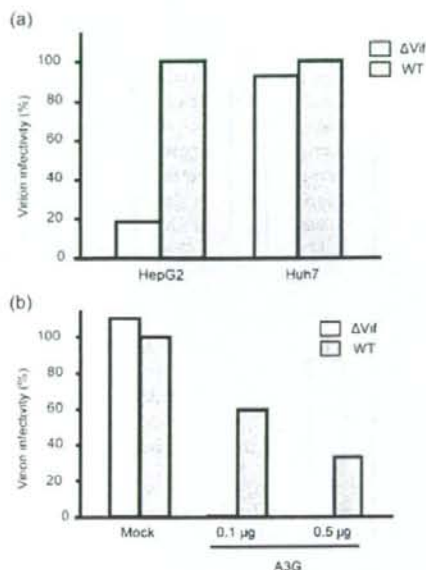

Fig. 4. Infectivity of HIV-1 virions produced from HepG2 and Huh7 cell lines. (a) Wild-type and mutant viruses lacking Vif protein produced from the two cell lines were examined for infectivity as described in Methods. The relative infectivity of the wild-type is shown. (b) Effect of APOBEC3G (A3G) expression on infectivity. HIV-1 virions produced by Huh7 cells co-transfected with the indicated number of APOBEC3G expression plasmid were used for measurement of infectivity.

similar to that of the wild-type virus (Fig. 4a). Transient expression experiments showed that the expression of APOBEC3G in Huh7 cell lines reduced infectivity of wild-type HIV-1 produced in these cell lines in a dose-dependent manner (Fig. 4b). Infectivity of Vif-deficient HIV-1 was reduced to almost undetectable levels (Fig. 4b). Thus, APOBEC3G effectively suppressed the production of infectious HIV in these cell lines.

Both IFN-α and -γ induce APOBEC3G mRNA expression and hypermutation of HBV genomes and reduce replication of HBV

We treated HepG2 cell lines stably transfected with 1.4 genome length construct HBV (Tsuge *et al.*, 2005) with either IFN-α or -γ to examine their influence on the expression of APOBEC3G mRNA and G to A hypermutation of HBV genomes. Chronological studies showed that the core-associated HBV DNA in the stably HBV-producing cell line gradually decreased until 36 h after IFN-α treatment (Fig. 5a). Expression levels of APOBEC3G mRNA, but not those of APOBEC3F, increased in this cell line at 12 h after the IFN treatment (Fig. 5a). Hypermutated genomes in this cell line increased with time until 36 h after IFN-α treatment.

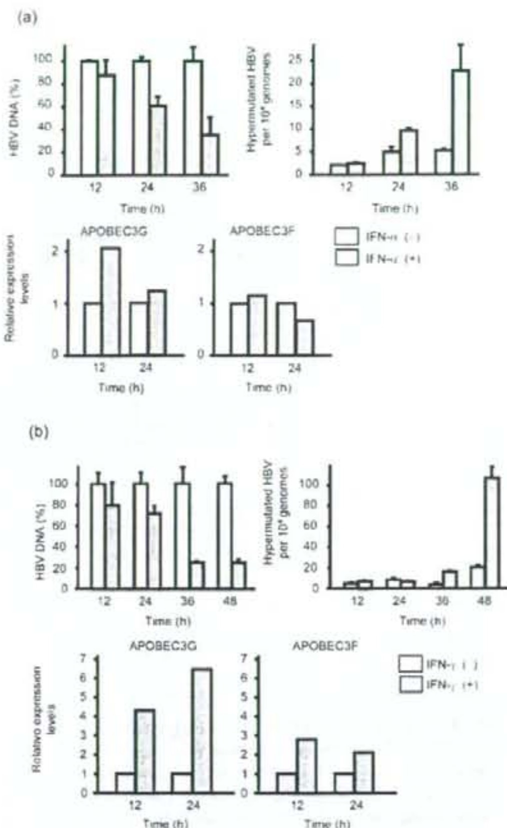

Fig. 5. Effects of IFN-α and -γ on HBV-producing cells. (a) The IFN-α-treated and -untreated HBV-producing T23 cell line was harvested at the indicated time after IFN treatment and examined for the number of core-associated HBV DNA, the number of hypermutated genome and mRNAs of APOBEC3G and APOBEC3F. (b) IFN-γ-treated and -untreated HBV-producing T23 cell line was examined as described in (a). Results are means ± SD values of three independent experiments.

Similarly, the core-associated HBV DNA decreased gradually to about 20 % of the levels in untreated cells after IFN-γ treatment (Fig. 5b). The increase in APOBEC3G mRNA expression was more prominent after IFN-γ than after IFN-α treatment. The level of APOBEC3F mRNA was also about double that of untreated cells. G to A hypermutation of HBV genomes increased markedly with time after IFN-γ treatment (Fig. 5b).

We further examined the effect of IFN on reduction of HBV replication and induction of hypermutation by comparing the effects of different doses of IFN-α and -γ. Both IFN-α

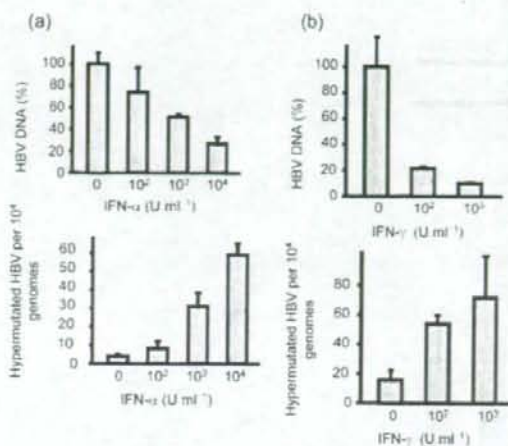

Fig. 6. Dose-dependent reduction of HBV replication and hypermutation of genomic sequences. HBV-producing cell line T23 was harvested after (a) IFN-α and (b) IFN-γ treatment for 72 h. The number of core-associated HBV DNA and the number of hypermutated genomes were measured. Results are means ± SD values of three independent experiments.

and -γ treatment decreased core-associated HBV DNA in a dose-dependent manner (Fig. 6). Hypermutation of HBV genomes also increased with higher doses of IFN (Fig. 6).

Expression of APOBEC3G increases hypermutation of the HBV genome

To confirm that the increase in hypermutation of the HBV genome is induced by the effect of APOBEC3G, we performed expression experiments of APOBEC3G and its deaminase function-deficient mutants into HepG2 cell lines and measured the number of hypermutated HBV genomes. Transient expression experiments showed that the number of HBV DNA was decreased by co-transfection of APOBEC3G in HepG2 cells (Fig. 7a). 3D-PCR and detection with HA-yellow agarose gel electrophoresis showed the presence of heavily hypermutated genomes (Fig. 7b). No amplification was observed at the 81 °C denaturation temperature (data not shown). Quantitative analysis showed an about 334-fold increase in hypermutated genomes compared with mock-transfected control cells (Fig. 7c). However, the proportion of hypermutated genomes was 9.68 % (968 in 10^4 genomes).

To confirm the effect of APOBEC3G on HBV hypermutation, we transfected wild-type and inactive mutants of APOBEC3G (Fig. 8a, b) into Huh7 cells. Wild-type APOBEC3G effectively induced hypermutation of HBV genomes and reduced the replication of HBV. In contrast, insufficient deaminase activity in the E67Q mutant induced less hypermutation of HBV genomes than in the wild-type. No increase in hypermutation was observed in cell lines transfected with deamination-defective E259Q and E67Q/E259Q mutants, although the number of HBV replication was reduced in these cells (Fig. 8a). We observed similar reduction in HBV replication by transient transfection of APOBEC3F. Induction of hypermutation by APOBEC3F was less efficient than by wild-type and the E67Q mutant of

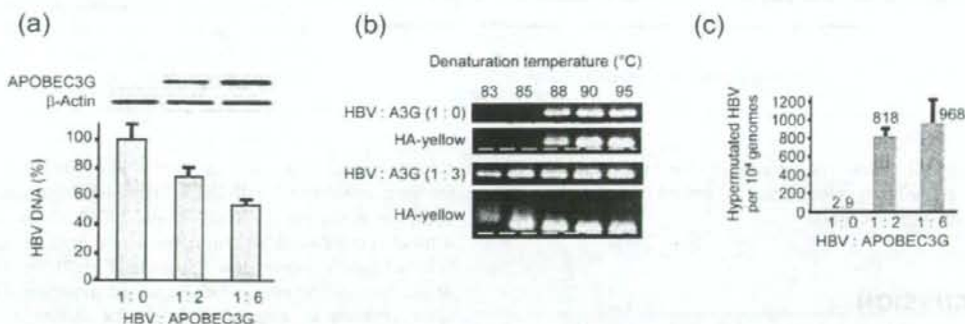

Fig. 7. Effects of APOBEC3G expression on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with pcDNA3/HA-A3G into HepG2 cells. At 72 h after transfection, the cells were harvested. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti-β-actin antibody. (b) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. Hypermutated genomes in the presence or absence of APOBEC3G-HA were amplified by 3D-PCR. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (c) Quantification analysis of hypermutated genomes by real-time 3D-PCR. Results are means ± SD values of three independent experiments.

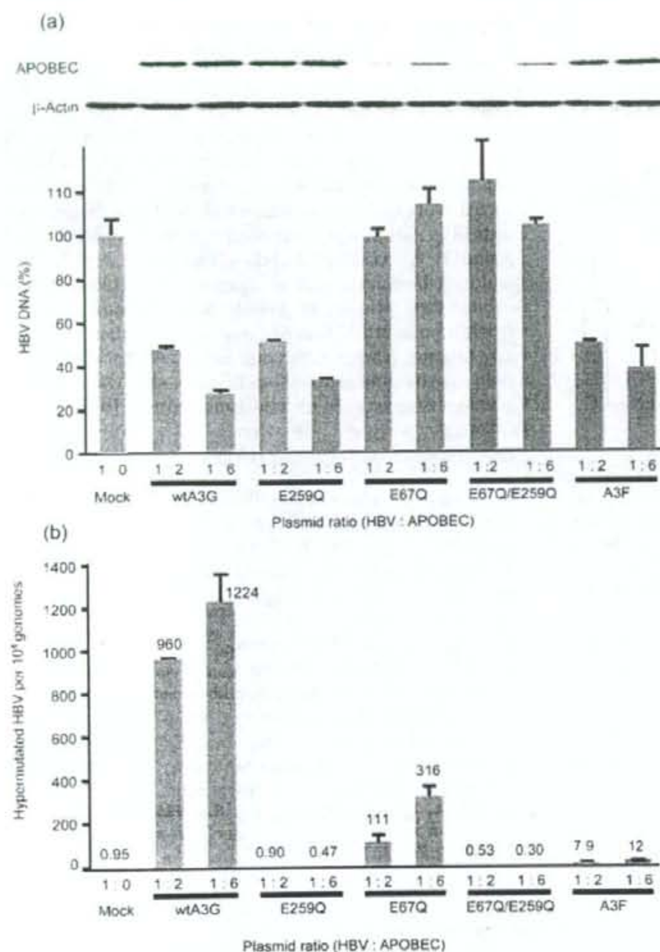

Fig. 8. Effect of APOBEC proteins on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with wild-type, enzymically impaired APOBEC3G mutants (E67Q, E259Q, E67Q/E259Q) and APOBEC3F into Huh7 cells (plasmid ratio HBV : APOBEC = 1 : 2 or 1 : 6). The cells were harvested at 96 h after transfection. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti-β-actin antibody. (b) Quantification of hypermutated genomes by real-time 3D-PCR. Results are means ± SD values of three independent experiments.

APOBEC3G. These results suggest that hypermutation of HBV contributes very little to reduce the number of replicative intermediate.

DISCUSSION

Induction of G to A hypermutation in HIV has been reported as part of host innate immunity against virus infection (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003; Sheehy *et al.*, 2002). We and others have reported the presence of hypermutated genomes of HBV in serum samples of chronically infected patients and in HepG2 cell lines (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005; Rosler *et al.*, 2004). Hypermutation of HBV was induced in hepatocytes (Noguchi *et al.*, 2005), and expression of APOBEC proteins in liver cell-derived cell lines increased hypermutation (Suspene *et al.*, 2005b; Rosler *et al.*, 2004). However, the estimated number of hypermutated genomes in chronically infected patients is very low (Noguchi *et al.*, 2005; Suspene *et al.*, 2005b). The reason for the partial hypermutation of HBV remains an enigma. It might be due to the low expression levels of APOBEC proteins in liver cells (Jarmuz *et al.*, 2002). Alternatively, rapid packaging of pregenome RNA into capsid might prevent access of APOBEC3G to the first strand DNA. Furthermore, rapid degradation of edited HBV genomes by uracil DNA glycosylase in liver cells might also explain the low number of hypermutated genomes.

The mechanism that controls the activities of APOBEC proteins to cause hypermutation has not been analysed until

recently. Tanaka *et al.* (2006) reported that IFN-α increases the expression levels of APOBEC3G mRNA. They reported the presence of ISRE elements in the promoter region of APOBEC3G and that the promoter was activated by IFN-α. However, they did not examine the occurrence of G to A hypermutation in their experiments. Moreover, Peng *et al.* (2006) showed that IFN-α and -γ cooperatively induce APOBEC3G expression and that the inhibition of HIV production by a small number of IFN is cancelled by a small interfering RNA (siRNA) against APOBEC3G. More recently, Bonvin *et al.* (2006) demonstrated that IFN-α induces transcription of APOBEC proteins. They showed that IFN treatment increased APOBEC3B, -3C, -3F and -3G mRNAs, particularly when they used primary cultured hepatocytes. They also reported that they were able to detect hypermutated genomes after transfection of APOBEC3 plasmids, but did not measure the direct effect of IFN on G to A hypermutation.

These studies did not analyse quantitatively the increase in hypermutation of viral genomes. The studies that analysed the expression of APOBEC protein and reduction of HBV DNA also did not analyse quantitatively the number of hypermutated genome (Suspene *et al.*, 2005a; Noguchi *et al.*, 2005; Turelli *et al.*, 2004a, b; Rosler *et al.*, 2005). In the present study, we developed a method that accurately measures the level of hypermutation using real-time PCR. It is often difficult to design a primer set and a probe to detect G to A hypermutation because they are located in a region with many G residues, but the primer and probe sequences should not contain any. It is thus possible that we did not see any C to T substitution because we did not design a primer–probe set to detect this substitution. We also tried to select such a primer–probe set applicable for all genotypes of HBV, but were able to select only one suitable for genotype C.

Using this method, we demonstrated that both IFN-α and -γ increased G to A hypermutation of the HBV genome. Although the expression levels of APOBEC3G increased after IFN treatment, we did not observe an apparent shift of preferred dinucleotide sequence of APOBEC proteins from 3F to 3G. This is probably because the increase in APOBEC3G is only slight (Fig. 5).

The exact mechanism by which IFNs activate the transcription of APOBEC3G is unknown. Furthermore, what kind of sensor(s) detects HBV infection and how the signal is communicated for the production of IFNs and subsequent induction of effector molecules have not been analysed yet. Although the importance of the IFN system in eliminating HBV and its possible mechanism have been reported (Wieland *et al.*, 2004a, b, 2005), further studies are needed to fully describe the mechanism of action of IFNs including the activation of APOBEC3G.

We also demonstrated that the number of hypermutated genomes increased with the expression of APOBEC3G and APOBEC3F (Fig. 8), but not in deaminase-inactive mutants, as demonstrated previously in HIV studies (Shindo *et al.*, 2003; Newman *et al.*, 2005). However, these mutants also reduced the replication of HBV almost to the wild-type level. This suggests that the contribution of hypermutation of HBV to the reduction of virus replication is only minimal and supports the previous report that showed that APOBEC3G reduced the replication of HBV through inhibition of packaging of the pregenome (Turelli *et al.*, 2004a). However, the effect of hypermutation on infectivity of the virus should be investigated further. The effects of APOBEC proteins, including other family members, especially under physiological conditions, should also be examined further. Whether any HBV protein inhibits deamination of the genomic DNA awaits further investigation. Furthermore, the mechanism that enables HBV to cause chronic infection, especially escape from innate antiviral immunity, should also be clarified in order to control chronic HBV infection and reduce HBV-related morbidity.

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

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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 $\geq$1.0 for HBsAg and anti-HCV Ab were defined as positive. An anti-HCV Ab titer of $\geq$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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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: Positive	Negative	Concordance (%)
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.

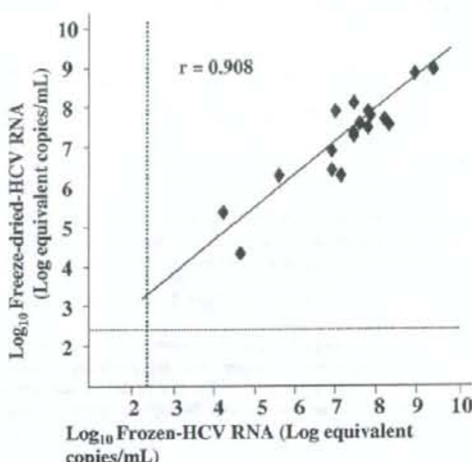

FIG. 1. Correlation of HCV RNA levels in frozen and freeze-dried serum samples. The correlation between $\log_{10}$-transformed HCV RNA quantities for pairs of frozen and freeze-dried serum samples from 18 cases analyzed by real-time PCR was significant ($r = 0.908$, $P < 0.0001$).

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.

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

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

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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).

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

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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′-TTTGGGCATGGACATTGAC-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: Source	Strain: Type	S331/rtA181 mutation	Lamivudine IC_{50} (μM)	Resistance (fold)
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′-GGTGAACAATGTTCCGGAGAC-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

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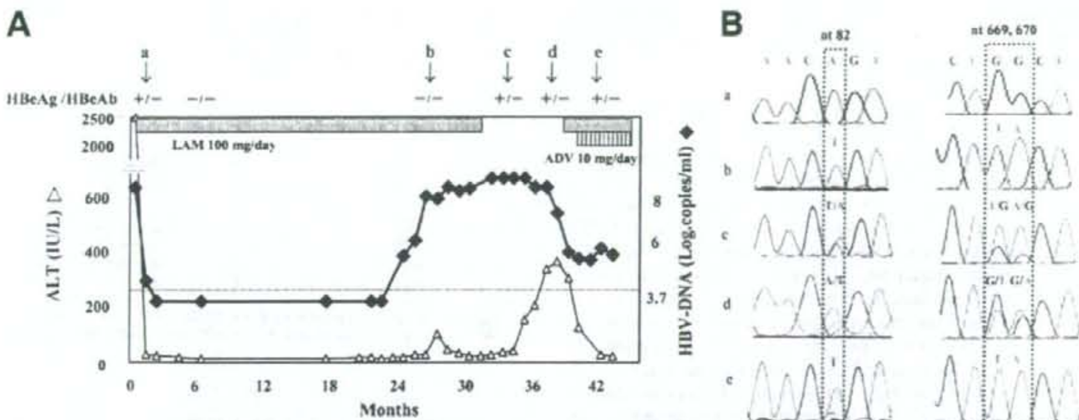

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis 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′-ACCACTGAACAAATGGCACTAGTAAGCTGA-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.

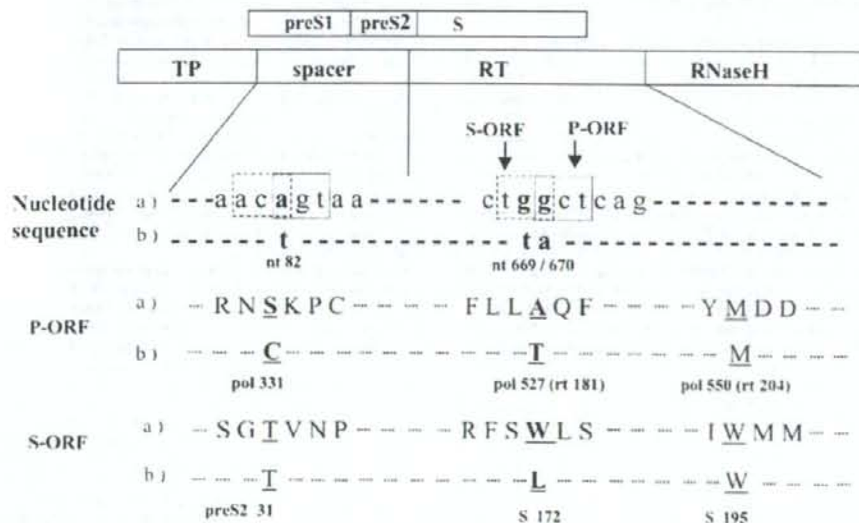

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were 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.

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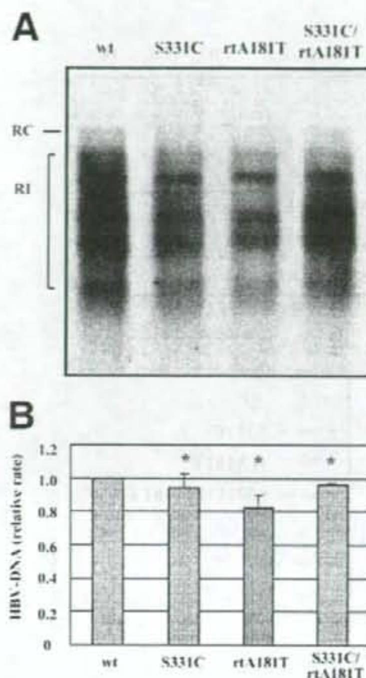

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates 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

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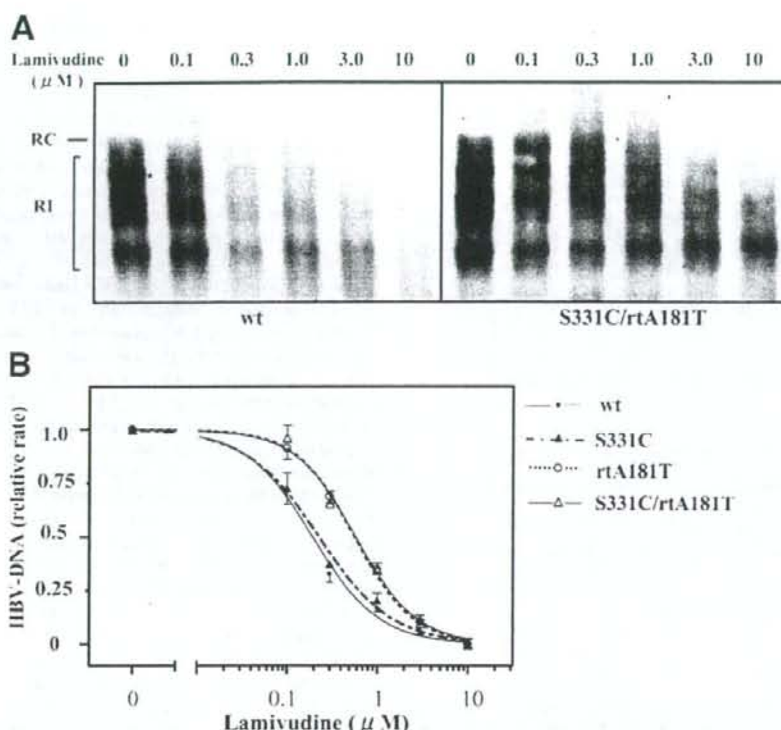

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (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).

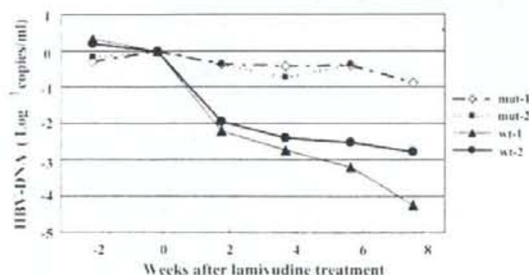

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

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

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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.

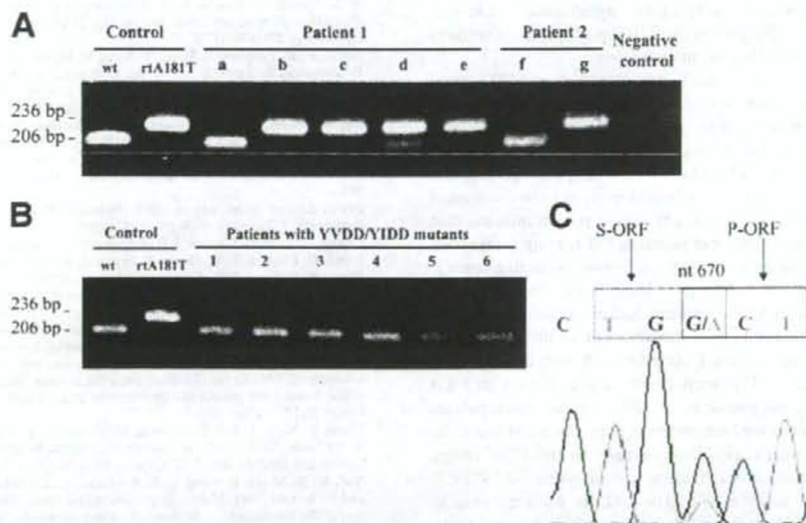

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. 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).

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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.

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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.

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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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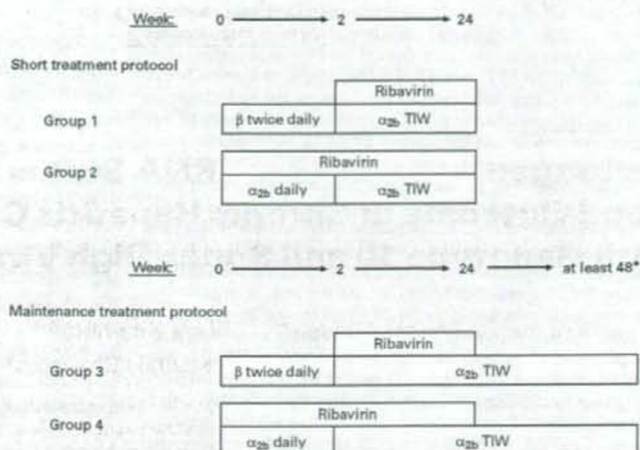

Fig. 1. Study design showing the two different protocols of IFN therapy. * Maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

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

charge explained the purpose and method of the clinical trial as well as potential adverse events during the twice-daily IFN-β induction. The physicians also explained the information including the result of clinical trials of combination therapy for 48 weeks in other countries, such as the SVR rate, HCV-RNA relapse rate and adverse events. After giving sufficient informed consent, the patients themselves decided whether or not to be treated by twice-daily IFN-β induction and also decided whether or not to be treated by additional IFN monotherapy to sustain a negative HCV-RNA result for 48 weeks. According to the patients' decision, four therapeutic groups were divided as follows:

Short Treatment Protocol: 40 patients were treated by this protocol for 24 weeks. 13 patients were treated by 3 MU of IFN-β twice-daily administered for 2 weeks followed by the combination therapy for 22 weeks (group 1). 27 patients were treated by the standard combination therapy for 24 weeks (group 2).

Maintenance Treatment Protocol: 38 patients were treated by this protocol. 13 patients were treated by 3 MU of IFN-β twice-daily administered for 2 weeks followed by the combination therapy (group 3). 25 patients were treated by the standard combination therapy (group 4). For consistency with current guidelines, patients who were HCV-RNA-positive by PCR at month 6 were removed from the study and considered as non-responders. The patients who had an undetectable HCV-RNA load in serum at month 6 had an additional minimum of 24 weeks' IFN monotherapy as maintenance treatment. The maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

The study was approved by the Institutional Review Boards of the participating clinical sites before study initiation, and the study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Virological Response to IFN

The virological response to IFN was determined by measuring serum HCV-RNA levels with the Amplicor HCV monitor assay at days 2, 3, 8, 15, 29 and every 28 days thereafter. Negative samples on the Amplicor HCV monitor assay were re-examined by the Amplicor qualitative assay, which has a detection limit of HCV-RNA of 0.2 KIU/ml. SVR was defined as a negative serum HCV-RNA during the 6 months following completion of IFN administration. All patients other than those with SVR were considered to be non-responders.

Histological Analysis

All patients underwent liver needle biopsy under sonographic guidance in the 3 months prior to the start of IFN administration. Baseline liver histology of chronic hepatitis was classified, based on the extent of fibrosis, into five stages (F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis)), and based on activity into four grades (A0 (no activity), A1 (mild activity), A2 (moderate activity), or A3 (severe activity)), according to the method of Desmet et al. [29].

Statistical Analysis

Baseline clinical characteristics were compared between the treatment groups using Fisher's exact test or the Mann-Whitney U-test. Treatment efficacy was analyzed by Fisher's exact test. p values <0.05 were considered statistically significant.

Results

Characteristics of the Patients

There were no significant differences in the general characteristics of the patients in demographic, biochemical, virological and histological features between the β-induction group (group 1) and standard combination group (group 2) in the short treatment protocol. There were no significant differences in the background characteristics between the β-induction group (group 3) and standard combination group (group 4) in the maintenance treatment protocol. Among the four therapeutic groups, background characteristics were also not significant, except the history of previous IFN monotherapies: the rate of previous IFN monotherapies in the short standard combination group (group 2) was significantly lower compared with other therapeutic groups ($p < 0.05$) (table 1).

HCV-RNA Clearance

HCV-RNA negativity and the week after starting therapy are shown in table 2. 96% (25/26) of the β-induction group (groups 1 and 3) had undetectable HCV-RNA load in serum 24 weeks after starting therapy. In comparison, 79% (41/52) of the standard combination group (groups 2 and 4) had undetectable HCV-RNA load in serum 24 weeks after starting therapy. There was a significant difference in the HCV-RNA status at 24 weeks between the β-induction group (groups 1 and 3) and the standard combination group (groups 2 and 4) ($p< 0.05$). Of the patients who received maintenance IFN monotherapy, 39% (5/13) in the β-induction group (group 3) and 43% (9/21) in the standard combination group (group 4) had detectable HCV-RNA during IFN monotherapy (breakthrough). The residual patients completed IFN monotherapy to sustain a negative HCV-RNA PCR profile for 48 weeks. In the patients with a negative HCV-RNA status for 48 weeks, 25% (2/8) in the β-induction group (group 3) and 33% (4/12) in the standard induction group (group 4) had re-appearance of HCV-RNA after IFN monotherapy. The periods of IFN maintenance monotherapy were 32.4 ± 6.2 weeks in the β-induction group (group 3) and 38.5 ± 6.9 weeks in the standard combination group (group 4) ($p < 0.05$).

HCV-RNA Dynamics and the Time of HCV-RNA Negativity

The first and second phase of HCV-RNA dynamics are shown in figure 2. An early significant decline in HCV-RNA was observed in the β-induction group (groups 1

Table 1. Baseline characteristics of the patients according to four therapeutic groups

	Short treatment protocol (n = 40)		Maintenance treatment protocol (n = 38)		p value
	β-induction group (group 1, n = 13)	standard combination group (group 2, n = 27)	β-induction group (group 3, n = 13)	standard combination group (group 4, n = 25)	
Mean age, years[a]	55.8 ± 5.6	54.6 ± 10.3	54.0 ± 9.2	56.7 ± 10.4	n.s.
Male:female	7:6	13:14	10:3	18:7	n.s.
Basal WBC, $\times 10^3/mm^3$	4.7 ± 1.4	4.5 ± 1.5	4.7 ± 1.3	4.9 ± 1.6	n.s.
Basal Hb, g/dl	14.4 ± 1.3	14.6 ± 1.0	15.2 ± 1.0	14.8 ± 1.1	n.s.
Basal ALT, IU/l[a]	72.4 ± 36.1	68.9 ± 31.7	73.8 ± 40.1	62.7 ± 26.2	n.s.
Platelets, $\times 10^4/mm^3$ [a]	16.4 ± 4.7	14.4 ± 4.4	16.7 ± 5.8	15.2 ± 3.7	n.s.
Serum HCV-RNA, KIU/ml	>500	>500	>500	>500	n.s.
Histological findings[b]					
Staging 0	0	0	0	0	n.s.
Staging 1	5	10	7	13	n.s.
Staging 2	4	7	3	8	n.s.
Staging 3	4	10	3	4	n.s.
Staging 4	0	0	0	0	n.s.
Grade 0	0	0	0	0	n.s.
Grade 1	4	9	4	10	n.s.
Grade 2	8	17	8	13	n.s.
Grade 3	1	1	1	2	n.s.
History of previous IFN monotherapies	6	5*	7	13	n.s.

[a] Data are mean ± SD. [b] Classified by the method of Desmet et al. [29]. n.s. = Not significant.
* The rate of previous IFN monotherapies in short standard combination group was significantly lower compared with other therapeutic groups ($p < 0.05$).

Table 2. HCV-RNA disappearance and the week after starting therapy

Weeks	β-Induction group (groups 1 and 3, n = 26)	Standard combination group (groups 2 and 4, n = 52)	p value
2	12% (3/26)	4% (2/52)	0.191
4	35% (9/26)	10% (5/52)	<0.01
8	62% (16/26)	29% (15/52)	<0.01
12	73% (19/26)	52% (27/52)	0.059
16	96% (25/26)	69% (36/52)	<0.01
20	96% (25/26)	77% (40/52)	<0.05
24	96% (25/26)	79% (41/52)	<0.05

and 3) on days 7 and 14 in the standard combination group (groups 2 and 4). Twice-daily administration of IFN-β accelerated HCV-RNA decline in the second phase against IFN/ribavirin combination therapy. As a result of early viral decline, HCV-RNA disappearance was attained in a shorter period in the β-induction group (groups 1 and 3) (table 2). That was significant with the standard combination group (groups 2 and 4) at weeks 4, 8, 16, 20 and 24. The mean time to the first negative HCV-RNA PCR result was 8.4 ± 6.2 weeks in the β-induction group (groups 1 and 3) and 14.5 ± 6.9 weeks in the standard combination group (groups 2 and 4) ($p < 0.01$).

Virological Response

Table 3 shows SVR rates. In patients who received the short treatment protocol, SVR was observed in 5 of 13 patients (38%) in the β-induction group (group 1) and in 3 of 27 patients (11%) in the standard combination group (group 2) ($p < 0.05$). In the patients who received the maintenance treatment protocol, SVR was observed in 6 of 13 patients (46%) in the β-induction group (group 3) and in 8 of 25 patients (32%) in the standard combination group (group 4) (NS).

Adverse Events

Table 4 summarizes the laboratory abnormalities and adverse events recorded for 24 weeks after initiation of IFN therapy. There were no patients with leukocyte counts $<1{,}000/mm^3$, hemoglobin concentrations