TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

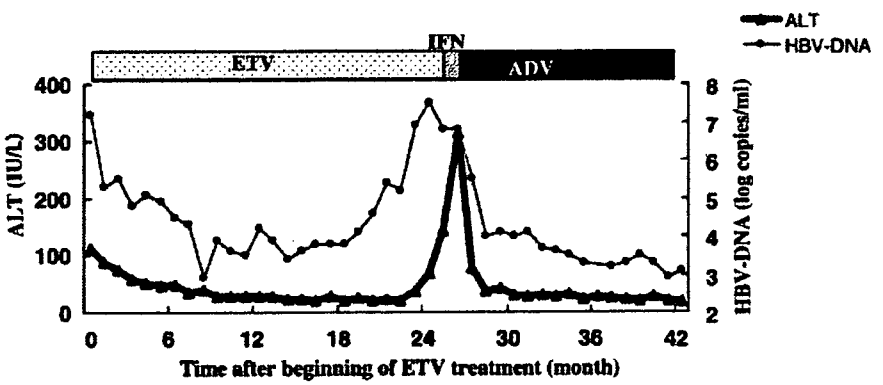

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

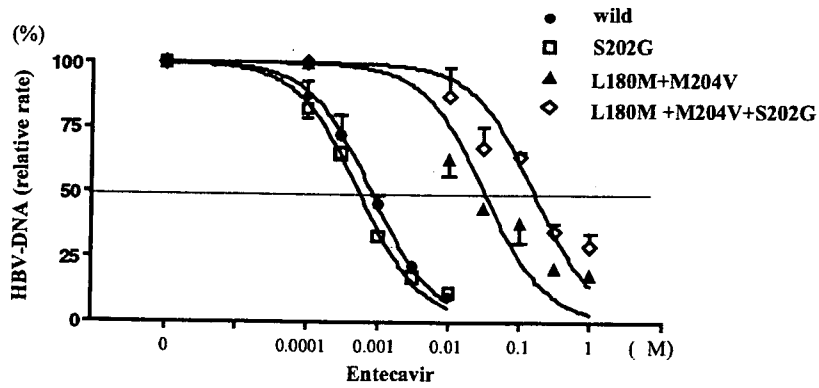

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,000**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

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REFERENCES

Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 27:1670–1677.

Bruix J, Llovet JM. 2003. Hepatitis B virus and hepatocellular carcinoma. J Hepatol 39:S59–S63.

Colonno RJ, Rose R, Baldick CJ, Leveine S, Pokornowski K, Yu CF, Walsh A, Fang J, Hsu M, Mazzucco C, Eggers B, Zhang S, Plym M, Klesczewski K, Tenney DJ. 2006. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44:1656–1665.

Delaney WE, Yang H, Westland CE, Das K, Arnold E, Gibbs CS, Miller MD, Xiong S. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J Virol 77:11833–11841.

Ganem D, Prince AM. 2004. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 350:1118–1129.

Gunther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, Will H. 1998. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: Frequency and functional consequences of PCR-introduced mutations. J Clin Microbiol 36:531–538.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. 1998. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 339:61–68.

Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 36:687–696.

Lee YS, Suh DJ, Lim YS, Jung SW, Lee HC, Chung YH, Lee YS, Yoo W, Kim SO. 2006. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology 43:1385–1391.

Levine SD, Hernandez G, Yamanaka S, Zhang R, Rose R, Weinheimer S, Colonno RJ. 2002. Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob Agents Chemother 46:2525–2532.

Maecellin P, Asselah T. 2005. Resistance to adefovir: A new challenge in the treatment of chronic hepatitis B. J Hepatol 43:920–923.

Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC. 1997. Lamivudine therapy for chronic hepatitis B: A six-month randomized dose-ranging study. Gastroenterology 113:1258–1263.

Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, Carrilho FJ, Omata M. 2001. The polymerase L528M mutation cooperates

with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 107:449–455.

Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. 2007. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. Hepatology 45:307–313.

Sherman MC, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska, A, Martin P, Goodman Z, Colonno R, Cross A, Denisky G, Kreter B, Hindes R. 2006. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology 130:2039–2049.

Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 30:743–748.

Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno RJ. 2004. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrob. Agents Chemother. 48:3498–3507.

Tenney DJ, Rose RE, Baldick CJ, Levine SM, Pokornowski KA, Walsh AW, Yu CF, Zhang S, Mazzucco CE, Eggers B, Hsu M, Plym MJ, Poundstone P, Yang J, Colonno RJ. 2007. Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. Antimicrob Agents Chemother 51:902–911.

Villet S, Ollivet A, Pichoud C, Barraud L, Villeneuve JP, Trepo C, Zoulim F. 2007. Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. J Hepatol 46:531–538.

Yim HJ, Hussain M, Liu Y, Wong SN, Fung SK, Lok SF. 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 44:703–712.

Serum HBV RNA is a Predictor of Early Emergence of the YMDD Mutant in Patients Treated with Lamivudine

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Lamivudine (LAM) is a nucleoside analogue widely used for the treatment of chronic hepatitis B virus (HBV) infection. Emergence of resistant strains with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of reverse transcriptase is a serious problem in patients on LAM therapy. The amount of covalently closed circular DNA in the serum is reported to be higher in patients who develop YMDD mutants than in those without mutants. However, there is no useful serum marker that can predict early emergence of mutants during LAM therapy. Analysis of patients who were treated with entecavir (n = 7) and LAM (n = 36) showed some patients had high serum levels of HBV RNA. Median serum levels of HBV RNA were significantly higher in patients in whom the YMDD mutant had emerged within 1 year (n = 6, 1.688 log copies/ml) than in those in whom the YMDD mutant emerged more than 1 year after treatment (n = 12, 0.456 log copies/ml, $P = 0.0125$) or in whom the YMDD mutant never emerged (n = 18, 0.688 log copies/ml, $P = 0.039$). Our results suggest that HBV RNA is a valuable predictor of early occurrence of viral mutation during LAM therapy. (HEPATOLOGY 2007;45:1179-1186.)

The hepatitis B virus (HBV) is a member of the hepadnaviridae family. Worldwide, approximately 350 million people are estimated to be chronically infected with HBV.[1] Patients with chronic HBV infection develop chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for approximately 1 million deaths per year.[2] Recently, inhibitors of reverse transcriptase have been developed and widely used for patients with chronic HBV infection. Lamivudine (LAM), a cytosine nucleoside analogue, was first developed as an antiviral agent against HIV and later was used effectively against HBV because HBV also uses reverse transcriptase for replication.[3,4] Because LAM suppresses HBV replication, patients who are treated with LAM show a decreased level or disappearance of HBV DNA in serum and hepatitis B e antigen, normalization of serum alanine aminotransferase (ALT) level, and histological improvement.[5-12] However, discontinuation of therapy often leads to reactivation of HBV.[6,8,13,14] Therefore, long-term therapy is necessary for many patients with chronic HBV infection. During long-term LAM therapy, drug-resistant mutants with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif emerge, resulting in expression of HBV DNA increasing again and in worsening of hepatitis.[6,10,15-18] Moreover, some patients develop a severe flare-up of hepatitis that could lead to fatal hepatic failure. Therefore, prediction of the emergence of YMDD mutants is an important issue.

In our hunt for useful serum markers to detect the early emergence of YMDD mutants, we noticed some patients who showed a discrepancy in the expression of HBV DNA measured by the transcription-mediated amplifica-

Abbreviations: cccDNA, covalently closed circular DNA; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; PCR, polymerase chain reaction; RT, reverse transcription; TMA-HPA, transcription-mediated amplification and hybridization protection assay; YMDD, tyrosine-methionine-aspartate-aspartate.

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Table 1. Clinical Characteristics of the 3 Groups

	Group A	Group B	Group C
Number	6	12	18
Age, median (range)	50 (37-67)	49 (31-66)	49 (27-68)
Sex (M:F)	3:3	9:3	13:5
Observation period (months)	34.5 (13-58)	38 (16-64)	34 (13-58)
Time before emergence of mutants (months)	8.5 (4-11)	19 (13-36)	
HBV DNA (LGE/ml)	7.8 ± 0.95	6.13 ± 0.84	6.64 ± 1.63
Hbe-antigen-positive	4 (66.7%)	6 (50%)	10 (55.6%)
Hbe-antibody-positive	1 (16.7%)	6 (50%)	9 (50%)
ALT (U/l)	136.1 ± 122.8	114.5 ± 104.1	129.8 ± 206.4

Group A: patients who showed early emergence of the mutants (within 1 year).
Group B: patients who developed resistance after 1 year of LAM therapy.
Group C: patients in whom mutants did not develop.

tion and hybridization protection assay (TMA-HPA) and that measured by the Amplicor HBV Monitor test. Because the former method detects both HBV DNA and HBV RNA, we thought that the difference in measurement by the 2 methods was a result of the presence of a large amount of HBV RNA.[19-21] We thus studied patients with chronic HBV infection who were being treated with LAM or entecavir (ETV) for the presence of HBV RNA. We also assumed that the presence of a large amount of HBV RNA would indicate that transcription and virus particle formation were still active in such patients. We thus assessed the value of this indicator in the prediction of the emergence of YMDD mutants during LAM therapy.

Patients and Methods

Patients. We studied 36 patients with chronic hepatitis B who were being treated with LAM from 2001 to 2006 at Hiroshima University Hospital, Kawakami Clinic, and Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital. We also analyzed 7 patients who were being treated with ETV from 2004 to 2006 at Hiroshima University Hospital. No patients showed clinical signs of cirrhosis or hepatocellular carcinoma. They were not treated with other antiviral agents, corticosteroids, or immunosuppressant drugs during LAM/ETV therapy. The LAM-treated patients were 25 men and 11 women whose median age was 52 years (range 27-68 years; Table 1). They were divided into 3 groups (groups A, B, and C) according to how long it took for YMDD mutants to appear. Group A (n = 6) was composed of patients who showed early emergence of the mutants (within 1 year); group B (n = 12) had patients who developed resistance after 1 year of LAM therapy; and group C (n = 18) was composed of patients who did not show resistance to LAM therapy. Each of the 36 patients received 100 mg of LAM daily for 4-58 months (median, 21.5 months). All patients continued LAM therapy throughout the course of the study. Patients in the ETV group were 6 men and 1 woman whose median age was 37 years (32-50 years). They received 0.01-0.5 mg of ETV daily for 21-28 months (median, 25 months), and all patients continued ETV therapy throughout the course of the study. Blood samples were obtained from patients of both groups just before commencement of antiviral therapy and every 4 weeks during therapy. Informed consent was obtained from each patient.

Quantification of HBV DNA. HBV DNA serum level was determined by using the TMA-HPA (Fujirebio Inc., Tokyo, Japan) and the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the former assay is $10^{3.7}$-$10^{8.7}$ genome equivalents (GE)/ml (3.7-8.7 LGE/ml),[22] whereas the range of the latter test was $10^{2.6}$-$10^{7.6}$ copies/ml (2.6-7.6 log copies/ml).[23] These quantitative assays of HBV DNA were performed at the Special Reference Laboratory (Tokyo, Japan).

Extraction of Nucleic Acid of HBV and Reverse Transcription. Nucleic acid was extracted from 100 μL of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL of H_2O for DNA analysis or 8.8 μL of ribonuclease-free H_2O for RNA analysis. The latter solution was reverse-transcribed by using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan). In the next step, 25 pM of random primer was added to 8.8 μL of nucleic acid extract and heated at 65°C for 5 minutes. The samples were set on ice for 5 minutes. Then 4 μL of 5× reverse transcription (RT) buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor, and 100 units of M-MLV reverse transcriptase were added to each sample. The reaction mixture was incubated at 30°C for 10 minutes and 42°C for 60 minutes, followed by inactivation at 99°C for 5 minutes.

Quantitative Analysis of HBV DNA by Real-Time Polymerase Chain Reaction. One microliter of DNA solution or cDNA solution was amplified by real-time polymerase chain reaction (PCR) with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. Amplification was performed in a 25-μL reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of forward primer (5′-TTTGGGGCATGGACATTGAC-3′, nucleotides 1893-1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029-2049), and 1 μL of DNA or cDNA solution. After incubation for 2 minutes at 50°C, the sample was heated for 10 minutes at 95°C for denaturing, followed by a PCR cycling program consisting of 40 2-step cycles of 15 seconds at 95°C and 60 seconds at 60°C. The lower detection limit of this assay was 10^3 copies/ml.

Confirmation of Presence of HBV RNA in Serum by RNase Digestion. To confirm the presence of HBV RNA, nucleic acid extracted from the serum samples by SMITEST (Genome Science Laboratories, Tokyo) was digested with 1 μg/μL of RNase A (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 60 minutes, digested with proteinase K (New England Biolabs Inc., Ipswich, MA) at 37°C for 60 minutes, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in water. Treated nucleic acid with or without RNase was analyzed by real-time PCR after reverse transcription with a random primer and reverse transcriptase, as already described.

Detection of YMDD Mutant. Mutations in the YMDD motif of reverse transcriptase of HBV were examined by PCR with peptide nucleic acid clamping, as described previously.[24]

Statistical Analysis. Differences between groups were examined for statistical significance using the Student t test, and correlations of parameters were examined by the Spearman's rank correlation. A difference with a P value less than 0.05 was considered statistically significant. All statistical analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC).

Results

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test During ETV Therapy. High expression of HBV RNA was initially observed by measuring HBV nucleic acid with the TMA-HPA and HBV DNA with the Amplicor HBV monitor test. As shown in Fig. 1, expression of HBV nucleic acid was higher than HBV DNA during the initial 6 months of ETV therapy. We assumed that the discrepancy in the measurements by these 2 methods was a result of the large amount of HBV RNA in the serum because the TMA-HPA measures both HBV DNA and HBV RNA, whereas the Amplicor HBV monitor test detects only HBV DNA. We measured the HBV nucleic acid levels in the 7 patients who received ETV therapy 3 and 6 months after the start of therapy. The HBV nucleic acid levels of all 7 patients determined by the TMA-HPA were 10-100 times higher than those determined by the Amplicor HBV Monitor test except for 2 patients who received a small amount (0.01 mg) of ETV (Fig. 2). The small dif-

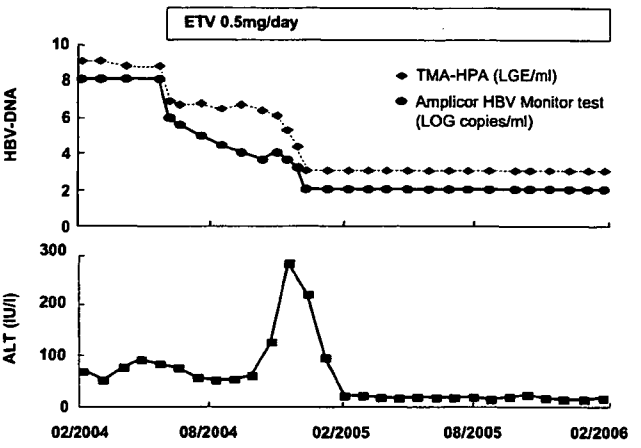

Fig. 1. Time courses of serum HBV DNA and ALT levels of patients treated with ETV. Expression of HBV nucleic acids determined by the TMA-HPA was higher than that determined by the Amplicor HBV Monitor test soon after beginning administration of ETV. The discrepancy was less marked when both measurements were low and when both were negative.

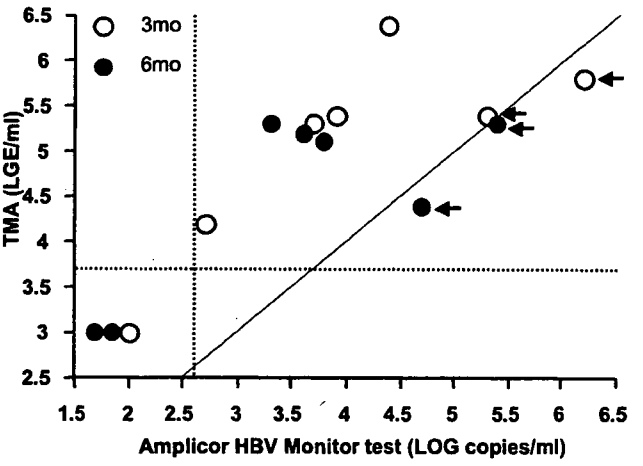

Fig. 2. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during ETV therapy. Serum samples obtained from the 2 patients who received low-dose ETV (0.01 mg) are indicated by arrows. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

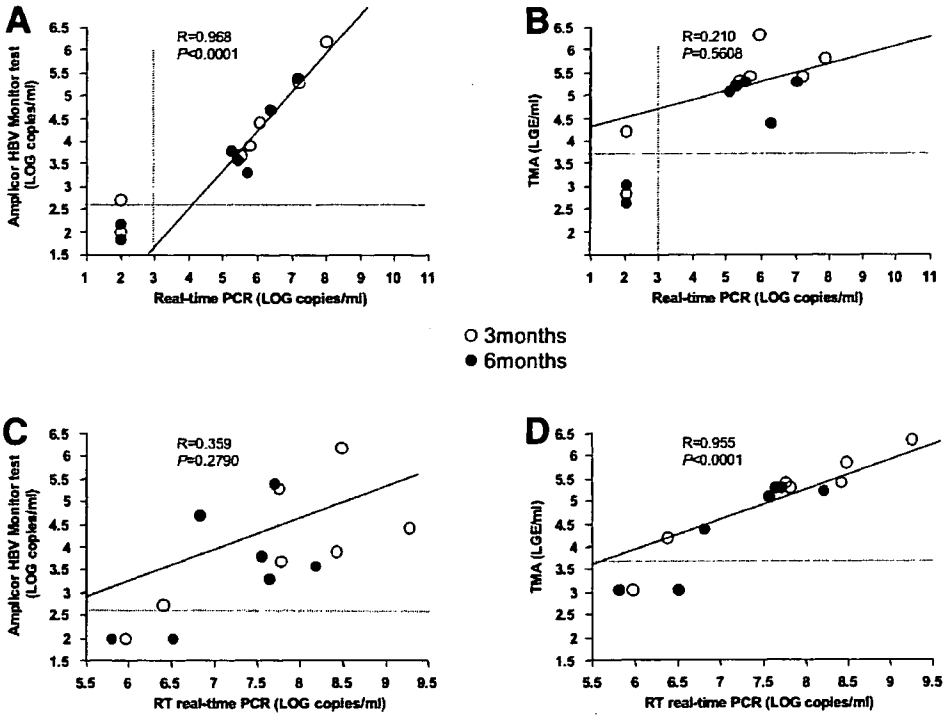

Fig. 3. Correlation between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of ETV therapy. (A) Correlation between HBV DNA level determined by Amplicor HBV Monitor test and that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA and of HBV DNA determined by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined by real-time RT-PCR. (D) Correlation of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

ference in nucleic acid level of these patients is probably a result of the small effect of the small amount of ETV.

Comparisons of HBV Nucleic Acid and DNA Values Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with ETV. We measured HBV DNA by in-house real-time PCR and HBV nucleic acid by real-time RT-PCR using serum samples obtained from the patients after 3 and 6 months of ETV therapy and compared these values with those obtained by the TMA-HPA and the Amplicor monitor test. HBV DNA determined by real-time PCR correlated well with that obtained by the Amplicor HBV Monitor test ($r = 0.968$, $P < 0.0001$; Fig. 3A), but not with HBV nucleic acid determined by the TMA-HPA ($r = 0.210$, $P = 0.5608$; Fig. 3B). Expression of HBV DNA determined by the in-house real-time PCR assay was $10^{1.5}$-10^{2} higher than that determined by the Amplicor HBV Monitor test. We confirmed the accuracy of our assay using limiting dilution and detection with nested PCR assay. When we diluted the standard samples used in our in-house assay to 1 copy/μL, we detected them by nested PCR using 1 μL of such samples. Three of the 10 (30%) samples tested positive by nested PCR. We thus conclude that our assay accurately measure the amount of HBV DNA in serum.

To examine if measurement by the TMA-HPA reflected the total amount of HBV RNA and HBV DNA in serum samples, we performed real-time RT-PCR using serum samples obtained from patients after 3 and 6 months of ETV therapy. In contrast to the values determined by real-time PCR without RT, the measurement of HBV nucleic acid determined by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test ($r = 0.359$, $P = 0.2790$; Fig. 3C), but did correlate well with that obtained with the TMA-HPA ($r = 0.955$, $P < 0.0001$; Fig. 3D). These results show that the TMA-HPA measures both HBV DNA and HBV RNA in serum. To further confirm the presence of HBV RNA, we digested 3 nucleic acid samples arbitrarily picked from serum samples obtained from patients treated by lamivudine for 3 months, by RNase A. As shown in Fig. 4, RNase treatment reduced the amount of HBV DNA detected by real-time RT-PCR to about 1% of that originally detected.

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test during LAM Therapy. We then investigated the levels of HBV DNA in serum samples obtained from 36 patients after 3 and 6 months of LAM therapy. In some patients, HBV DNA was already negative after 3 and 6 months of therapy (Fig. 5). Similar to the results obtained from patients treated with ETV, comparisons of values obtained from patients who showed measurable HBV DNA levels revealed that HBV nucleic acid levels determined by the TMA-HPA tended to be higher than those determined by the Amplicor HBV Monitor test (Fig. 4).

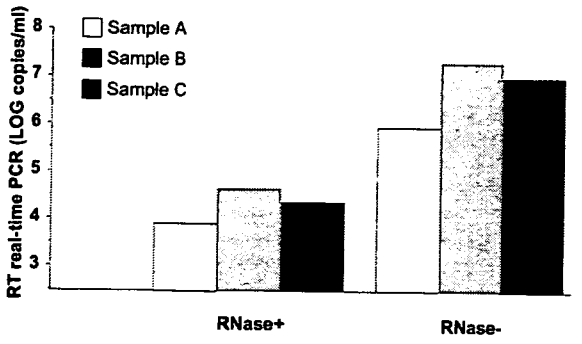

Fig. 4. Presence of HBV RNA confirmed by RNA treatment of 3 nucleic acid samples (samples A-C) obtained from patients after 3 months of LAM therapy. Extracted nucleic acid samples with or without RNase digestion were further digested by proteinase K and ethanol-precipitated after phenol/chloroform extraction. The amount of HBV DNA in each sample was then measured by real-time RT-PCR.

Comparisons of HBV Nucleic Acid Values and HBV DNA Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with LAM. We measured HBV nucleic acid and DNA levels by the same 4 methods and investigated the correlations between them after 3 and 6 months of LAM therapy (Fig. 6). HBV DNA levels determined by real-time PCR correlated better with those determined by the Amplicor HBV Monitor test (r = 0.653, P = 0.0083; Fig. 6A) than with those determined by the TMA-HPA (r = 0.456, P = 0.1173; Fig. 6B). Similarly, measurement of HBV nucleic acid by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test (Fig. 6C), but showed better correlation with that obtained by the TMA-HPA (r = 0.452, P = 0.0907, and r = 0.675, P = 0.0114, respectively; Fig. 6D). These results also showed that the TMA-HPA detects both HBV RNA and HBV DNA.

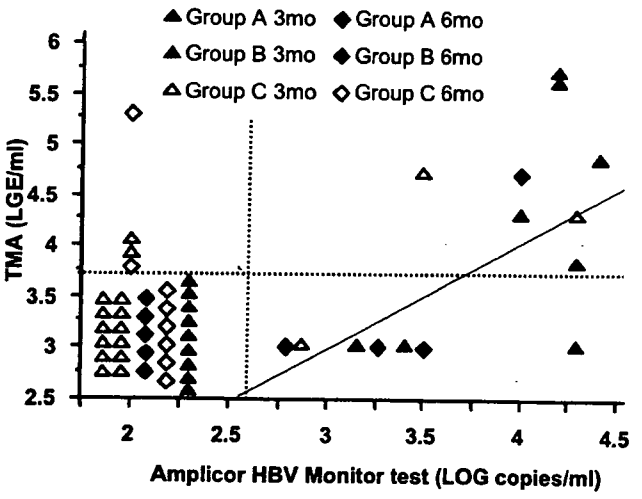

Fig. 5. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during LAM therapy. During ETV therapy the TMA-HPA showed higher expression of HBV DNA in patients regardless of the presence of the mutation than did the Amplicor HBV Monitor test. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

HBV RNA in Serum after 3 Months of LAM Therapy Is Higher in Patients Who Showed Early Emergence of YMDD Mutants. In LAM-treated patients, it was assumed that a high serum level of HBV RNA was a marker of the active transcription form of covalently closed circular DNA (cccDNA) and packaging of HBV RNA in the liver. We assumed that YMDD mutants easily emerged under such condition. We compared HBV RNA values (HBV nucleic acid determined by real-time RT-PCR minus HBV DNA determined by real-time PCR) in patients who showed early emergence of mutants (within 12 months) with those who showed late emergence of mutants (more than 12 months) and those who did not show emergence of mutants (Table 1). As shown in Fig. 7, HBV RNA levels were significantly higher in patients who showed early emergence of mutants than the other 2 groups after 3 months of LAM therapy. There was no significant difference in the amount of HBV RNA between group A (patients who showed emergence of mutants within 12 months) and the other 2 groups at the beginning of LAM therapy (data not shown).

Discussion

In this study, we addressed the discrepant measurements of HBV nucleic acid by the TMA-HPA and the Amplicor Monitor test. The presence of HBV RNA in serum samples of patients with HBV infection has been previously reported.[19-21] Because the TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase,[22] we assumed that the discrepancy was a result of the presence of HBV RNA in the serum of LAM- and ETV-treated patients. The presence of HBV RNA in a patient treated with LAM was reported previously.[21] In that report, the authors mainly analyzed truncated HBV RNA, which they assumed was transcribed from the integrated genome.[20, 21] They showed a large difference between HBV DNA and truncated HBV RNA, which did not decrease during LAM therapy. We also detected HBV DNA and HBV nucleic acid by real-time PCR and real-time RT-PCR. The values determined by these 2 methods showed less than a 1 log difference (data not shown); we assume that the effect of truncated HBV RNA in serum was only minimal in our study. As we demonstrated in this study, HBV nucleic acid measured by real-time RT-PCR correlated with that determined by the TMA-HPA. This finding suggests that the

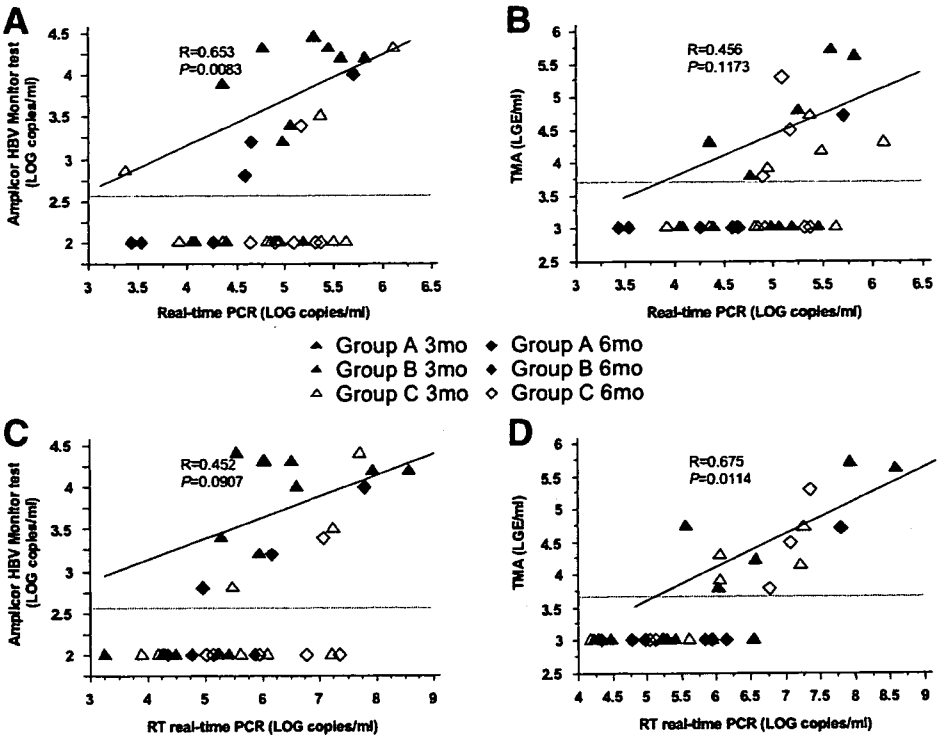

Fig. 6. Correlations between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of LAM therapy. (A) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA with HBV DNA by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined real-time RT-PCR. (D) Correlations of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

discrepancy in the values measured by the TMA-HPA and the Amplicor Monitor test is a result of the presence of HBV RNA in the serum.

We showed that a large amount of HBV RNA in the serum was produced during the early stage of ETV (Fig. 1) and LAM treatments (within 6 months). Because ETV and LAM work only on reverse transcription, it is difficult to conceive that the level of transcription from the cccDNA was altered by these drugs. Thus, the slow decrease in HBV RNA seems to reflect that a certain amount of cccDNA still existed in the liver and that the virus replication machinery was still actively operational. This is consistent with previous reports that showed that the amount of cccDNA in the liver tissues[25, 26] and in serum,[26] which correlated well with intrahepatic cccDNA,[27] reflected the effect of LAM and is a marker for cessation of therapy without viral level increasing again after stopping the therapy.

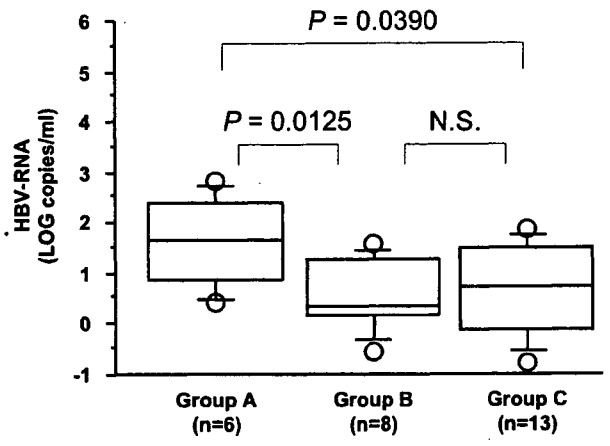

Fig. 7. Box plots of HBV RNA levels of patients in group A (patients who showed emergence of the mutants within 1 year, group B (those who developed resistance after 1 year of LAM therapy), and group C (patients who did not show resistance to LAM therapy). HBV RNA level represents the difference between HBV nucleic acid level determined by real-time RT-PCR minus HBV DNA level determined by in-house real-time PCR. Nine samples that tested negative for in-house real-time PCR were omitted from the analysis (4 samples of group B and 5 samples of group C).

Whether a large amount of HBV RNA originates from a large amount of cccDNA template in hepatocytes or from active transcription (or both) is actually unknown. However, it is assumed that the probability of developing mutants is high in patients who have large amounts of HBV RNA. We thus analyzed the amount of HBV RNA in patients treated with LAM and compared it in patients who showed early emergence of mutants and those who did not. As expected, the amount of HBV RNA in the serum was significantly higher in patients who showed early emergence of mutants than in those who showed late emergence and those who did not show emergence of mutants.

Using complex analysis, previous studies identified several factors predictive of emergence of YMDD mutants such as HBV genotype,[28] ALT level,[29, 30] HBV DNA level

before therapy,[28,30-32] degree of decline of HBV DNA level during therapy,[33,34] presence of hepatitis B e antigen,[17,29,31,32,35] presence of core promoter mutations,[36] deletion of pre-S region,[37] and HBV core–related antigen.[38] We also showed that a slow decrease in HBV nucleic acid measured by the TMA-HPA is a marker of early emergence of mutants. Our finding is important because this assay is routinely used in daily clinical practice. However, the results did not reach statistical significance, probably because of the small number of patients analyzed in our study and the low sensitivity of the assay (detection limit 3.7 log copies/ml). We assume that a sensitive measurement of HBV RNA is useful for predicting the emergence of mutants. Development of such an assay is needed for the proper treatment of patients using different nucleotide and nucleoside analogues. Mechanisms that control transcription of HBV from cccDNA deserve further investigation in order to develop more effective therapies for HBV infection.

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References

1. Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:362-366.
2. Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 1988;61:1942-1956.
3. Chang CN, Skalski V, Zhou JH, Cheng YC. Biochemical pharmacology of (+)-and (+)- 2′,3′-dideoxy-3′-thiacytidine as anti-hepatitis B virus agents. J Biol Chem 1992;267:22414-22420.
4. Benhamou Y, Dohin E, Lunel-Fabiani F, Poynard T, Huraux JM, Katlama C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995;345:396-397.
5. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657-1661.
6. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61-68.
7. Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, et al. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 1999;30: 743-748.
8. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-1263.
9. Dienstag JL, Schiff ER, Mitchell M, Casey DE Jr, Gitlin N, Lissoos T, et al. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. HEPATOLOGY 1999; 30:1082-1087.
10. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, et al. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000;119:172-180.
11. Suzuki Y, Arase Y, Ikeda K, Saitoh S, Tsubota A, Suzuki F, et al. Histological improvements after a three-year lamivudine therapy in patients with chronic hepatitis B in whom YMDD mutants did not or did develop. Intervirology 2003;46:164-170.
12. Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003;124:105-117.
13. Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, et al. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology 1997;113:1258-1263.
14. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. HEPATOLOGY 2000;32:803-806.
15. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. HEPATOLOGY 1996;24:711-713.
16. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA–dependent DNA polymerase confers resistance to lamivudine *in vivo*. HEPATOLOGY 1996;24:714-717.
17. Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. HEPATOLOGY 1998;27:1711-1716
18. Lau DT, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, et al. Long-term therapy of chronic hepatitis B with lamivudine. HEPATOLOGY 2000;32:828-834.
19. Köck J, Theilmann L, Galle P, Schlicht HJ. Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus. HEPATOLOGY 1996;23:405-413.
20. Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. Clin Cancer Res 2001;7:2005-2015
21. Zhang W, Hacker HJ, Tokus M, Bock T, Schröder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. J Med Virol 2003;71:24-30.
22. Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, et al. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 1999;37: 310-314.
23. Ranki M, Schätzl HM, Zachoval R, Uusi-Oukari M, Lehtovaara P. Quantification of hepatitis B virus DNA over a wide range from serum for studying viral replicative activity in response to treatment and in recurrent infection. HEPATOLOGY 1995;21:1492-1499.
24. Ohishi W, Chayama K. Rare quasispecies in the YMDD motif of hepatitis B virus detected by polymerase chain reaction with peptide nucleic acid clamping. Intervirology 2003;46:355-361.
25. Sung JJ, Wong ML, Bowden S, Liew CT, Hui AY, Wong VW, et al. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterology 2005;128: 1890-1897.
26. Yuen MF, Wong DK, Sum SS, Yuan HJ, Yuen JC, Chan AO, et al. Effect of lamivudine therapy on the serum covalently closed-circular (ccc) DNA of chronic hepatitis B infection. Am J Gastroenterol 2005;100:1099-1103.
27. Wong DK, Yuen MF, Yuan H, Sum SS, Hui CK, Hall J, et al. Quantification of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. HEPATOLOGY 2004;40:727-737
28. Zollner B, Petersen J, Puchhammer-Stockl E, Kletzmayr J, Sterneck M, Fischer L, et al. Viral features of lamivudine resistant hepatitis B genotypes A and D. HEPATOLOGY 2004;39:42-50.
29. Nafa S, Ahmed S, Tavan D, Pichoud C, Berby F, Stuyver L, et al. Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. HEPATOLOGY 2000;32:1078-1088.

30. Yuen MF, Sablon E, Hui CK, Yuan HJ, Decreamer H, et al. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. HEPATOLOGY 2001;34:785-791.
31. Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 2003;46:182-189.
32. Sun J, Wang Z, Ma S, Zeng G, Zhou Z, Luo K, et al. Clinical and virological characteristics of lamivudine resistance in chronic hepatitis B patients: A single center experience. J Med Virol 2005;75:391-398.
33. Puchhammer-Stockl E, Mandl CW, Kletzmayr J, Holzmann H, Hofmann A, Aberle SW, et al. Monitoring the virus load can predict the emergence of drug-resistant hepatitis B virus strains in renal transplantation patients during lamivudine therapy. J Infect Dis 2000;181:2063-2066.
34. Zollner B, Schafer P, Feucht HH, Schroter M, Petersen J, Laufs R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J Med Virol 2001;65: 659-663.
35. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
36. Lok AS, Hussain M, Cursano C, Margotti M, Gramenzi A, Grazi GL, et al. Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e antigen-negative patients receiving lamivudine therapy. HEPATOLOGY 2000;32:1145-1153.
37. Tanaka Y, Yeo AE, Orito E, Ito K, Hirashima N, Ide T, et al. Prognostic indicators of breakthrough hepatitis during lamivudine monotherapy for chronic hepatitis B virus infection. J Gastroenterol 2004;39:769-775.
38. Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, et al. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. Liver Int 2006;26:90-96.

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Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**

Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAGCGTCTAGC-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 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGGCTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

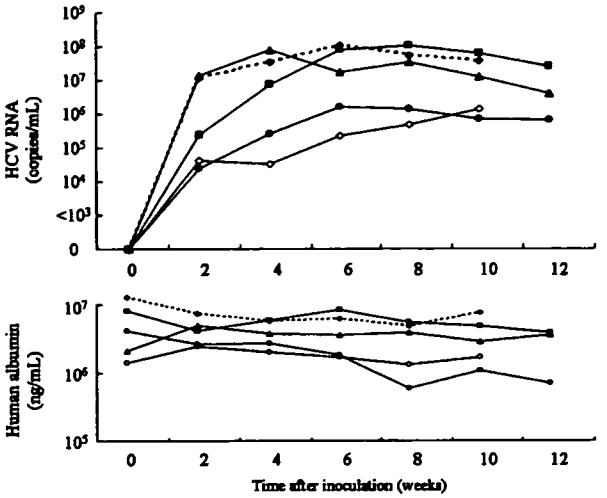

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).

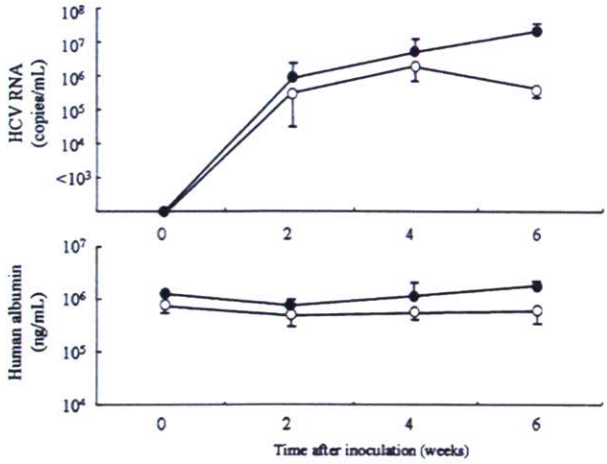

Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean ± S.D.

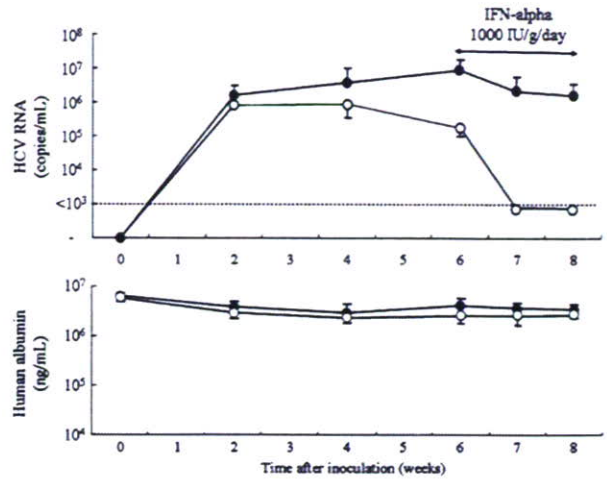

Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μl) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean ± S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment reduced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

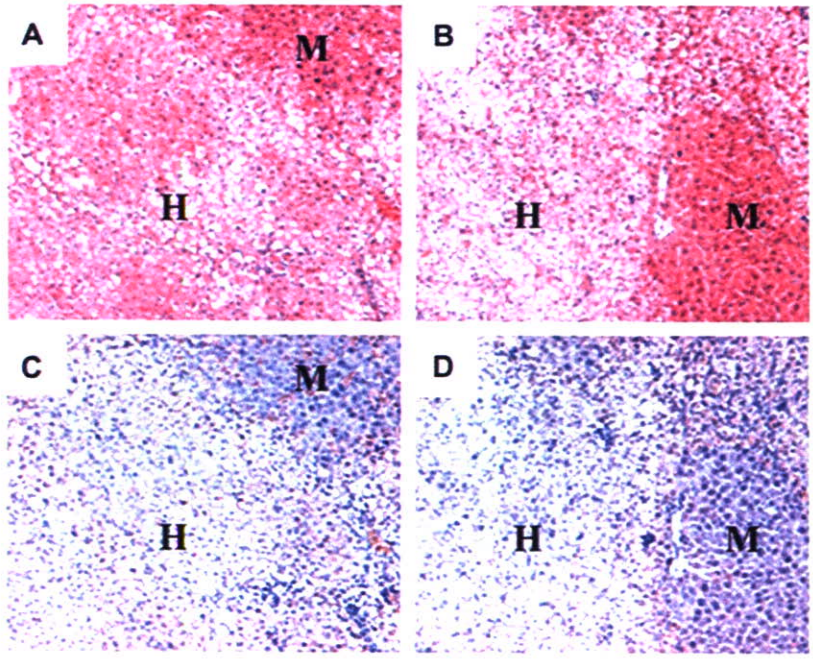

Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, ×100.)

human hepatocytes in mice infected with each genotype after 2-week IFN-treatment (Fig. 4). These results suggest that the decrease in HCV is due to the direct anti-viral effect of IFN and not induced by liver cell damage. The difference in the virus titer and susceptibility to IFN are considered to be due to the characteristics of the genotypes.

4. Discussion

In this study, we established a reverse genetics system of HCV genotype 1a and 2a clones using human hepatocyte chimeric mice. The HCV genotype 2a clone, JFH-1, has remarkable features, i.e., infects cultured Huh7 cell line as well as establish infection in chimeric mouse [7]. It has been reported that HCV genotype 1a clone, H77-S, also infects Huh7 cell line and produces infectious virion [14]. In the present study, we intrahepatically inoculated genotype 1a infectious clone, CV-H77C. As reported in chimpanzee [13,15–17], we were able to establish genotype 1a infection using human hepatocyte chimeric mice. Using this technique, it is hoped that we can conduct further experiments in the future using genetically engineered HCV clones. Experiments using chimeric clone described by Lindenbach et al. [7] should also provide further information regarding the variable replication property of HCV genomes. Modifying genomes with nucleotide substitutions allowed examination of the functions of HCV peptides as we showed with HBV [12].

As reported recently by Kneteman et al. [10], the mouse model system is useful for evaluating the effect of anti-HCV drugs such as IFN, protease inhibitors and polymerase inhibitors. As we showed in this study, the response to IFN therapy varied according to HCV genotype. Further experiments are necessary to determine whether differences in response to IFN are due to the different replication ability (replication level of genotype 2a clone was slightly lower than that of genotype 1b, see Figs. 2 and 3) or differences in genotypes, as has been reported in clinical studies [18]. As we showed in this study (Fig. 4), there is no hepatocyte damage or inflammation in the liver of the infected chimeric mouse. Thus, this model is suitable for the study of mechanisms involved in HCV replication and IFN resistance.

The intrahepatic injection method used in this study simplified our experiments using genetically engineered virus. This is particularly important in studies of protease inhibitors and polymerase inhibitors because HCV will easily develop resistance against these small molecule agents.

Previous studies identified amino acid sequences that correlate with different susceptibilities of genotype 1b HCV against IFN therapy, namely, interferon sensitivity determining region [19] and the PKR-eIF2 phosphorylation homology domain [20,21]. To elucidate such issues, we are currently trying to establish genotype 1b infection system using the method described in this paper.

In summary, we showed in the present study the successful application of a genetically engineered HCV in human hepatocyte chimeric mice. Using this mouse model, we showed that genotypes 1a and 2a HCV clones exhibit different susceptibilities to IFN-alpha therapy. Our mouse model seems useful for the study of HCV virology and resistance of HCV against IFN and for the development of new anti-HCV therapy.

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References

[1] WHO. (1999) Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J. Viral. Hepat. 6, 35–47.

[2] Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K. and Purcell, R.H. (1990) Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12, 671–675.

[3] Niederau, C., Lange, S., Heintges, T., Erhardt, A., Buschkamp, M., Hurter, D., Nawrocki, M., Kruska, L., Hensel, F., Petry, W. and Haussinger, D. (1998) Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 28, 1687–1695.

[4] Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales Jr., F.L., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. and Yu, J. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347, 975–982.

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

[6] Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R. and Liang, T.J. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

[7] Lindenbach, B.D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A.J., McKeating, J.A., Lanford, R.E., Feinstone, S.M., Major, M.E., Leroux-Roels, G. and Rice, C.M. (2006) Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. USA 103, 3805–3809.

[8] Shimizu, Y.K., Weiner, A.J., Rosenblatt, J., Wong, D.C., Shapiro, M., Popkin, T., Houghton, M., Alter, H.J. and Purcell, R.H. (1990) Early events in hepatitis C virus infection of chimpanzees. Proc. Natl. Acad. Sci. USA 87, 6441–6444.

[9] Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L. and Kneteman, N.M. (2001) Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7, 927–933.

[10] Kneteman, N.M., Weiner, A.J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z.J., Zhu, Q., Hashash, A., Kline, J., His, B., Schiller, D., Douglas, D., Tyrrell, D.L. and Mercer, D.F. (2006) Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. Hepatology 43, 1346–1353.

[11] Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T. and Yoshizato, K. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165, 901–912.

[12] Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y., Ochi, H., Chayama, K., Tateno, C. and Yoshizato, K. (2005) Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology 42, 1046–1054.

[13] Yanagi, M., Purcell, R.H., Emerson, S.U. and Bukh, J. (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc. Natl. Acad. Sci. USA 94, 8738–8743.

[14] Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T. and Lemon, S.M. (2006) Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA 103, 2310–2315.
[15] Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M. and Rice, C.M. (1997) Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 277, 570–574.
[16] Yanagi, M., StClaire, M., Emerson, S.U., Purcell, R.H. and Bukh, J. (1999) In vivo analysis of the 3′ untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. Proc. Natl. Acad. Sci. USA 96, 2291–2295.
[17] Beard, M.R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D.V. and Lemon, S.M. (1999) An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. Hepatology 30, 316–324.
[18] McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S. and Albrecht, J.K. (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N. Engl. J. Med. 339, 1485–1492.
[19] Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F. and Sato, C. (1996) Mutations in the non-structural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. 334, 77–81.
[20] Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N. and Lai, M.M. (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285, 107–110.
[21] Chayama, K., Suzuki, F., Tsubota, A., Kobayashi, M., Arase, Y., Saitoh, S., Suzuki, Y., Murashima, N., Ikeda, K., Takahashi, N., Kinoshita, M. and Kumada, H. (2000) Association of amino acid sequence in the PKR-eIF2 phosphorylation homology domain and response to interferon therapy. Hepatology 32, 1138–1144.

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Humanization of Excretory Pathway in Chimeric Mice with Humanized Liver

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The liver of a chimeric urokinase-type plasminogen activator (uPA)$^{+/+}$/severe combined immunodeficient (SCID) mouse line recently established in Japan could be replaced by more than 80% with human hepatocytes. We previously reported that the chimeric mice with humanized liver could be useful as a human model in studies on drug metabolism and pharmacokinetics. In the present study, the humanization of an excretory pathway was investigated in the chimeric mice. Cefmetazole (CMZ) was used as a probe drug. The CMZ excretions in urine and feces were 81.0 and 5.9% of the dose, respectively, in chimeric mice and were 23.7 and 59.4% of the dose, respectively, in control uPA$^{-/-}$/SCID mice. Because CMZ is mainly excreted in urine in humans, the excretory profile of chimeric mice was demonstrated to be similar to that of humans. In the chimeric mice, the hepatic mRNA expression of human drug transporters could be quantified. On the other hand, the hepatic mRNA expression of mouse drug transporters in the chimeric mice was significantly lower than in the control uPA$^{-/-}$/SCID mice. In conclusion, chimeric mice exhibited a humanized profile of drug excretion, suggesting that this chimeric mouse line would be a useful animal model in excretory studies.

Key Words: **biliary excretion; CMZ; renal excretion; transporter.**

To clarify the pharmacokinetics of a drug is helpful to evaluate its therapeutic and toxicological effects. The pharmacokinetics is mainly determined by absorption, distribution, metabolism, and excretion. A drug is mostly eliminated by biliary and urinary excretion. To elucidate the excretory pathway of a drug is important for understanding the pharmacokinetics and toxicity. One of the major determinants of the excretory pathway of a drug is thought to be its molecular weight (Hirom *et al.*, 1972). In general, a drug with high molecular weight is mainly excreted via the bile duct. Recently, several adenosine 5′-triphosphate-binding cassette (ABC) transporters including P-glycoprotein (P-gp) and multidrug resistance–associated protein 2 (MRP2) have been shown to be relevant to biliary excretion (Chandra and Brouwer, 2004; Faber *et al.*, 2003). Various drug interactions in excretory pathways have been reported. Quinidine decreased the biliary clearance of digoxin by 42% in normal healthy human volunteers due to the inhibition of P-gp encoded by *multidrug resistance 1* (*MDR1*) gene (Angelin *et al.*, 1987). Probenecid decreased the clearance of irinotecan hydrochloride and increased the area under the curve of its metabolite by the inhibition of MRP2 in rats (Horikawa *et al.*, 2002). Thus, it is important to predict the drug interactions in excretion for avoiding adverse reactions.

In drug development, experimental animals have been used to predict the human excretory pathway of a drug candidate in preclinical studies. However, urinary recovery has been reported to be different between species in terms of ^{14}C-labeled compounds such as *N*-(2,6-dichlorobenzoyl)-4-(2,6-dimethoxyphenyl)-L-phenylalanine and zenarestat (Tanaka *et al.*, 1992; Tsuda-Tsukimoto *et al.*, 2005). The mechanism of such species differences is unclear, but may be partly due to differences in metabolism and excretion. For patient with liver or renal disease, it may be desirable to select a drug in consideration of its excretory pathway. Therefore, the elucidation of human excretory profile is necessary for preventing the condition to be worsened.

The livers of a chimeric urokinase-type plasminogen activator (uPA)$^{+/+}$/severe combined immunodeficient (SCID) mouse line established by Tateno *et al.* (2004) could be replaced by more than 80% with human hepatocytes. We previously investigated the expression of human drug metabolizing enzymes in the liver of the chimeric mice (Katoh *et al.*, 2004, 2005a,b,c). The purpose of the present study is to clarify the excretory profile of a drug and the effect of the replacement with human hepatocytes on drug excretion in the chimeric mice. The clarification of humanized excretion as well as metabolism in the chimeric mice can support an evaluation of the toxicity,

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TABLE 1
Chimeric Mice Used in Excretion Study

Number	hAlb[a] (mg/ml)	RI[b] (%)
1	4.3	60
2	7.9	80
3	8.0	80
4	9.2	80
5	9.8	80

[a]The hAlb concentration in blood of chimeric mice.
[b]RI was calculated as described by Tateno *et al.* (2004).

especially in liver toxicity. Cefmetazole (CMZ), which is one of the cephalosporin antibiotics, was used as a probe in this excretory study because CMZ is excreted in an unchanged form in humans and rodents. The excretory pathways of CMZ in humans and rats were different. Urinary excretion was dominant in humans (Ko *et al.*, 1989; Welage *et al.*, 1990), whereas biliary excretion was dominant in rats (Murakawa *et al.*, 1980). However, CMZ excretion in mice has not been studied. In our preliminary study, biliary excretion was major in mice as well as rats. Therefore, in the present study, the chimeric mice were employed to investigate the humanized type of excretion.

MATERIALS AND METHODS

Materials. CMZ and cefazolin were purchased from Sigma-Aldrich (St Louis, MO). All primers shown in Tables 2 and 3 were commercially synthesized at Hokkaido System Science (Sapporo, Japan). Human P-gp, human MRP2, rat breast cancer resistance protein (Bcrp), and rat bile salt export pump (Bsep) membranes were obtained from GenoMembrane (Yokohama, Japan) and ABC Transporter ATPase Assay Reagents Kit was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the analytical or highest grade commercially available.

Animals. Animal maintenance and treatment were conducted in accordance with the National Institute of Health Guide for Animal Welfare in Japan, and the present study was approved by the Ethics Committees of Kanazawa University and the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. ICR mice (8 weeks old) were purchased from SLC Japan (Hamamatsu, Japan). Cryopreserved human hepatocytes from a donor (9-month-old, white, male) were purchased from In Vitro Technologies (Catonsville, MD). The chimeric mice were generated by the method described previously (Tateno *et al.*, 2004). Briefly, uPA$^{+/+}$/SCID mice at 20–30 days after birth were injected with human hepatocytes through a small left flank incision into the inferior splenic pole. When necessary, the chimeric mice were treated ip with nafamostat mesilate as described by Tateno *et al.* (2004). The treatment with nafamostat mesilate was discontinued more than 12 h before the excretion study. The concentration of human albumin (hAlb) in the blood of the chimeric mice and the replacement index (RI; the rate of replacement from mice to humans) were measured using an enzyme-linked immunosorbent assay and immunohistochemistry with anti-human specific cytokeratin 8 and 18 antibodies, respectively (Tateno *et al.*, 2004). There was a good correlation between the hAlb concentration and RI (Tateno *et al.*, 2004). The chimeric mice used in the present study were 12- to 17-week-old and exhibited more than 5 mg/ml hAlb concentrations or 60% of RI. The control uPA$^{-/-}$/SCID mice were obtained as previously reported (Tateno *et al.*, 2004).

TABLE 2
Sequence of Primers for Human Drug Transporters Used in the Present Study

Primer	Sequence
MDR1 S	5′-GGTACCATACAGAAACTCTT-3′
MDR1 AS	5′-TACAACTTCACCAAATGTG-3′
BSEP S	5′-TGAGCCTGGTCATCTTGTG-3′
BSEP AS	5′-TCCGTAAATATTGGCTTTCTG-3′
MRP2 S	5′-ACTATGGGCTGATATCCAG-3′
MRP2 AS	5′-CAGAATTCATCACAAACGCA-3′
BCRP S	5′-GCAGACTTCTTCTTGGACA-3′
BCRP AS	5′-TGATGACAGAAGGAGGTGGT-3′
OCT1 S	5′-GTACCTGTGGTTCACGGACT-3′
OCT1 AS	5′-CCAGGCAGATCATTTGTATTG-3′
OATP1B1 S	5′-GGGATCTCTGTTTTCTAAAATG-3′
OATP1B1 AS	5′-TCCAGCACATGCAAAGACAG-3′
OATP1B3 S	5′-GTCCAGTCATTGGCTTTGCA-3′
OATP1B3 AS	5′-TTATTTGGATTTTTCGGCAAG-3′

Note. S, sense primer; AS, antisense primer.

CMZ excretion in ICR mice. To clarify CMZ excretion in mice and evaluate the pathway of administration, ICR mice were ip ($n = 6$) and iv ($n = 6$) administered with CMZ in phosphate-buffered saline (PBS) (pH 7.4) at a dose of 25 mg/kg. Urine and feces samples were collected during 24 h after the CMZ administration. The samples were frozen at − 20°C until analysis.

CMZ excretion in chimeric mice. Chimeric mice ($n = 5$, Table 1) and control uPA$^{-/-}$/SCID mice ($n = 7$) were ip administered with CMZ in PBS (pH 7.4) at a dose of 25 mg/kg. Urine and feces samples were collected during 24 h after the CMZ administration. The samples were frozen at − 20°C until analysis.

Quantification of CMZ. The CMZ in urine and feces was quantified using high-performance liquid chromatography (HPLC) (Welage *et al.*, 1990). Urine samples (0.1 ml) were diluted with 0.4 ml of distilled water. Feces samples were homogenized by Polytoron homogenizer (OMNI international, Marietta, GA) with distilled water and centrifuged at 1,500 × g for 10 min. The diluted

TABLE 3
Sequence of Primers for Mouse Drug Transporters Used in the Present Study

Primer	Sequence
mdr1 S	5′-TACGACCCCATGGCTGGATC-3′
mdr1 AS	5′-GGTAGCGAGTCGATGAACTGG-3′
bsep S	5′-AAAATAATCCTGGAGTACTA-3′
bsep AS	5′-AGCCTTCTCCAGAATTTC-3′
mrp2 S	5′-GAGGCTACAGTCGATAACGA-3′
mrp2 AS	5′-ATAGTTCAATACGTAGAAGAT-3′
bcrp S	5′-GCGGATTTTTTCCTTGATGT-3′
bcrp AS	5′-AAAGAGGTAACATAGACTGG-3′
oct1 S	5′-GTATCTATGGTTCTCTTGTG-3′
oct1 AS	5′-AAGACAAGCGAGGGTCACA-3′
oatp1b2 S	5′-ATATGTAGACCTGAGAAGTG-3′
oatp1b2 AS	5′-ATGCTTCTCAGAGACCATAG-3′

Note. S, sense primer; AS, antisense primer.

TABLE 4
Hepatic mRNA Expression of Human Transporters in Chimeric Mice with Humanized Liver

Human transporter	Relative mRNA expression
MDR1	2.39 ± 0.90
BSEP	0.41 ± 0.11
MRP2	0.73 ± 0.17
BCRP	5.84 ± 3.49
OCT1	2.19 ± 0.59
OATP1B1	1.58 ± 0.36
OATP1B3	0.58 ± 0.13

Note. Data represent the mean ± SD of seven mice. Relative mRNA expression was calculated as described in "Materials and Methods" section.

TABLE 5
Hepatic mRNA Expression of Mouse Transporter in Chimeric Mice with Humanized Liver

Mouse transporter	Relative mRNA expression
mdr1	2.03 ± 0.61
bsep	0.27 ± 0.18
mrp2	0.16 ± 0.10
bcrp	0.10 ± 0.07
oct1	0.04 ± 0.03
oatp1b2	0.05 ± 0.04

Note. Data represent the mean ± SD of seven mice. Relative mRNA expression was calculated as described in Materials and Methods section.

urine (0.5 ml) and the supernatant of the feces (0.5 ml) samples were treated with 0.5 ml of 0.5% trichloroacetic acid in methanol containing cefazolin (20 nmol) as an internal standard. After incubation at − 20°C for 20 min, the mixture was centrifuged at 15,000 × g for 10 min. Then the supernatant was diluted 1:1 with 0.1 M citrate buffer (pH 5.4). Aliquots of 50 µl of the sample were injected to the HPLC system with a C_{30} 5-µm analytical column (Develosil, 4.6 × 150 mm; Nomura Chemical, Aichi, Japan). The mobile phase was acetonitrile:0.01 M citrate buffer (pH 5.4) = 8:92 (vol/vol), and the flow rate was 1.2 ml/min. The column temperature was 35°C. The eluate was monitored at 254 nm.

Hepatic RNA extraction and real-time RT-PCR. Human and mouse transporter mRNAs in chimeric mice were quantified by real-time RT-PCR. Total hepatic RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNAs were synthesized as described previously (Iwanari *et al.*, 2002). The sequences of primers for human or mouse transporters are shown in Tables 2 and 3, respectively. PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA). After initial denaturing at 95°C for 30 s, amplification was started by denaturation at 94°C for 4 s, and then annealing and extension was performed simultaneously. The conditions of the one-step annealing and extension were as follows: human MDR1, human BCRP, human organic anion transporting polypeptide (OATP) 1B1, mouse mdr1, mouse mrp2, mouse organic cation transporter 1 (oct1), and mouse oatp1b2: 64°C for 20 s for 45 cycles; human MRP2: 64°C for 20 s for 35 cycles; human BSEP, human OCT1, human OATP1B3, and mouse bcrp: 66°C for 20 s for 45 cycles; mouse bsep: 60°C for 20 s for 45 cycles.

Amplified products were monitored directly by measuring the increase in the dye intensity of SYBR Green I (Molecular Probes, Eugene, OR) that binds to double-strand DNA amplified by PCR. The copy number of mRNA in the cDNA sample was calculated using the standard amplification curve. It was confirmed that the primers for human and mouse transporters used in this study did not cross-react with mouse mRNA and human mRNA, respectively. The primer set of mouse mdr1 reacts with both mdr1a and mdr1b. For the investigation of mRNA expression of human and mouse transporters, the numbers of the chimeric mice and control uPA$^{-/-}$/SCID mice used were seven and five, respectively (Tables 4 and 5). For human transporter, the mRNA expression level in chimeric mice was calculated as the relative mRNA expression to the donor hepatocytes (Table 4). For mouse transporter, the mRNA expression level in chimeric mice was calculated as relative mRNA expression to control uPA$^{-/-}$/SCID mice (Table 5).

Drug-stimulated transporter ATPase activity assay. The drug-stimulated transporter ATPase activity was measured using ABC Transporter ATP Assay Reagents Kits as described by Ohashi *et al.* (2006) with slight modifications. Briefly, human P-gp, human MRP2, rat Bsep, and rat Bcrp membranes (20 µg) were preincubated at 37°C for 5 min in 40 µl of reaction buffer and CMZ in the presence or absence of 500 µM sodium orthovanadate in 96-well plates. The reaction was initiated by the addition of 20 µl of 12mM MgATP solution and was terminated 30 min later by the addition of 30 µl of stop solution (10 wt/vol% lithium lauryl sulfate). Two hundred microliters of detection reagent (8% ascorbic acid, 0.8% ammonium molybdate, and 3 mM zinc acetate) was added and incubated at 37°C for 20 min. The inorganic phosphate complex was detected by its absorbance at 665 nm and was quantitated by comparing the absorbance with a phosphate standard. The vanadate-sensitive ATP hydrolysis was determined by subtracting the value obtained with the vanadate-coincubated membrane fraction from vanadate-free membrane fraction.

RESULTS

Excretion of CMZ in ICR Mice

By iv administration of CMZ, the means of the 24-h cumulative urinary and fecal excretions were 32.4 ± 4.7 and 63.1 ± 6.6% of the dose, respectively. On the other hand, by ip administration of CMZ, the means of the 24-h cumulative urinary and fecal excretions were 36.9 ± 9.3 and 56.7 ± 8.5% of the dose, respectively. The biliary excretion of CMZ was clarified to be dominant in mice. The 24-h urinary and fecal excretions were similar between iv and ip administrations.

Excretion of CMZ in Chimeric Mice

The recovery of CMZ in urine and feces up to 24 h in chimeric mice is shown in Figure 1. In all of the chimeric mice, the excretion of CMZ in urine was significantly higher than that in feces. On the other hand, in control uPA$^{-/-}$/SCID mice, the excretion of CMZ in urine was significantly lower than that in feces. The means ± SD of the 24-h cumulative urinary and fecal excretions were 81.0 ± 9.5 and 5.9 ± 4.7% of the dose, respectively, in chimeric mice and 23.7 ± 8.8 and 59.4 ± 11.0% of the dose, respectively, in control uPA$^{-/-}$/SCID mice. The means of the total CMZ recovery were 86.9 ± 9.4 and 83.2 ± 13.3% of the dose in chimeric mice and the uPA$^{-/-}$/SCID mice, respectively.