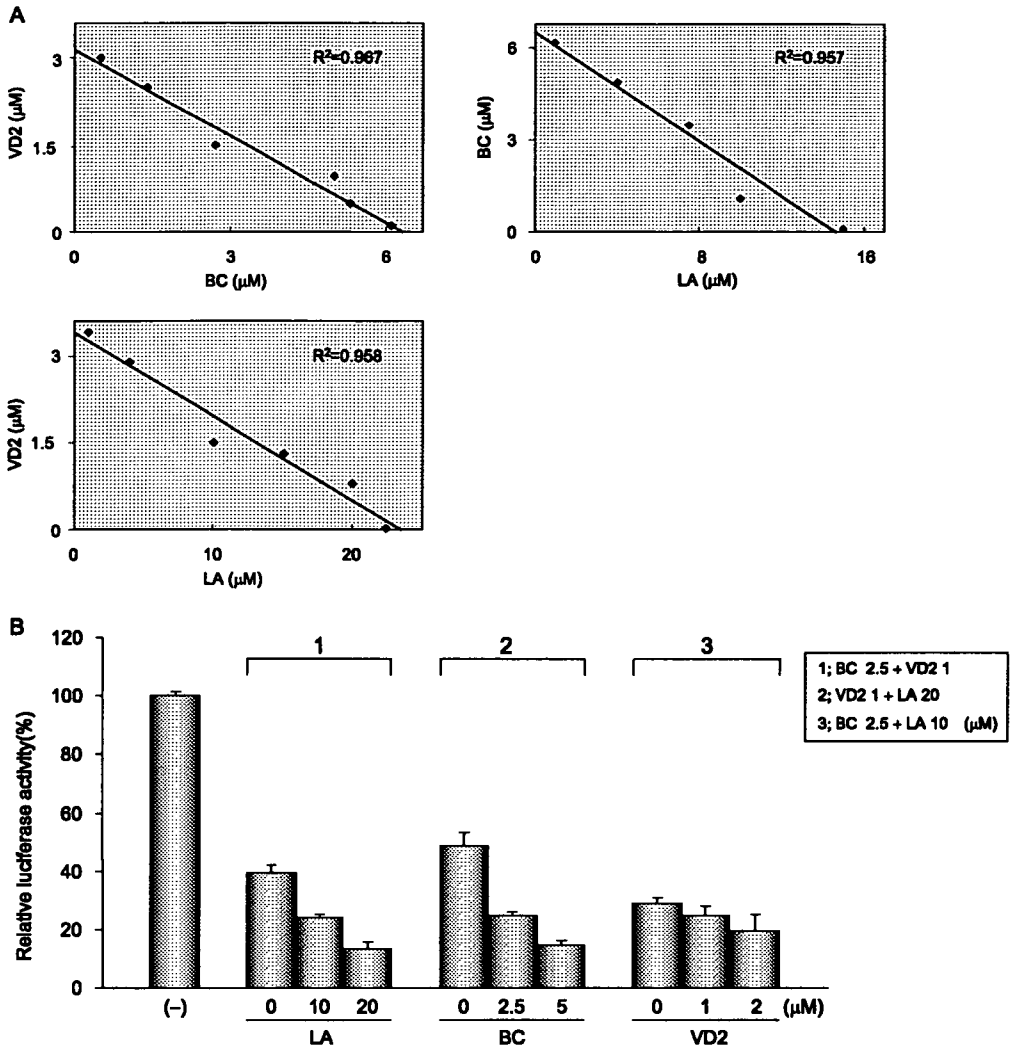


FIG. 6. Effects of treatment with each combination or all of the three nutrients on HCV RNA replication. (A) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with each combination of three nutrients, BC (0, 0.5, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.1, 0.5, 1, 1.5, 2, and 3 μM), 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 (≈EC_{20}) in addition to 1 μM VD2 (≈EC_{30}) plus 20 μM LA (≈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%

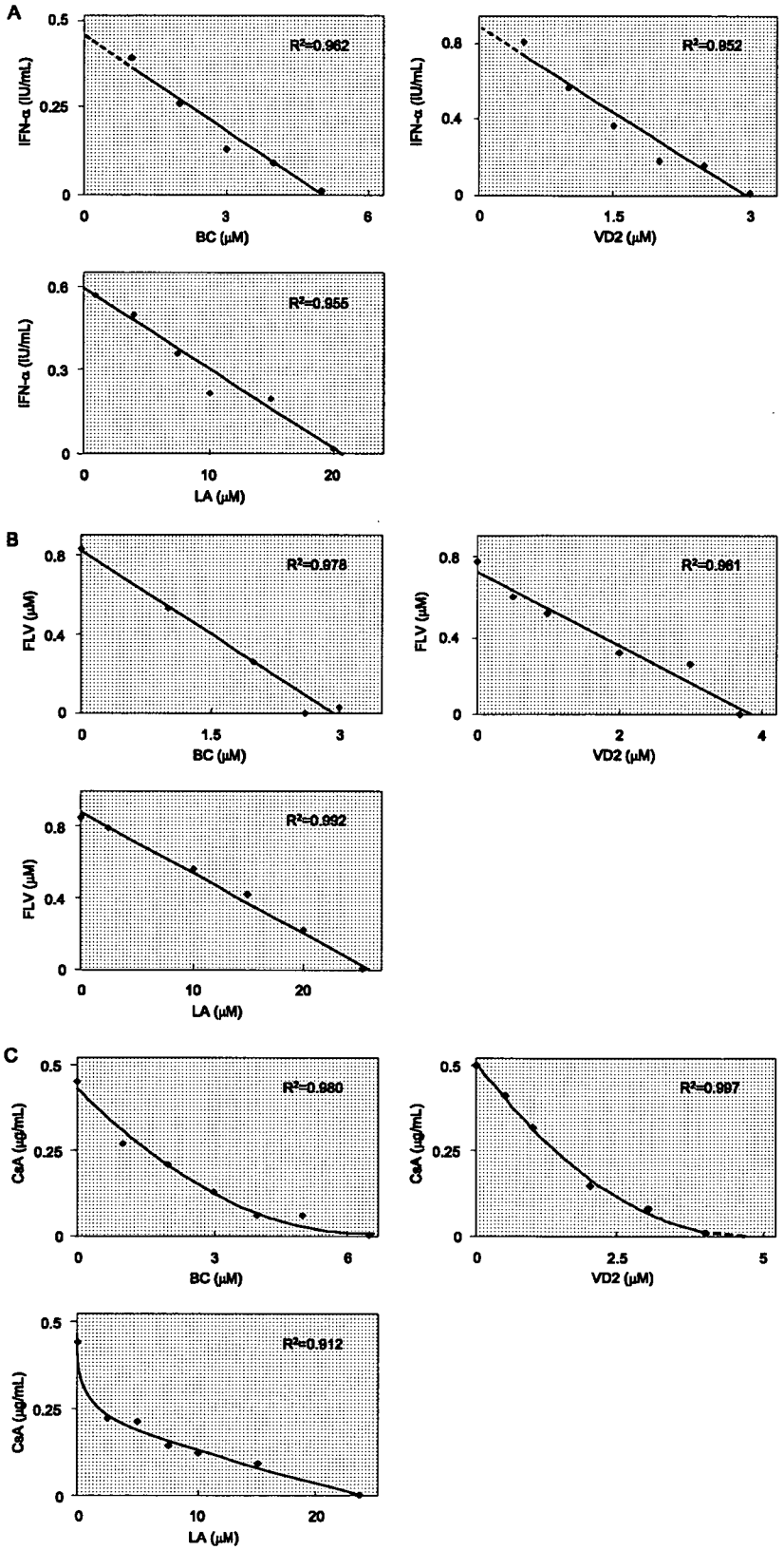


FIG. 7. Additive inhibitory effects of each of three nutrients in combination with IFN-α or FLV on HCV RNA replication, and synergistic effects observed with Cs. (A to C) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with BC (0, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.5, 1, 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.