JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2006, p. 4593–4595 Vol. 44, No. 12
0095-1137/06/$08.00+0 doi:10.1128/JCM.00621-06

Feasibility of Freeze-Dried Sera for Serological and Molecular Biological Detection of Hepatitis B and C Viruses▽

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Received 22 March 2006/Returned for modification 20 August 2006/Accepted 30 September 2006

We compared hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus (HCV) antibody, and HCV RNA quantification in frozen and freeze-dried serum samples to assess the usefulness of freeze-dried sera for detection of HBV and HCV. The results indicated that freeze-dried sera as well as frozen sera can be useful for serological and molecular biological analyses of HBV and HCV.

Freeze-dried sera are generally used as standards for hematobiochemical tests. In frozen and freeze-dried sera, protein, lipid, and electrolyte levels remain relatively stable, but enzyme levels show a greater degree of variation in freeze-dried sera than frozen sera (6). Nevertheless, freeze-dried sera can be stored at room temperature for a long time and are therefore easier to handle than frozen sera.

For the study of hepatitis B virus (HBV), dried blood spot (DBS) samples have been used for detecting hepatitis B virus surface antigen (HBsAg) and antibody to hepatitis B core antigen (2, 8). Recently, DBS samples allowed the development of a simple, sensitive, and appropriate test for quantifying HBV DNA and studying HBV genetic variants (5). As for hepatitis C virus (HCV), dried sera are used for the test of anti-HCV antibody (Ab) (2), and DBS samples allowed the development of a simple, sensitive, and reliable test for detection and genotyping of HCV RNA (1, 7). However, there is no report on their usefulness in HCV RNA quantification. We conducted serological and molecular biological tests to detect HBV and HCV using frozen and freeze-dried serum samples to determine the feasibility of freeze-dried sera.

The Atomic Bomb Casualty Commission established the Adult Health Study (AHS) longitudinal cohort in 1958; since then, the Atomic Bomb Casualty Commission and its successor, the Radiation Effects Research Foundation (RERF), have examined about 20,000 atomic-bomb survivors and controls biennially in outpatient clinics in Hiroshima and Nagasaki. We selected at random 12 consecutive HBsAg-positive and 25 consecutive anti-HCV Ab-positive individuals among 6,121 AHS longitudinal cohort subjects who underwent hepatitis screening from 1993 through 1995. Their serum samples were stored by both freezing and freeze-drying methods.

First, the procedure used for the preparation of frozen serum samples was as follows: Blood obtained from the AHS subjects was kept at room temperature for 20 min. Serum was then divided into four equal parts and stored in 1.5-ml polypropylene tubes at −80°C until use. These samples were thawed by leaving them at room temperature for 30 min and mixed well by inversion before use. Second, the procedure used for the preparation for freeze-dried serum samples was as follows. A 0.4-ml portion of the serum was separated as mentioned above and stored in a glass tube at −80°C. After 1 week of storage, the samples were freeze-dried using a freeze-dryer, sealed, and stored at room temperature (20 to 25°C) until use. These samples were reconstituted by the volumetric method using diethyl pyrocarbonate-treated Milli-Q water and mixed well before use.

The tests for HBsAg and anti-HCV Ab using fresh serum samples in hepatitis screening from 1993 through 1995 were described previously (3, 4). In screening tests, an anti-HCV Ab titer of $\geq 2^{12}$ was defined as a high titer. In the present study, HBsAg and anti-HCV Ab were measured by enzyme immunoassay (EIA) (International Reagents Corporation, Kobe, Japan) and second-generation EIA (International Reagents Corporation), respectively. Measured values of ≥1.0 for HBsAg and anti-HCV Ab were defined as positive. An anti-HCV Ab titer of ≥50 was defined as a high titer.

Serum RNA was extracted from 100 μl of frozen or reconstituted freeze-dried serum samples using SepaGene RV-R (SankoJunyaku Co., Tokyo, Japan). The prepared RNA was reverse transcribed with random primers (6-mer) and reverse transcriptase (ReverTra Ace; Toyobo Co., Tokyo, Japan). HCV RNA was quantitated by real-time PCR using fluorescence resonance energy transfer probes. Primers and probes were designed within a highly conserved 5′ untranslated region (UTR) and also targeted homologous regions of genotypes 1a, 1b, 2a, and 2b. The oligonucleotide sequences of the primers were as follows: HCVNC2, 5′-CCTGTGAGGAACTACTGTC-3′, and HCVNC1, 5′-CAACACTACTCGGCTAGCAGTC-3′. The hybridization probes were as follows. Probe NCJ-LC (5′-GAACCGGTGAGTACACCGGAAT) was labeled at the 5′ end with the fluorophore Red 640 and phosphorylated at the 3′ end. Another probe, NCJ-FL (5′-GGGAGAGCCATAGTGGTCTGC), was labeled with fluorescein isothiocyanate at the 3′ end. PCR was performed in a total volume of 20 μl,

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▽ Published ahead of print on 11 October 2006.

containing 5 mM $MgCl_2$, 6 pmol of NCJ-LC, 4 pmol of NCJ-FL, 10 pmol of the two PCR primers, 2 μl of LightCycler-FastStart DNA Master hybridization probe mix (Roche Diagnostics Co.), and 1 μl of synthesized cDNA solution. The PCR cycling program consisted of an initial denaturing step at 95°C for 10 min and 50 amplification cycles of 95°C for 15 s, 55°C for 6 s, and 72°C for 10 s. Once the threshold was chosen, the point at which the amplification plot crossed the threshold was defined as the threshold cycle (C_T). The calculated C_T value is predictive of the quantity of target RNA copies. The standard curve was calculated using serially diluted plasmids containing nucleotide sequences of the HCV 5′ UTR, to obtain control fragments for determination of HCV copy numbers. All assays were conducted in duplicate.

The positive-negative results of HBsAg in frozen and freeze-dried serum samples were consistent with results using fresh serum samples. The concordance in measurement of anti-HCV Ab among fresh, frozen, and freeze-dried serum samples was not complete but was satisfactory. Both frozen and freeze-dried serum samples of one case tested negative for anti-HCV Ab, despite testing positive in the 1993-1995 hepatitis screening. One freeze-dried serum sample of another case tested positive for anti-HCV Ab, despite testing negative in the 1993-1995 screening (Table 1). For these two patients with discrepant results, the specimen yielding a positive result contained only low titers of anti-HCV Ab; subsequent testing for HCV RNA by quantitative or qualitative PCR was negative in both cases (data not shown). Furthermore, 86% (18/21) of the fresh serum samples yielding high anti-HCV Ab titers by passive hemagglutination also yielded high anti-HCV Ab titers on subsequent testing of both frozen and freeze-dried serum samples by EIA; frozen and freeze-dried samples from the remaining three cases produced low-positive results. These results might be due to decay of the antibody during storage or differences in criteria for high antibody titer between the previous and present kits. However, the results for high anti-HCV Ab titers were completely consistent in frozen and freeze-dried serum samples.

TABLE 1. Comparison of detection of HBsAg and anti-HCV Ab in frozen, freeze-dried, and fresh serum samples

Substance tested, sample type, and EIA result[a]	No. of fresh serum samples with PHA[b] result		Concordance (%)
	Positive	Negative	
HBsAg			
Frozen			
Positive	12	0	100
Negative	0	25	
Freeze-dried			
Positive	12	0	100
Negative	0	25	
Anti-HCV Ab			
Frozen			
Positive	24	0	97
Negative	1[c]	12	
Freeze-dried			
Positive	24	1[c]	95
Negative	1[c]	11	

[a] HBsAg was measured by EIA; anti-HCV Ab was measured by second-generation EIA.
[b] HBsAg was measured with a reverse passive hemagglutination (PHA) test kit; anti-HCV Ab was measured with a second-generation PHA test kit.
[c] HCV infection status was negative with quantitative or highly sensitive qualitative PCR.

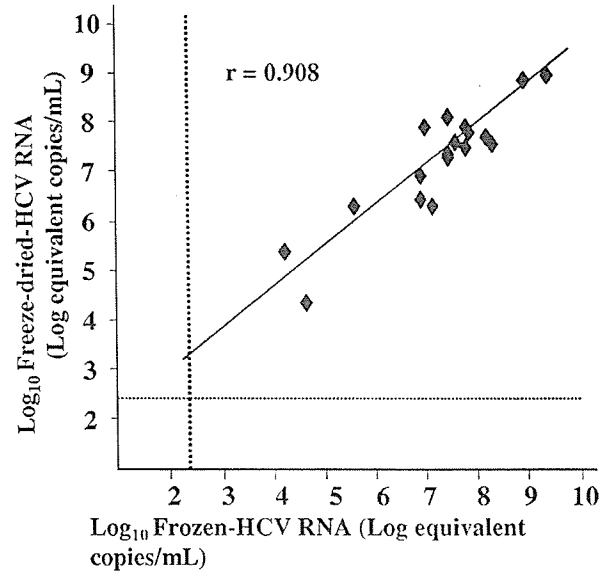

FIG. 1. Correlation of HCV RNA levels in frozen and freeze-dried serum samples. The correlation between $\log_{10}$-transformed HCV RNA quantities for pairs of frozen and freeze-dried serum samples from 18 cases analyzed by real-time PCR was significant ($r = 0.908$, $P < 0.0001$).

A linear relationship in the range of 2.0×10^2 to 2.0×10^9 equivalent copies/ml was observed between C_T values and quantity of RNA copies ($r > 0.99$) (data not shown). Real-time PCR detected HCV RNA in 18 of 25 frozen and freeze-dried serum samples from anti-HCV-positive cases in the 1993-1995 hepatitis screening. The correlation between HCV RNA concentration in frozen and freeze-dried serum samples was significant ($r = 0.908$, $P < 0.0001$) (Fig. 1).

The intra-assay variability was determined by assaying two frozen serum samples containing HCV RNA of genotype 1b and 2a (respectively, 9×10^5 and 1.3×10^6 copies/ml) 10 times in a single day, and the respective coefficients of variation (CVs) were 6.2% and 2.9%. The respective interassay CVs calculated by assaying each of these serum samples once a day for 10 days were 3.6% and 4.3%.

On the whole, results for fresh, frozen, and freeze-dried serum samples for HBsAg and anti-HCV Ab demonstrated very good agreement, indicating that these methods and storage conditions are appropriate for serological assays of HBV and HCV. Furthermore, results of a newly developed highly sensitive and high-range HCV RNA quantitative assay for frozen and freeze-dried serum samples showed good correlation. We expected that the PCR products of HCV RNA would vary depending on storage method and conditions; however, the results showed no marked differences during 10 years of storage. The use of sera of AHS subjects stored from 1969 can further advance the study of the evolution of HBV/HCV as well as the natural history of viral liver diseases.

We thank Toshinori Kurisu for collection and processing of the data.

The RERF, Hiroshima and Nagasaki, Japan, is a private, nonprofit foundation funded by the Japanese Ministry of Health, Labor and Welfare (MHLW) and the U.S. Department of Energy (DOE), the latter through the National Academy of Sciences. This publication was supported by RERF Research Protocol RP 1-04 and by a Scientific Research Grant from the MHLW.

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Hiroshima J. Med. Sci.
Vol. 55, No. 3, 71~77, September, 2006
HIJM 55–11

Suppression of Interferon-related Promoter Activation by Hepatitis C Virus Proteins Expressed in Cultured Cells

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ABSTRACT

Interferon is important for anti-viral defense of the host. The E2, NS3/4A, and NS5A proteins of hepatitis C virus (HCV) have recently been reported to confront anti-viral action induced by interferon. However, roles of the individual HCV proteins in anti-interferon action are still not well understood. We have isolated an HCV strain, HCV-K, from a patient with acute hepatitis. Nucleotide sequencing of the entire genomic DNA of HCV-K revealed that the isolate belongs to the genotype 1b, which is generally resistant to interferon therapy. In the present study, we expressed individual HCV-K proteins in mammalian cells and investigated effects of the proteins on interferon signal transduction. The results showed that the core, E1, NS4A, and NS4B proteins suppressed activation of interferon stimulation responsive element (ISRE) and gamma activation sequence (GAS) reporters. These results suggest that multiple HCV proteins have a function in suppression of the anti-viral effect by interferon and may indicate a novel role of E1 and NS4B proteins in interferon antagonism.

Key words: *Hepatitis C Virus, Interferon, ISRE, GAS*

It is estimated that hepatitis C virus (HCV) has infected at least 170 million people in the world. About 70% of patients with acute HCV infection progress to chronic HCV infection. Furthermore, 30–40% of them suffer from liver cirrhosis 20–30 years later, and some of them develop hepatocellular carcinoma. No vaccine effective for HCV has been developed so far. Currently, the only approved therapy for HCV is interferon (IFN)-α or pegylated-IFN-α (PEG-IFN-α) in monotherapy or in combination with rebavirin[13]. However, its effectiveness is limited depending on the viral genotype and initial virus quantity[15,19]. Although genotypes 2a and 2b are generally susceptible to IFN therapy, genotype 1b, which is prevalent in Japan, is highly resistant to the therapy. The reason for this is not clear.

IFN is a host key player to confront virus infection. Cells sense virus infection through Toll-like receptors or intracellular sensors, RIG-I or mda5, and further activate transcription factors IRF-3 and NF-κB, subsequently activating the IFN-β promoter. Induced IFN-β activates the interferon stimulation responsive element (ISRE) through the IFN-α/β receptor and the Jak-Stat pathway in an autocrine or paracrine fashion. The activation induces production of massive IFN-α through IRF-7 induction by the ISRE activation and results in successive amplified production of anti-viral effectors such as protein kinase R (PKR) and oligoadenyl synthase [reviewed in][4,5,10].

HCV is a positive single-stranded RNA virus belonging to the family *Flaviviridae*. It contains ca. 9600 nucleotides and encodes an approximately 3000-amino-acid-long polyprotein, which is cleaved into at least 10 proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) through the activity of cellular peptidases as well as viral encoded proteases[2,12].

Recently, HCV proteins have been shown to antagonize the IFN system. Aizaki et al[1] demonstrated that expression of the HCV 1b non-struc-

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tural proteins NS3, NS4 and NS5, or NS5A alone in HepG2 cells rendered the cells sensitive to encephalomyocarditis virus (EMCV) infection after IFN-α treatment. The structural proteins core, E1 and E2, however, did not have such antagonizing activity, suggesting that the HCV non-structural proteins, NS4 and NS5, antagonized IFN-induced anti-viral action. On the other hand, Keskinen et al[11] observed that U-2 OS cells inducibly expressing the core protein, the NS4B protein or a polyprotein containing the core, E1 and E2 proteins was partially sensitive to vesicular stomatitis virus (VSV) infection after IFN-α treatment. Thus, the results obtained from the HCV 1b genotype are controversial.

In the present study, we obtained an HCV genomic cDNA of genotype 1b from a patient with acute hepatitis, and we transiently expressed nine out of the ten HCV proteins in HeLa cells and HuH-7 cells from cDNA and examined effects of the respective HCV proteins on IFN-related promoters systematically to investigate mechanisms of the resistance of HCV 1b to IFN therapy.

MATERIALS AND METHODS

Cells and viruses

Human hepatoma-derived HuH-7 cells and human cervical cancer-derived HeLa cells were propagated in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. African green monkey kidney-derived CV1 cells were grown in MEM supplemented with 10% fetal calf serum.

Plasmid preparation

The HCV-K strain, 1b genotype, was isolated from a patient suffering from acute hepatitis in Hiroshima University Hospital. A full-length genomic cDNA clone of the HCV-K strain was constructed by using RT-PCR and connecting at restriction enzyme sites (DDBJ/EMBL/GenBank accession number AB249644). The DNA fragments encoding individual proteins were amplified by using the HCV-K genomic cDNA as a template with simultaneous addition of the start codon with a Kozak translation start consensus sequence, 5'-ACCATG-3', and a stop codon, 5'-TAG-3', at respective 5' and 3' ends.

For the cDNAs of NS2, NS3, NS4A, NS4B, NS5A, and NS5 proteins, an HA-tag, N'-YPYDVPDYA-C', was attached at the C terminus of each protein. The regions of E1 and E2 were extended toward the N terminus to involve a hydrophobic peptide functioning as a signal sequence. Amino acid numbers of amplified regions were as follows: 1–191 for C, 155–383 for E1, 340–746 for E2, 810–1026 for NS2, 1027–1657 for NS3, 1658–1711 for NS4A, 1712–1972 for NS4B, 1973–2419 for NS5A, and 2420–3010 for NS5B.

The amplified fragments were subcloned into the multicloning site of pKS336, a eukaryotic pol II expression vector possessing the human elongation factor promoter and a blastocidin-*S* acetyl transferase gene[20]. For a control, the cDNA of heat shock protein 70 (Hsp70) 1a with the C-terminal HA tag was also inserted into the vector.

Immunofluorescent staining

CV1 cells were transfected with expression vectors by using FuGENE6 (Roche Diagnostics) and fixed with 0.5% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min at 24 hr after transfection. The cells were then treated with 100 mM glycine in PBS and 0.1% Triton X-100 in PBS and incubated with either an anti-HA monoclonal antibody (262K, Cell Signaling Technology), an anti-core monoclonal antibody (10G5H4, a gift from T. Fujiwara), an anti-NS5A monoclonal antibody (B7, a gift from T. Fujiwara), an anti-E1 monoclonal antibody (ViroStat) or an anti-E2 goat polyclonal serum (Biodesign International). The cells were subsequently incubated with an Alexa 488-conjugated anti-mouse or anti-goat IgG antibody (Molecular Probes) and observed under a fluorescent microscope (TE2000-S, Nikon).

Immunoprecipitation and SDS-PAGE

Subconfluent CV1 cells were transfected with plasmids, metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 min at 24 hr post transfection, and solubilized with a RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Proteins were immunoprecipitated with a specific antibody and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 10% or 15% gel and then visualized using a BAS2000 Bio-imaging Analyzer (Fuji Film) as described previously[21].

Reporter assay

A reporter assay was performed basically as described by Gotoh et al[8]. HuH-7 and HeLa cells in a 24-well plate were transfected with 0.3 μg/well of a firefly luciferase ISRE reporter plasmid, pISRE-Luc (Clontech), 0.03 μg/well of a

ABBREVIATIONS

GAS, gamma activation sequence; IRF, interferon regulatory factor; ISRE, interferon-stimulated responsive element; Jak, Janus kinase; mda5, melanoma differentiation-associated gene 5; NF-κB, nuclear factor κB; RIG-I, retinoic acid-inducible gene I; SeV, Sendai virus; Stat, signal transducer and activator of transcription; TLR, Toll-like receptor; TRIF, TIR-domain-containing adaptor inducing IFN.

Renilla luciferase-expressing plasmid under the control of thymidine kinase promoter, pRL-TK (Promega), and 0.3 μg/well of a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, cells were incubated with 1000 IU/ml human IFN-α (Mochida Pharmaceutical Co.) for 6 hr and then harvested for measuring luciferase activity. Firefly luciferase reporter activity in the cell extracts was normalized to Renilla luciferase activity or in some cases to protein contents. Fold induction of the ISRE promoter was calculated by dividing the relative luciferase activity of IFN-α-treated cells by that of mock-treated cells. Data represent the mean values of the normalized luciferase activities from triplicate samples. For measurement of GAS promoter activation, pGAS-Luc (Clontech) was used as a reporter plasmid and 5 ng/ml recombinant human IFN-γ (Strathmann Biotec) was used for stimulation.

For IFN-β promoter activation, 0.3 μg/well of pIFΔ (–125) lucter, which has a firefly luciferase gene under control of the human IFN-β promoter (provided by S. Goodbourn[18]), was introduced into cells in a 24-well plate together with 0.03 μg/well of pRL-TK and 0.3 μg/well of a plasmid expressing an HCV protein. After 24 hr, cells were treated with 100 μg/ml of poly I:C for 6 hr and harvested for luciferase assay.

RESULTS

Expression of HCV proteins derived from the HCV-K strain in mammalian cells

We initially examined the expression of HCV-K proteins in mammalian cells. The constructed expression plasmids were introduced into CV1 cells and stained with protein-specific or anti-HA tag antibody, followed by immunofluorescent staining (Fig. 1). The core, E1 and E2 proteins were found mainly in the peri-nuclear region, probably around the endoplasmic reticulum. The E1 and E2 proteins appeared to be delivered to the ER by the attached signal sequence. NS2, NS4B, NS5A, and NS5B were observed mainly in the cytosol with homogenous or patchy patterns. NS3 and NS4A appeared to reside mainly in the nucleus.

Transfected cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine, and proteins were immunoprecipitated with one of the E1-, E2-specific and anti-HA tag antibodies (Fig. 2). The results demonstrated that NS2, NS3, NS4A, NS4B, and NS5B appeared to migrate to the posi-

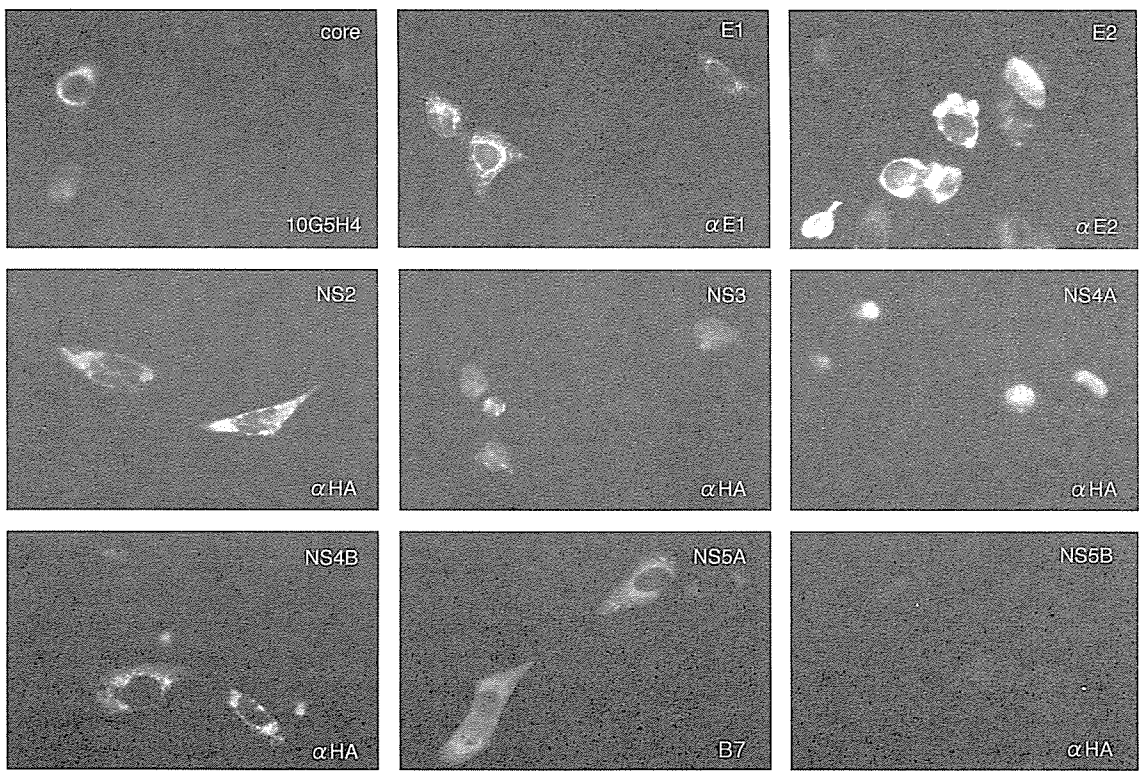

Fig. 1. Immunofluorescent staining of HCV-K proteins.
CV1 cells were transfected with an expression plasmid encoding either core, E1, E2, HA-tagged NS2, HA-tagged NS3, HA-tagged NS4A, HA-tagged NS4B, HA-tagged NS5A, or HA-tagged NS5B protein as indicated in the figure. After 24 hr, the cells were fixed and subjected to immunostaining with anti-HCV core monoclonal antibody (10G5H4), anti-HCV E1 monoclonal antibody (αE1), anti-HCV E2 goat polyclonal antibody (αE2), anti-HCV NS5A monoclonal antibody (B7), and anti-HA monoclonal antibody (αHA) together with an Alexa488-conjugated anti-mouse IgG or anti-goat IgG antibody. Primary antibodies used are also indicated in the figure.

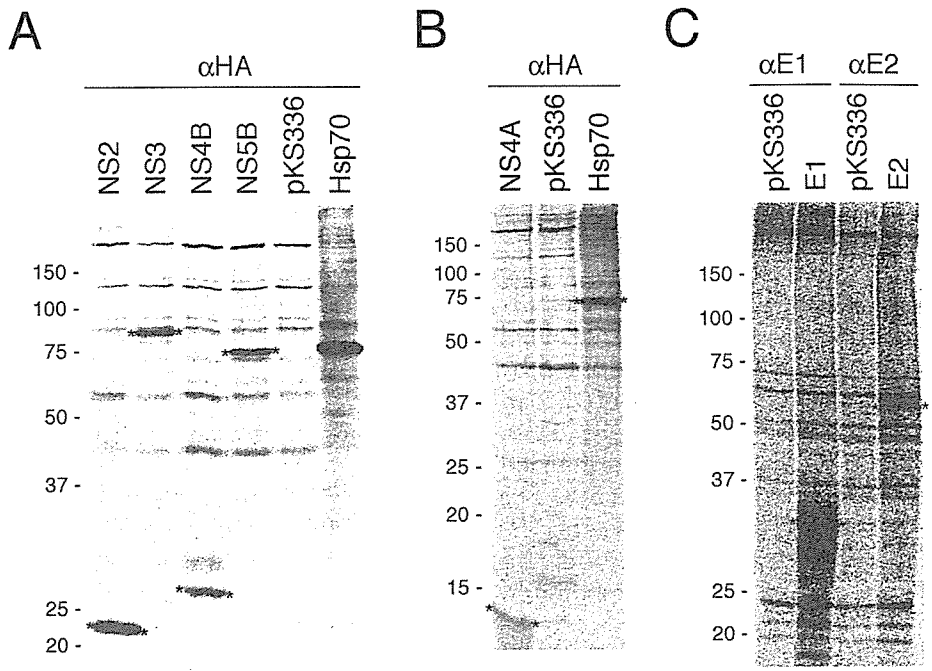

Fig. 2. Detection of HCV-K proteins in SDS-PAGE.
CV1 cells were transfected with plasmids expressing E1, E2, HA-tagged HCV proteins, NS2, NS3, NS4A, NS4B, HA-tagged Hsp70, and an empty vector (pKS336), and they were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min at 24 hr post transfection. The cells were then immunoprecipitated with an anti-HA antibody (αHA), an anti-E1 antibody (αE1) or an anti-E2 antibody (αE2) and analyzed by SDS-PAGE using 10% (A, C) and 15% (B) gels. Positions of molecular size markers are shown in the figure. Asterisks mark the migrating positions of individual expressed proteins.

tions of ca. 20, 80, 10, 30, and 70 kilodaltons, respectively (Figs. 2A, 2B). These are expected sizes from their respective amino acid numbers including an HA tag, 226, 640, 63, 270, and 600, and largely conformed to the reference[12]. The E1 and E2 proteins migrated to the positions of ca. 33 kilodaltons (in two bands) and 60 kilodaltons, respectively (Fig. 2C). These are consistent with the results of previous SDS-PAGE analysis of glycosylated E1 and E2[14,23]. The immunoprecipitated bands of core and NS5A were not clear because of overlapping of the bands with host proteins (data not shown). These results indicate that the HCV proteins, E1, E2, NS2, NS3, NS4A, NS4B, and NS5B, were expressed in mammalian cells and, as far as investigated, their size and antigenicity were authentic. Although the size of core and NS5A was not clear in electrophoresis, these proteins were thought to be expressed in cells based on the results of immunofluorescent experiment.

Effects of the HCV proteins on ISRE activation

We next examined ISRE promoter activation by IFN-α to investigate effects of the HCV proteins on IFN signaling. HuH-7 cells were transfected with pISRE-luc, pRL-TK, and one of the HCV protein expression plasmids, and levels of activation of ISRE-responsive reporter were measured (Fig. 3). SeV C protein, which is known to inhibit IFN

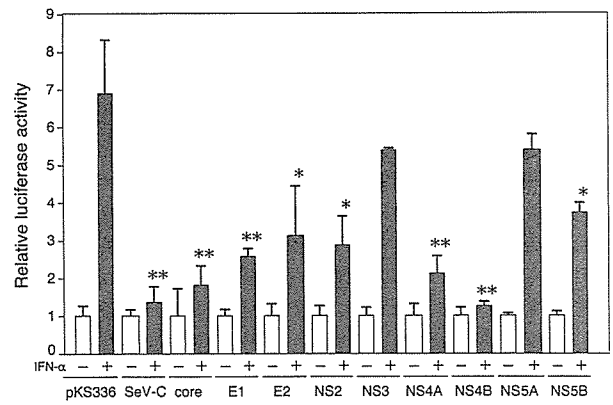

Fig. 3. Effects of HCV-K proteins on ISRE promoter activation by IFN-α.
HuH-7 cells were cotransfected with pISRE-Luc, pRL-TK, and a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, the cells were incubated with 1000 IU/ml IFN-α for 6 hr. The cells were then harvested and luciferase activity was measured. Firefly luciferase reporter activity was normalized to Renilla luciferase activity. Fold induction of ISRE promoter was calculated by dividing the relative luciferase activity of IFN-α-treated cells (closed bars) by that of mock-treated cells (open bars). Data represent the mean values of the fold inductions from triplicate samples. Asterisks indicate a significant difference from the IFN-α-treated control pKS336 sample (*$p < 0.05$, **$p < 0.01$, Fisher's PSLD test).

signal transduction[7,9], suppressed ISRE activation by IFN-α treatment. The core, E1, NS4A, and NS4B proteins significantly suppressed ISRE activation. The E2, NS2, and NS5B proteins moderately suppressed the activation, but NS3 and NS5A did not suppress the activation. Similar results were also obtained when HeLa cells were used (data not shown). We also investigated effects of the HCV proteins on IFN-γ signaling (Fig. 4). GAS activation by IFN-γ was also inhibited by all of the HCV proteins except NS3 and NS5A in HeLa cells. These results demonstrate that HCV proteins can disturb IFN-α and IFN-γ signal transduction.

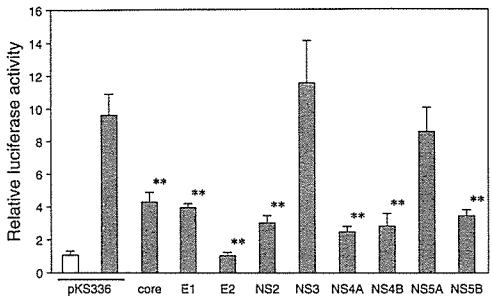

Fig. 4. Effects of HCV-K proteins on GAS promoter activation by IFN-γ.
HeLa cells were cotransfected with pGAS-Luc and a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, the cells were incubated with 5 ng/ml IFN-γ for 6 hr. The cells were then harvested and luciferase activity was measured. Firefly luciferase reporter activity in the cell extracts was normalized to protein contents. Data from IFN-γ-treated cells (closed bars) and mock-treated cells (open bars) are shown in the graph. Data represent the mean values of the normalized luciferase activities from 6 samples. Asterisks indicate a significant difference from the IFN-γ-treated control pKS336 samples (**$p < 0.01$, Fisher's PSLD test).

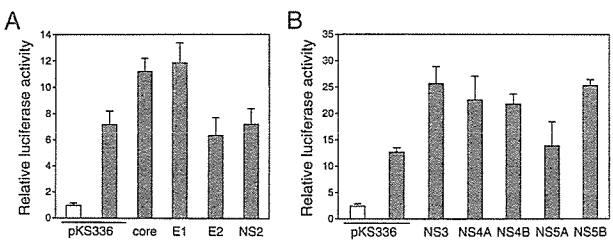

Fig. 5. Effects of HCV-K proteins on IFN-β promoter activation.
HeLa cells were cotransfected with pIFΔ (–125) lucter and a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, the cells were incubated with 100 μg/ml poly I:C for 6 hr. The cells were then harvested and luciferase activity was measured. Firefly luciferase reporter activity in the cell extracts was normalized to protein contents. Data from poly I:C-treated cells (closed bars) and mock-treated cells (open bars) are shown in the graph. Data represent the mean values of the normalized luciferase activities from 6 samples.

Effects of the HCV proteins on activation of IFN-β promoter

IFN-β promoter activation by poly I:C, which is a trigger of massive IFN-α production, was also examined in the presence of the HCV proteins (Fig. 5). None of the nine proteins investigated significantly suppressed IFN-β promoter activation. Some of the proteins such as core, E1, NS3, NS4A, NS4B and NS5B rather appeared to activate the IFN-β promoter.

DISCUSSION

In the present study, we constructed plasmids expressing individual HCV proteins on the basis of the HCV-K strain, belonging to genotype 1b, which is generally resistant to IFN treatment. We performed reporter assays after introduction of the plasmids into HuH-7 cells or HeLa cells, and we investigated effects of the proteins on IFN-related promoters. There was no difference in the IFN-β promoter activation induced by poly I:C possibly through TLR-3 and TRIF. However, the core, E1, NS4A and NS4B proteins suppressed activation of both of the ISRE and GAS reporters. These results demonstrate that HCV proteins can disturb IFN-α and IFN-γ signal transduction and may indicate that the HCV proteins disturb the factors commonly employed both in the ISRE and GAS activation such as Stat1 and Jak1.

E2 and NS5A proteins have been reported to counter anti-viral activity induced by IFN[6,24]. However, we did not observed suppression of ISRE activation by either NS5A or E2 in a reporter assay. Accordingly, the IFN antagonistic activity of NS5A and E2 proteins does not appear to be attributed to suppression of ISRE activation but to direct inhibition of an IFN effector, PKR. Indeed, it has been reported that the NS5A interferon sensitivity-determining region (ISDR: amino acids 2209–2248), which correlates with sensitivity to IFN treatment[3], directly interacts with PKR and inhibits its anti-viral activity[6,22]. Deletion of the ISDR or mutations in the ISDR abolished binding of NS5A with PKR[6,17,22]. On the other hand, E2 has an amino acid sequence called PKR-eIF2α phosphorylation homology domain (PePHD), which is similar to the target sequences for autophosphorylation of PKR and phosphorylation of eIF2α by PKR, and E2 inhibits PKR function *in vitro*[24].

The present study suggests that some of the HCV 1b proteins have anti-IFN activity probably through suppression of ISRE activity. The fact

that the Jak-Stat pathway has been shown to be suppressed in cultured cells expressing the entire HCV genome [for example][16] suggests that the HCV proteins have anti-IFN activity. Aizaki et al[1] demonstrated that expression of a polypeptide containing NS3, NS4, and NS5 proteins and the NS5A protein of an HCV 1b genotype strain canceled resistance to EMCV infection after IFN treatment. They also demonstrated that a polyprotein containing core, E1, E2, NS2, and NS3 did not have such activity. Anti-viral activity was not observed, however, when VSV was used as a challenge virus instead of encephalomyocarditis virus. On the other hand, Keskinen et al[11] reported that cells expressing the core protein, the NS4B protein, and a polyprotein containing core, E1, and E2 were partially sensitive to VSV infection after IFN-α treatment but that cells expressing the NS3A-NS4A protein and the NS5A protein were not. Differences in IFN-antagonistic activities so far reported in individual HCV proteins may be due to differences in virus strains, cells, and experimental systems. Of the two reports described above, our results are closer to the results presented in the latter, although the results for NS4A and NS5A are different. Disturbance of IFN-related promoters by E1 or NS4B of the HCV-K strain in the present study suggests novel functions of E1 and NS4B against the IFN system.

For the next step of these experiments, stable transformants expressing the HCV-K proteins should be established to clarify effects of the proteins on Jak-Stat signal transduction. Furthermore, it may be interesting to compare anti-IFN activities of HCV proteins in IFN-sensitive and IFN-resistant strains. In order to clarify the anti-interferon activity of HCV proteins more precisely, the use of a full-length genome replicon system or the use of a virus infection system should be considered in the future.

ACKNOWLEDGEMENTS

We thank Dr. T. Fujiwara (Ortho-Clinical Diagnostics K.K., Tokyo, Japan) for providing monoclonal antibodies against HCV proteins and Dr. S. Goodbourn (University of London, U.K.) for providing pIFΔ (–125) lucter. We also thank the staff of the Research Center for Molecular Medicine and the Analysis Center of Life Science, Hiroshima University for the use of their facilities. This work was support by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology.

(Received April 27, 2006)
(Accepted June 1, 2006)

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2006, p. 3867–3874 Vol. 50, No. 11
0066-4804/06/$08.00+0 doi:10.1128/AAC.00239-06

Emergence of a Novel Lamivudine-Resistant Hepatitis B Virus Variant with a Substitution Outside the YMDD Motif[▽]

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Received 24 February 2006/Returned for modification 21 April 2006/Accepted 1 September 2006

Lamivudine is a major drug approved for treatment of chronic hepatitis B virus (HBV) infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well-documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with an intact YMDD motif, which included an amino acid substitution, rtA181T, in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (W172L) in the overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive peptide nucleic acid-mediated PCR clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a threefold decrease in susceptibility to lamivudine in in vitro experiments in comparison with the wild type. In vivo analysis using human hepatocyte-chimeric mice confirmed the resistance of this mutant strain to lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with the HBs stop codon using restriction fragment length polymorphism PCR and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif.

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits HBV replication by interfering with RNA-dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

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[▽] Published ahead of print on 18 September 2006.

Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed (1, 9). In vitro analyses have confirmed that the rtL180M mutation augments the level of lamivudine resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly with or after emergence of YMDD motif mutants and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of the overlapping HBs gene transcription by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the

susceptibility to lamivudine in vitro. They also detected such mutations in virus from a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were mutated to T and A, respectively, and associated with the amino acid substitution rtA181T. The substitutions also induced the amino acid substitution W172L in the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitutions in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance to lamivudine in vitro. We also analyzed the resistance of this new strain in vivo using a human hepatocyte-chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence of resistance compared with lamivudine resistance has been reported for both of these two drugs (2, 5). We also developed a detection system to identify the novel and previously reported (7, 34) nucleotide substitutions to study the incidence of such mutations.

MATERIALS AND METHODS

Antiviral compounds. Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, United Kingdom). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and entecavir {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of virological markers. Hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and antibody against HBeAg (anti-HBe) were quantified by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using a Light Cycler (Roche, Mannheim, Germany). The primers used for amplification were 5′-TTTGGGCATGGACA TTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Cloning of HBV DNA and plasmid construction. HBV DNA was extracted from 100 μl of each serum sample by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunther et al. (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4-genome-length HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into plasmid vector pTRE (Takara Bio, Tokyo, Japan) (patient strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400 to 2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), and the resulting construct was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600 to 3215 and 1 to 1400) and cloned into pBlueScript SK+ (Stratagene, La Jolla, CA), and the resulting construct was named pB-1. The KpnI-BamHI fragment from pB-1 and the KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multicloning site and cloned into plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing a 1.4-genome-length wild-type genotype C HBV (wild-type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T patient and wild-type strains, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene).

TABLE 1. In vitro susceptibility of the S331/rtA181 mutant to lamivudine[a]

Strain		S331/rtA181 mutation	Lamivudine IC_{50} (μM)	Resistance (fold)
Source	Type			
Patient	WT	−/−	0.19 ± 0.01	1
	S331C	C/−	0.23 ± 0.01	1.2*
	rtA181T	−/T	0.58 ± 0.08	3**
	S331C/rtA181T	C/T	0.57 ± 0.06	3**
Laboratory	WT	−/−	0.23 ± 0.04	1
	S331C	C/−	0.3 ± 0.05	1.3*
	rtA181T	−/T	0.88 ± 0.2	3.9**
	S331C/rtA181T	C/T	0.98 ± 0.12	4.3**

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

The eight plasmids with and without amino acid substitutions in the spacer and reverse transcriptase domain are listed in Table 1.

Cell culture, transfection, and determination of IC_{50}. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 cells was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each antiviral drug, various concentrations of lamivudine, adefovir, and entecavir were added after 24 h to the culture plate containing the cells, and cells were harvested after 5 days. The medium containing the drugs was changed on days 1, 3, and 4. A plasmid encoding β-galactosidase (β-Gal) was cotransfected to adjust the transfection efficiency. The β-Gal enzyme assay was performed with a β-Gal enzyme assay system (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad Prism software (GraphPad Software, Inc.) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation. The cells were harvested at 3 or 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, and 0.5% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × *g*. The core-associated HBV genome was immunoprecipitated by mouse anticore monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate–proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using a Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGT GAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte-chimeric mice. Human hepatocyte-chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of in vivo growth potential and function. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured-cell-line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 μl of serum samples containing wild-type and newly identified drug-resistant strains. Serum samples obtained from mice were stored at −80°C before further analyses. After stable high-level HBV viremia was confirmed, the mice were administered food containing 30 mg of lamivudine/kg of body weight/day. The nucleotide sequences of wild-type and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP). HBV DNA extracted from serum samples were amplified by

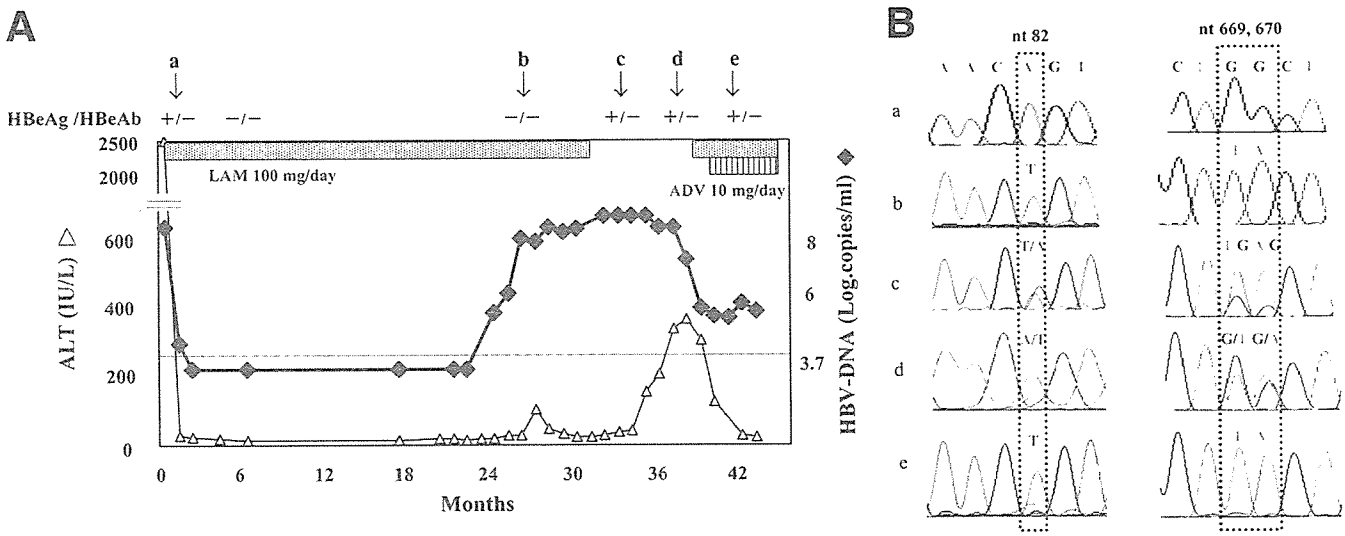

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Time points of serum sampling (see panel A) were as follows: (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before readministration of lamivudine, and (e) during adefovir and lamivudine therapy. Note that the wild type reappeared during the cessation of therapy (c and d), but it disappeared after readministration of the drug (e).

PCR using the primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCA CTGAACAAATGGCACTAGTAAGCTGA-3′. The reverse primer was designed to introduce an EspI site (GCTCAGC) into only wild-type sequences. The PCR was performed in a total volume of 25 μl, consisting of a reaction buffer (100 mmol/liter Tris-HCl [pH 8.3], 50 mmol/liter KCl, and 15 mmol/liter $MgCl_2$), 0.2 mmol/liter of each deoxynucleoside triphosphate, 1 μl of the DNA solution, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Gene Taq; Wako Pure Chemicals, Tokyo, Japan) with 0.2 μg of anti-*Taq* high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), and final extension at 72°C for 7 min. Two μl of PCR products was digested with 5 U of EspI and subjected to electrophoresis in a 3.5% agarose gel.

Statistical analysis. Data are expressed as means ± standard deviations (SD). Group comparisons were performed using the Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

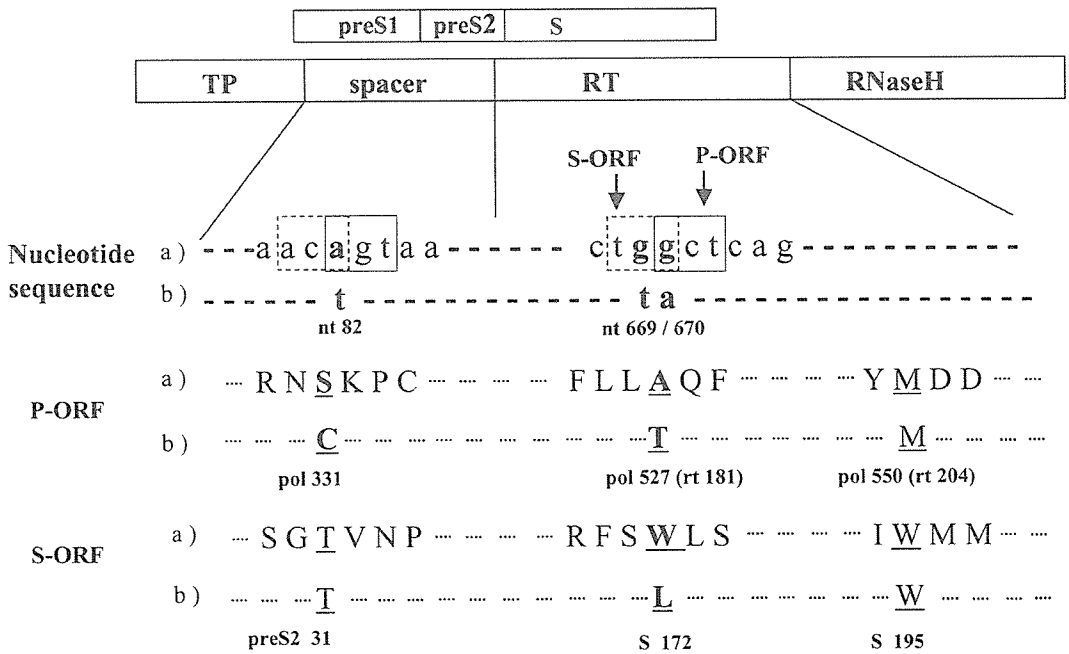

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were compared. See Fig. 1A for time points of serum sampling. Nucleotide sequence numbers are those of typical HBV (e.g., accession no. AB206816 [31]), which starts from a unique EcoRI site.

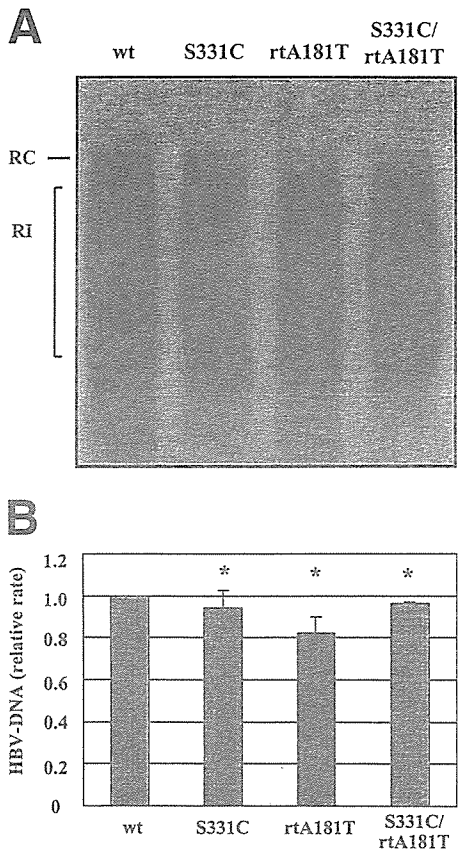

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means ± SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase (polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

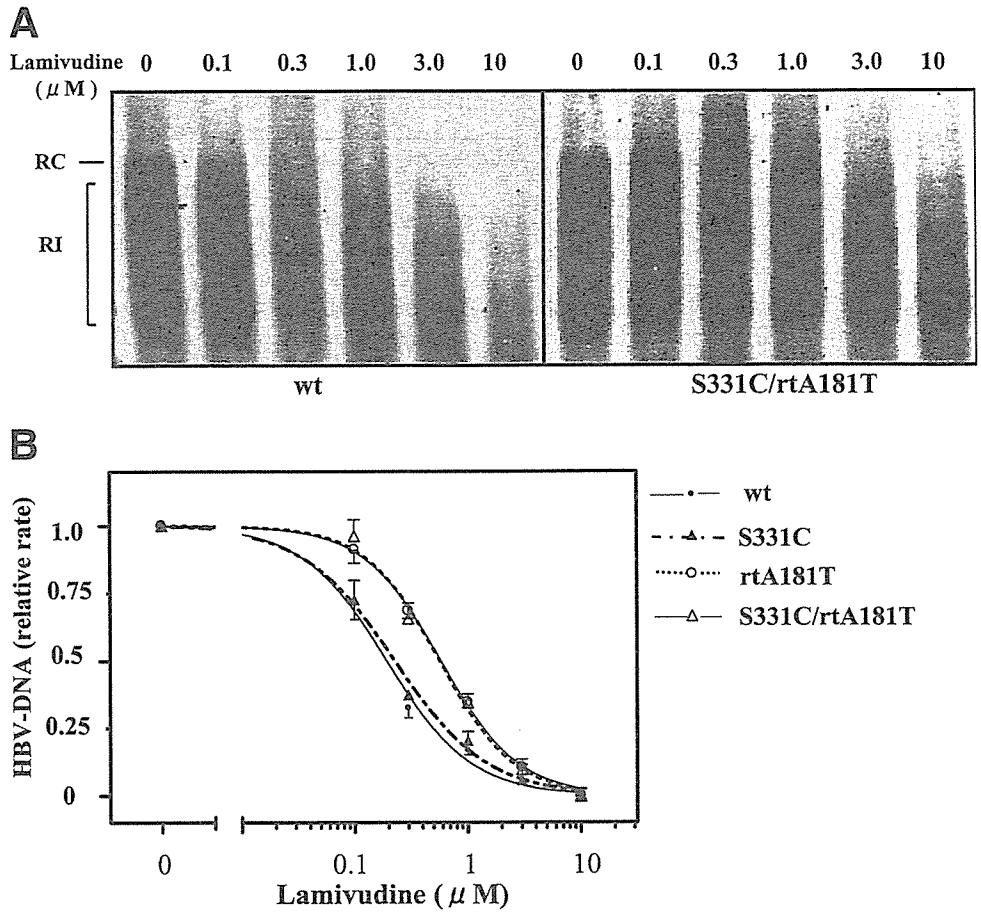

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50}s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means ± SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respectively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

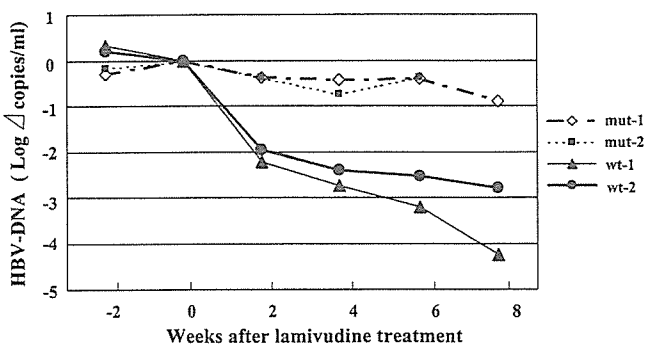

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50}s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the

TABLE 2. In vitro susceptibility of the S331/rtA181 mutant to lamivudine, adefovir, and entecavir[a]

Patient strain	S331/rtA181	Lamivudine		Adefovir		Entecavir	
		IC_{50} (μM)	Resistance (fold)	IC_{50} (μM)	Resistance (fold)	IC_{50} (nM)	Resistance (fold)
WT	−/−	0.19 ± 0.01	1	0.37 ± 0.1	1	0.19 ± 0.02	1
S331C/rtA181T	C/T	0.57 ± 0.06	3**	0.36 ± 0.08	0.98*	0.23 ± 0.05	1.2*

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

mutant strain had the rtA181T mutation with a truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The RFLP PCR analysis of these 39 samples showed that four contained a small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with a truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in eight patients who showed a poor response with lamivudine treatment (HBV viral load above 6.0 log copies/ml after 6 months of treatment). None of these patients tested positive for both of these mutations (data not shown).

DISCUSSION

In this study, we identified a novel lamivudine-resistant strain of HBV with an intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutants were not detected even by a sensitivity-enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One might assume that the compliance of the patient was poor. However, the patient was very punctual and confirmed that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine 3.0- to 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using human hepatocyte-chimeric mice. The amino acid substitution in the reverse transcriptase (RT) domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after those with the mutation in the YMDD motif (YIDD or YVDD) and took over them (34). There are two additional differences between the substitutions we identified and those described by Yeh et al. (34), as detailed below.

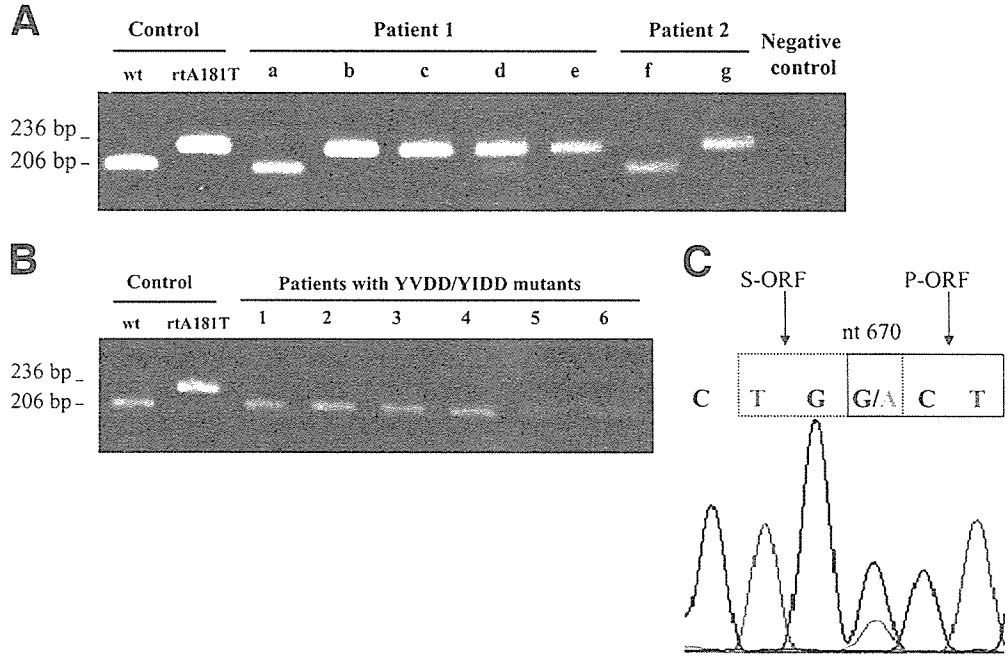

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. See Fig. 1A for the time points of serum sampling (a to e) for patient 1 and see Fig. 1B for a comparison with nucleotide sequence analyses. f and g indicate the time points before and after viral breakthrough for patient 2. (B) Agarose gel electrophoresis of RFLP PCR products using HBV DNA samples obtained from 39 patients who showed lamivudine breakthrough. Of the 39 samples, 35 were wild type (lanes 1 and 2). The remaining four samples (lanes 3 to 7) showed partial digestion, suggesting a mixture of wild-type and mutant strains. (C) Nucleotide sequence analysis of a sample by RFLP PCR suggested the presence of a wild-type–mutant mixture (lane 5 of panel B).

Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al. (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif in woodchuck hepatitis virus (WHV) treated with lamivudine was reported (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codons. These findings suggest that the mutant strains of HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought to replicate by using in vivo-supplied HBs antigen from wild-type strains as helper antigens. In contrast, the novel strain identified in this study had no premature termination of the HBs gene. The in vitro study suggested that the strain had a replication ability similar to that of the wild type. Furthermore, we also showed that the strain infected and reached a high viral load in human hepatocyte-chimeric mice. Although the inoculum contained only a small amount of wild-type strain (one of 12 clones), all clones obtained from mouse serum were mutant strains (rtA181T). Considering these results and the fact that the index patient showed high viral titers after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and has strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitutions in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19–21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after the patient restarted lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Fig. 3 and 4; Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V were reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one case in which the virus developed the rtA181T mutation during famciclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues, including lamivudine, famciclovir, and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchucks (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). The amino acid substitution at position 181 has to be further analyzed with regard to resistance to anti-HBV drugs.

The rtA181T mutation detection system using RFLP PCR developed in this study is a useful tool, as we were able to distinguish the wild type from all mutants with nucleotide substitutions in a given region. The system also enabled us to monitor the fluctuation of the wild-type/mutant ratio during therapy against HBV (Fig. 1 and 6). The incidence of rtA181T mutants with an intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamivudine breakthrough and were positive for YMDD mutants were found to have small amounts of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to monitor these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and that the incidence of disease chronicity is higher than for other genotypes (26). It is thus expected that an increasing number of the sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.

ACKNOWLEDGMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank Hiromi Ishino, Asako Kozono, Kana Kunihiro, Rie Akiyama, Yoshiko Seo, Yoshiko Nakata, Eiko Miyoshi and Kiyomi Toyota for their excellent technical assistance.

This work was supported in part by grants-in-aid for scientific research and development from the Ministry of Education, Sports, Culture, and Technology and the Ministry of Health, Labor and Welfare.

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Original Paper

Intervirology

Intervirology 2006;49:362–369
DOI: 10.1159/000095156

Received: September 28, 2005
Accepted after revision: November 30, 2005
Published online: August 21, 2006

Prolonged Negative HCV-RNA Status Led to a Good Outcome in Chronic Hepatitis C Patients with Genotype 1b and Super-High Viral Load

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Key Words

IFN-α_{2b} + ribavirin, combination therapy · Hepatitis C virus genotype 1b · High viral load · IFN-β, induction therapy

Abstract

Objective: We examined whether a sustained negative HCV-RNA status for 48 weeks affects the outcome in patients with genotype 1b and super-high viral load, and also investigated whether the outcome is affected by the induction therapy of twice-daily pre-administrated interferon (IFN)-β. ***Methods:*** 78 eligible patients were divided into four groups. 40 were patients assigned to the short treatment protocol. 13 patients received 3 MU IFN-β twice daily for 2 weeks followed by IFN-α_{2b} + ribavirin for 22 weeks (β-induction group: group 1). 27 patients received IFN-α_{2b} + ribavirin for 24 weeks (standard combination group: group 2). 38 patients were assigned to the maintenance treatment protocol. All of the 13 in the β-induction group (group 3) and 21 of 25 patients in the standard combination group (group 4) who were negative HCV-RNA PCR at week 24 had IFN monotherapy to maintain a negative HCV-RNA result for 48 weeks. ***Results:*** An HCV-RNA-negative status at week 24 was observed in 96% (25/26) of groups 1 and 3 versus in 79% (41/52) of groups 2 and 4 ($p < 0.01$). The sustained virological response (SVR) was 38% (5/13) in group 1 and 11% (3/27) in group 2 ($p < 0.05$). In the maintenance treatment, SVR was observed in 46% (6/13) of group 3 and 32% (8/25) of group 4 (NS). ***Conclusions:*** A sustained negative HCV-RNA status for 48 weeks might be associated with viral elimination in patients with genotype 1 and super-high viral load.

Introduction

Hepatitis C virus (HCV) infection is estimated to affect 170 million individuals worldwide [1], including 2 million people in Japan [2]. Chronic HCV infection often progresses into liver cirrhosis including the development of associated complications such as gastroesophageal varices and hepatocellular carcinoma over the course of 20–50 years [3–6]. Interferon (IFN) is the only effective treatment for HCV infection, and is widely used. The beneficial effects of IFN in patients with chronic HCV infection have been clearly defined and include decreases in serum transaminase concentration, eradication of the virus, and improvement of liver histology [7–10]. However, a sustained virological response (SVR) is rarely obtained by

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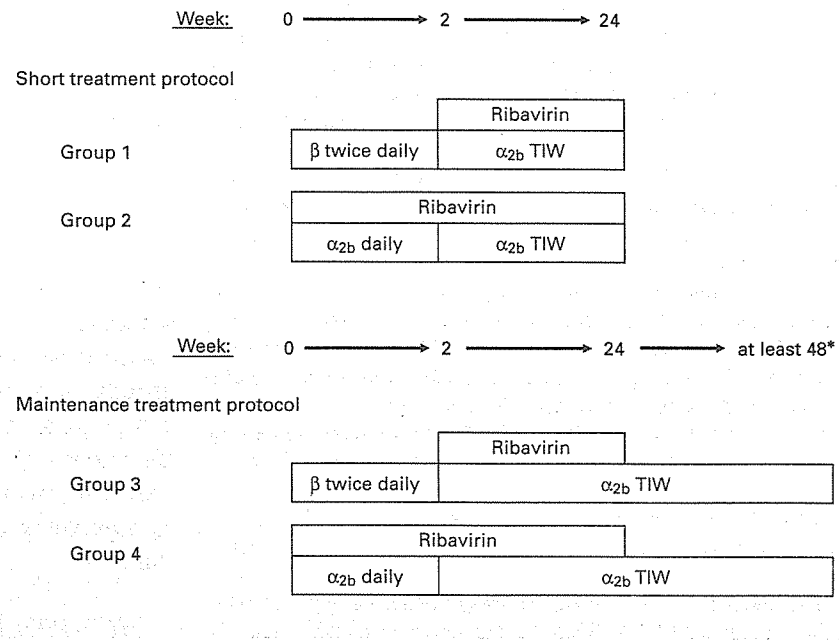

Fig. 1. Study design showing the two different protocols of IFN therapy. * Maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

IFN monotherapy in poor response categories (cirrhosis, high viral load, genotype 1/4) [11–16]. Recent advances of various IFN treatments such as consensus IFN, ribavirin combination, and pegylated IFN can achieve a relatively high SVR in those patients [17–25].

In Japan, the patients with genotype 1 and high viral load is most prevalent [26]. The oral administration of ribavirin has been permitted for only 24 weeks by medical insurance until December 2004 [27]. Because the relapse rate is higher in combination therapy only for 24 weeks [20, 22], we conducted prolonged IFN monotherapy after ribavirin combination. Recently, it was reported that not only the treatment duration but also the duration of therapy with an undetectable HCV-RNA load are associated with the probability of a long-term antiviral response during pegylated IFN/ribavirin combination therapy, and that patients infected with genotype 1 would require a continuous non-detectable viral load in serum for 36 weeks to attain 90% probabilities of SVR [28]. In this study, we designed a clinical trial consisting of combination therapy followed by prolonged IFN monotherapy, which was continued for 48 weeks from the time of the first negative HCV-RNA PCR result for HCV genotype 1 patients with high viral load. We also investigated whether the outcome of IFN therapy is affected by the induction therapy of twice-daily pre-administered IFN-β.

Materials and Methods

Patients

A total of 78 adult patients were recruited for this study. All patients were infected with HCV genotype 1b and had super-high viral load (>500 KIU/ml) as determined by Amplicor HCV monitor assay (Roche Molecular Diagnostics Co., Tokyo, Japan). The detection range of the assay was between 0.5 and 500 KIU/ml (a standard sample containing 10^5 copies/ml of HCV was assigned a titer of 10^5 IU/ml). Patients eligible for study participation were required to satisfy the following criteria: (1) aged from 20 to 65 years; (2) a recent liver biopsy within 3 months of the start of therapy; (3) diagnosis of chronic hepatitis by the conventional classification; (4) positive for HCV-RNA of genotype 1b in serum within 3 months in titers of >500 KIU/ml by the Amplicor HCV monitor assay; (5) abnormal serum alanine aminotransferase levels for >6 months; (6) leukocyte count >3,000/mm^3, platelets >100,000/mm^3; (7) serum bilirubin <2.0 mg/dl; (8) lack of liver cirrhosis, hepatocellular carcinoma, autoimmune hepatitis, alcoholic liver disease and any other chronic liver diseases (positive for serological markers of hepatitis B virus); (9) lack of psychiatric illnesses, including depression, or conditions affecting the bone marrow, alimentary, cardiovascular or pulmonary systems, and (10) no immunosuppressive or antiviral therapy within 6 months prior to entry.

IFN Protocol

Patients were treated with the combination therapy of IFN and ribavirin: 6–10 million units (MU) of IFN-α_{2b} subcutaneously administered three times weekly; oral ribavirin administered twice daily at a total dose of 600 or 800 mg for patients whose weight was less or more than 60 kg, respectively. The IFN therapy protocol is described in figure 1. At the start of the therapy, the physicians in