Table 3 Clinical backgrounds of patients who spontaneously cleared HCV in HIV-infected patients

Patient no.	Age	Sex	Transmission route	Observation period (years)	HCV-RNA (KIU/mL)	HCV genotype	HIV-RNA ($\times10^2$/mL)	WBC (/μL)	CD4+ T cells (/μL)	Platelets ($\times10^4$/mL)	ALT (U/l)	HAART
1	33	M	Transfusion	8.8	290	ND	200 000	4500	5	26.3	21	Yes
2	31	M	MSM	2.3	Positive†	ND	13 000	5760	931	22.7	29	Yes
3	27	M	Transfusion	9.3	>850	3a	180 000	4000	51	10.1	84	Yes
4	53	M	Transfusion	4.5	Positive†	1a	20 000	4800	296	35.4	24	No
5	22	M	Transfusion	7.8	220	ND	990	5500	125	33.1	44	Yes

†Positive: HCV-RNA was positive by qualitative PCR, but was not quantitatively determined.
ALT, aminotransferase; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; ND, not determined; WBC, white blood cells.

Table 4 Changes in clinical parameters and IFN treatment in HIV–HCV co-infected patients

	Outcome of IFN treatment	Number	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	Ascites/ encephalopathy	HCC
IFN-treated patients		60	9.5 ± 5.0	0.05 ± 0.42	0.08 ± 0.38*	0.06 ± 1.13	0	1
	SVR	26	9.1 ± 4.4	0.13 ± 0.59	(−) 0.02 ± 0.08*	0.14 ± 0.76*	0	1
	ETR	11	14.6 ± 7.0	(−) 0.07 ± 0.14	0.51 ± 1.04	0.07 ± 1.50	0	0
	NR	23	7.4 ± 2.0	0.01 ± 0.30	0.09 ± 0.30	(−) 0.18 ± 0.32	0	0
Non-IFN-treated patients		98	8.2 ± 8.2	(−) 0.80 ± 0.82	0.15 ± 0.15	(−) 0.94 ± 0.95	6	2
All		158	8.7 ± 4.7	(−) 0.45 ± 2.93	0.13 ± 0.52	(−) 0.59 ± 3.78	6	3

*$P < 0.05$ versus patients without IFN treatment.
†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years).
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).
§⊿Platelet: changes in platelet count ($\times10^4$/μL)/observation period (years).
ETR, end of treatment virological response; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; NR, no virological response; SVR, sustained virological response.

Table 5 Changes in clinical parameters and HAART in HIV–HCV co-infected patients

	Number	Age	Sex (M : F)	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	IFN	Ascites/ encephalopathy	HCC
HAART (+)	234	37.8 ± 10.4	227:7	8.4 ± 4.2	(−) 0.002 ± 0.18	0.13 ± 0.53	(−) 0.40 ± 3.71	143 (61.1%)	6	5
HAART (−)	58	38.1 ± 10.5	58:0	9.8 ± 6.0	(−) 0.14 ± 0.18	0.03 ± 0.25	(−) 1.40 ± 3.30	30 (51.7%)	3	2

†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years).
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).
§⊿Platelet: changes in platelet count ($\times 10^4$/(L)/observation period (years).
HAART, highly active anti-retroviral therapy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

of ribavirin/IFN combination therapy in HIV–HCV co-infected patients in this study.

The response rate to ribavirin/IFN combination therapy was 31.4% in total, and 15.3% in patients with HCV genotype 1, which are comparable rates to those achieved in previous studies on HIV–HCV co-infected patients in Western countries.[7] The low response rate in HIV–HCV co-infected patients compared with HCV mono-infected patients[12] may be attributed to several factors: impaired immune response, high HCV loads and viral quasi-species caused by frequent chances of transmission. Of these, high viral loads may be essential, because Table 2 shows that patients with genotype 1 HCV achieved SVR even by IFN monotherapy if their viral loads were low. In the era of IFN monotherapy, patients with favorable conditions were treated first of all: pretreatment viral loads in patients who received IFN monotherapy were lower than those who received PEG-IFN–ribavirin combination therapy. This may be the reason why the efficacy of PEG-IFN–ribavirin combination therapy was lower than that with IFN monotherapy in this study.

The serum bilirubin concentrations and platelet counts were improved in the patients who achieved SVR by IFN treatment. Although the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients, the overall benefit of IFN treatment on liver function may be similarly expected in the patients who achieved SVR. HAART showed no impact on the liver function in HIV–HCV co-infected patients. Improvement of liver function can be expected only in IFN-treated patients, although there is a possibility that only patients with preserved liver function were able to receive IFN treatment. Given that liver disease is the major life-threatening factor in HIV-infected patients, IFN treatment should be considered in the early stage of HIV–HCV co-infection.

It should be noted that nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was much younger than that of HCV mono-infected patients with the same complications.[9] This finding is compatible with reports from Western countries showing a faster progression of fibrosis[13] and earlier development of HCC.[14] A possibly interesting finding is that five patients (approximately 3% of patients whose serum HCV-RNA level was serially determined) cleared HCV-RNA from the serum without IFN treatment. Previous reports showed that some HIV-infected patients could spontaneously clear HCV-RNA.[15–17] The clearance of HCV among patients with chronic HCV infection is rare, although it has been

reported in Japan.[18] Three of the five patients had high HCV loads and low $CD4^+$ T-lymphocyte counts, which are generally thought to be unfavorable for spontaneous HCV clearance. A difference in immune status of HIV-infected patients from HCV mono-infected patients may be involved in such an observation, although further studies are awaited.

In summary, our study demonstrated that approximately 20% of HIV-infected patients are co-infected with HCV. Some of the HIV–HCV co-infected patients had advanced liver disease such as ascites, encephalopathy or HCC at a younger age than HCV mono-infected patients, suggesting that the progression of liver disease may be more rapid in HIV–HCV co-infected patients than in HCV-mono-infected ones. Treatments with regimens including IFN, which may improve liver function and decrease liver-related death, should be considered in HIV–HCV co-infected patients.

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CLINICAL INVESTIGATION

Transarterial Infusion Chemotherapy Using Cisplatin-Lipiodol Suspension With or Without Embolization for Unresectable Hepatocellular Carcinoma

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Abstract We evaluate the long-term prognosis and prognostic factors in patients treated with transarterial infusion chemotherapy using cisplatin-lipiodol (CDDP/LPD) suspension with or without embolization for unresectable hepatocellular carcinoma (HCC). Study subjects were 107 patients with HCC treated with repeated transarterial infusion chemotherapy alone using CDDP/LPD (adjusted as CDDP 10mg/LPD 1ml). The median number of transarterial infusion procedures was two (range, one to nine), the mean dose of CDDP per transarterial infusion chemotherapy session was 30 mg (range, 5.0–67.5 mg), and the median total dose of transarterial infusion chemotherapy per patient was 60 mg (range, 10–390 mg). Survival rates were 86% at 1 year, 40% at 3 years, 20% at 5 years, and 16% at 7 years. For patients with >90% LPD accumulation after the first transarterial infusion chemotherapy, rates were 98% at 1 year, 60% at 3 years, and 22% at 5 years. Multivariate analysis identified >90% LPD accumulation after the first transarterial infusion chemotherapy ($p = 0.001$), absence of portal vein tumor thrombosis (PVTT; $p < 0.001$), and Child-Pugh class A ($p = 0.012$) as independent determinants of survival. Anaphylactic shock was observed in two patients, at the fifth transarterial infusion chemotherapy session in one and the ninth in the other. In conclusion, transarterial infusion chemotherapy with CDDP/LPD appears to be a useful treatment option for patients with unresectable HCC without PVTT and in Child-Pugh class A. LPD accumulation after the first transarterial infusion chemotherapy is an important prognostic factor. Careful consideration should be given to the possibility of anaphylactic shock upon repeat infusion with CDDP/LPD.

Keywords Hepatocellular carcinoma · Transcatheter arterial chemoembolization · Cisplatin-lipiodol suspension · Arterial infusion chemotherapy · Prognosis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1–4]. Recent advances in imaging and treatment modalities have resulted in a number of improvements in the prognosis of patients with HCC. Patients with small HCCs, for example, are commonly treated by surgical resection and locoregional therapy such as percutaneous ethanol injection (PEI), microwave coagulation therapy, laser ablation, and radiofrequency (RF) ablation, and these treatments are often associated with a satisfactory long-term prognosis [5–9]. However, these locoregional therapies are not suitable in all patients, mainly due to the presence of a large tumor, multiple HCC tumors, or a serious underlying chronic liver disorder.

Since the development of transcatheter arterial embolization (TAE) for HCC [10–12], intra-arterial treatments have been widely used for patients with unresectable HCC.

Among these, transcatheter arterial chemoembolization (TACE) using anticancer drugs mixed with lipiodol (LPD; Lipiodol Ultrafluide; Laboratoire Guerbet, Aulnay-Sous-Bois, France), which remains selectively in tumor tissue for extended periods of time, has now become one of the most effective treatment modalities for patients with unresectable HCC [13–27]. Randomized controlled trials recently confirmed the survival benefits of TACE in such patients [28, 29].

Various anticancer drugs have been used as TACE agents in the treatment of HCC, including doxorubicin hydrochloride (ADM) [13–16], epirubicin hydrochloride [17], mitomycin C (MMC) [13, 16], zinostatin stimalamer (SMANCS) [27], and cisplatin (*cis*-diaminedichloroplatinum; CDDP) [30–33]. However, the most effective of these anticancer drugs and protocols against HCC has yet to be identified. In particular, little or no information is available on the effects of TACE-CDDP/LPD on prognosis or on the factor(s) predictive of a response.

We conducted a retrospective study to determine the long-term prognosis of patients who received transarterial infusion chemotherapy with CDDP/LPD for unresectable HCC and identified the factor(s) predictive of long-term prognosis.

Materials and Methods

Patients

From June 2000 to December 2007, 526 patients with naïve HCC were admitted to our hospital. Of these, 323 patients were treated with transarterial infusion chemotherapy, 68 with surgical resection, 5 with living-donor liver transplantation (LDLT), 54 with RF ablation, 13 with PEI, 4 with RF ablation and PEI, 32 with hepatic arterial infusion chemotherapy, 3 with systemic chemotherapy, and 24 with conservative therapy. Of the 323 patients treated with transarterial infusion chemotherapy, 91 were later treated with surgical resection, 41 with RF ablation, 35 with transarterial infusion chemotherapy combined with PEI, 7 with LDLT, 7 with radiotherapy, 32 with hepatic arterial infusion chemotherapy, and 3 with a combination of systemic chemotherapy, leaving 107 patients treated with transarterial infusion chemotherapy alone for enrollment in this retrospective cohort study. The study group consisted of 75 men and 32 women ranging in age from 42 to 92 years (median, 73 years). Tests were positive for hepatitis C virus in 82 patients (78.8%) and for hepatitis B virus in 7 patients (6.7%). Seventy-five patients were classified as having Child-Pugh class A (72.1%) disease and 29 as Child class B disease (27.9%). Median total bilirubin level was 1.0 mg/dl, and median serum albumin was 3.6 g/dl. Tumor staging was defined based on the tumor node metastasis staging system of the Liver Cancer Study Group of Japan (LCSGJ): stage I (fulfilling three intrahepatic conditions: solitary, <2 cm, no vessel invasion; $n = 9$ [9%]), stage II (two of the three intrahepatic conditions; $n = 41$ [38%]), stage III (one of the three intrahepatic conditions; $n = 53$ [50%]), stage IVa (none of the three intrahepatic conditions, with no distant metastases or any intrahepatic conditions with lymph node metastases), and stage IVb (any intrahepatic condition with distant metastases; stage IV, $n = 4$ [3%]) [34]. The median value of the maximum diameter of the main tumor was 30 mm (range, 6–130 mm). Forty-three (40%) patients had a solitary tumor, 35 (33%) had two or three tumors, and 29 (27%) had four or more tumors. The clinical characteristics of the study group are summarized in Table 1. The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics committee of our hospital, and written informed consent was obtained from all participating patients.

Preparation of Chemotherapeutic Agents

LPD was mixed at 1 ml per 10 mg CDDP powder. Because CDDP powder was not available for clinical use in Japan from June 2000 to December 2004, we prepared CDDP powder from a commercially available CDDP solution (Randa; Nippon Kayaku, Tokyo) as described in our previous study [35]. After it became available, from December 2004 to December 2007, we mixed CDDP powder with

Table 1 Characteristics of 107 patients who underwent repeated transarterial infusion chemotherapy using a cisplatin/lipiodol suspension for unresectable hepatocellular carcinoma

Age, yr[a]	73 (42–92)
Gender, male/female	75/32
Etiology, HCV/HBV/others	82/7/18
Child-Pugh class, A/B/C	75/29/3
T-bilirubin, mg/dl[a]	1.0 (0.2–5.4)
Albumin, g/dl[a]	3.6 (2.4–4.7)
Tumor stage, T1/T2/T3/T4	9/41/53/4
Tumor size, mm[a]	30 (6–130)
Tumor number, 1/2 or 3/>3	43/35/29
Tumor portal vein thrombus, present/absent	3/104
α-Fetoprotein, ng/ml[a]	32.2 (5–35,610)
Des-γ-carboxy prothrombin, mAU/ml[a]	167 (10–11,600)
TAE, with/without	62/45
Period of follow-up, mo[a]	13 (1–92)

Note: *HCV* hepatitis C virus, *HBV* hepatitis B virus, *TAE* transcatheter arterial embolization

[a] Data are median (range)

LPD (IA-call; Nippon Kayaku). The particle size of CDDP powder is 28.5 μm.

Imaging and Confirmation of Diagnosis

Pretreatment imaging studies included abdominal ultrasonography (US), contrast-enhanced dynamic CT, dynamic magnetic resonance (MR) imaging, digital subtraction angiography (DSA), angiography combined with CT during arterial portography (CTAP), and hepatic arteriography (CTHA). All tumors were diagnosed by distinctive findings on US, dynamic CT and/or dynamic MR imaging, DSA, CTAP, and CTHA. Diagnosis was confirmed by early enhancement in the arterial phase and hypoattenuation in the portal venous or equilibrium phase on contrast-enhanced dynamic CT or dynamic MR images, or by hypoattenuation on CTAP and hyperattenuation on CTHA. In addition, changes in serum tumor markers (α-fetoprotein [AFP] or des-γ-carboxy prothrombin) were used to support the imaging-based diagnosis.

Transarterial Infusion Chemotherapy with or Without Embolization

Transarterial infusion chemotherapy was performed through the femoral artery under local anesthesia using the technique of Seldinger. An angiographic catheter was inserted into the hepatic feeding artery of the segment or subsegments containing the target tumor under CT scan during hepatic arteriography and arterial portography. We used a CDDP/LPD suspension as an anticancer drug. The tumor vessels were evaluated by CTHA scans during hepatic arteriography. Dosage was based on tumor size, and injection was discontinued based on the full accumulation of iodized oil in the tumor vessels and the degree of visualization of the portal vein during injection on fluoroscopy. The accumulation of iodized oil in the tumor was evaluated by CTHA scan; if accumulation in the tumor was poor, other vessels were tested, and when a vessel was identified as a feeding vessel, CDDP/LPD was added to the infusion. CDDP/LPD was not injected into the right hepatic artery, left hepatic artery, or proper hepatic artery.

A gelatin sponge was used for embolization (Gelpart; Nippon Kayaku, Tokyo), cut into 1- or 2-mm cubes, depending on the vascular diameter. The gelatin sponge was used after arterial infusion chemotherapy in patients who had a membrane-covered lesion and a segmental lesion in the periphery. Most patients were treated by arterial infusion chemotherapy in principle, but a gelatin sponge was not used in all patients, particularly those with chronic liver failure. A gelatin sponge was not used on the right hepatic artery, left hepatic artery, or proper hepatic artery. The angiographic endpoint of gelatin sponge embolization was very mild embolization. Extrahepatic collateral arteries which supplied tumors were also embolized.

The fluid replacement volume was 3000 ml/day on the day of treatment and 1000 ml/day for the next 2 days.

Criteria for Evaluation of the Therapeutic Effect of Transarterial Infusion Chemotherapy with or Without Embolization

The efficacy of transarterial infusion chemotherapy was evaluated by CT at 3 months after treatment, as follows: when LPD was seen in >90% of the tumor, efficacy was considered grade I; in 50% to 90% of the tumor, grade II; and in <50% of the tumor, grade III [35]. Grading for LPD retention was based on quantitative measurement of tumor diameter in all tumors, based on the assumption that the tumor portion with retained LPD was necrotic tissue. The percentage of LPD accumulation in the target tumor was graded by two radiologists blinded to clinical status. Discrepancies between the two observers were resolved by adopting the lowest grade of assessment.

Follow-Up Protocol

Concentrations of serum tumor markers, including AFP and des-γ-carboxy prothrombin, were measured once a month after transarterial infusion chemotherapy; follow-up US was performed every 3 months; and CT or MR imaging was performed every 6 months. Patients showing an increase in tumor markers, diminution of LPD accumulation, or new nodules remote from the treated nodules were readmitted for an additional round of transarterial infusion chemotherapy using the same procedure. On follow-up, patients treated with transarterial infusion chemotherapy who did not show complete uptake of LPD (i.e., those classified as grade I), but did show the presence of a viable tumor, namely, by arterial phase enhancement on CT/MR, were retreated with transarterial infusion chemotherapy within 3–6 months of the first treatment. Patients with tumor progression, appearance of PVTT, and liver failure were excluded from TACE.

Complications

Major complications were defined in accordance with the definitions established by the Society of Interventional Radiology as hemorrhage requiring transfusion, liver abscess requiring percutaneous drainage, bile duct injury requiring biliary drainage, pleural effusion requiring thoracocentesis, hepatic failure, and death [36]. In all patients, the following laboratory tests were conducted before treatment and 1, 3, and 7 days after and 1 month after

treatment: serum transaminases, bilirubin, alkaline phosphatase, albumin, creatinine, and complete blood cell count. Adverse reactions were assessed using the National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 3.0) [37].

Statistical Analysis

Data were collected and calculated at the end of the study and statistically analyzed on April 1, 2008. Cumulative survival rate was calculated from the initial date of transarterial infusion chemotherapy therapy and assessed by the Kaplan–Meier life-table method, with differences evaluated by the log rank test. Univariate analysis of predictors of survival was assessed by the Kaplan–Meier life-table method, and differences were evaluated by the log rank test. Multivariate analysis of predictors of survival was assessed by the Cox proportional hazards model. Statistical significance was defined as a p value <0.05. We also calculated hazard ratios and 95% confidence intervals (95% CI). All p values <0.05 in two-tailed tests were considered significant. Variables that achieved statistical ($p < 0.05$) or marginal significance ($p < 0.10$) in univariate analysis were entered into a multiple Cox proportional hazards model to identify significant independent factors. Parameters used for the prediction of survival were LPD accumulation, tumor number, PVTT (present or absence), Child-Pugh class, AFP, age, gender, etiology, TAE (with or without embolization), and tumor size. All analyses were performed with SPSS software (version 16; SPSS, Chicago, IL).

Results

Therapeutic Effects of Transarterial Infusion Chemotherapy-CDDP/LPD

The median number of transarterial infusion chemotherapy procedures per patient was two (range, one to nine). The mean dose of CDDP per single session of transarterial infusion chemotherapy was 30 mg (range, 5.0–67.5 mg), and the median total dose of CDDP per patient was 60 mg (range, 10–390 mg). LPD accumulation was evaluated after the first transarterial infusion chemotherapy: grade I was recorded in 58 patients (55%), grade II in 36 (33%), and grade III in 13 (12%) (Table 2).

Survival Rates

Cumulative survival curves of patients treated with TACE using CDDP/LPD suspension for unresectable HCC. Survival rates were 86% at 1 year, 40% at 3 years, 20% at 5 years, and 16% at 7 years. No significant difference in overall survival was seen between patients with and those without embolization ($p = 0.20$) (Fig. 1). Survival rate in patients assessed as grade I was 98% at 1 year, 60% at 3 years, and 22% at 5 years. Respective rates, in contrast, were 68%, 52%, and 22% in those assessed as grade II and 48%, 20%, and 0% in those assessed as grade III (Fig. 2). The probability of survival correlated with the extent of LPD accumulation in grades I and III ($p < 0.05$). Representative examples of patients with grades I and II are shown in Fig. 3.

Table 2 Transarterial infusion chemotherapy with a cisplatin/lipiodol suspension

No. of procedures[a]	2 (1–9)
Mean dose of CDDP per single session, mg[a]	30 (5–67.5)
Total dose of CDDP per single case, mg[a]	60 (10–390)
LPD accumulation of transarterial infusion chemotherapy, grades I/II/III	55%/33%/12%

[a] Data are median (range)

We then investigated the relationship between survival after the initiation of transarterial infusion chemotherapy and various clinicopathological variables by univariate analysis. Results showed that survival correlated significantly with grade I ($p = 0.001$), and AFP < 200 ng/ml ($p = 0.013$) (Table 3). grade I, absence of PVTT, Child-Pugh class A, number of tumors = 1, and AFP < 200 ng/ml were then entered into the multiple Cox proportional hazard model, which identified grade I ($p = 0.001$), absence of PVTT ($p < 0.001$) and Child-Pugh class A

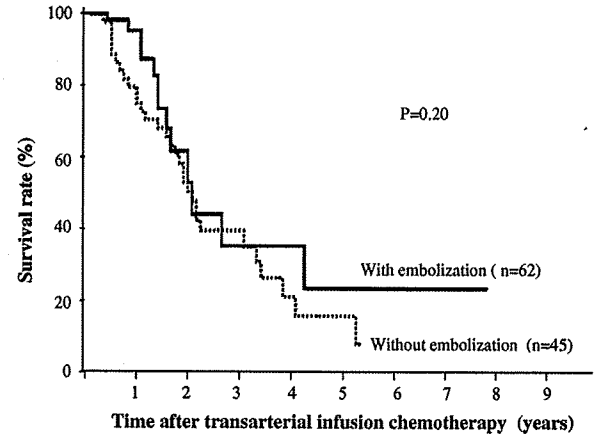

Fig. 1 Cumulative survival curves of patients treated with TACE using CDDP/LPD suspension for unresectable HCC. Survival rates were 96% at 1 year, 36% at 3 years, 24% at 5 years, and 24% at 7 years in with embolization groups and 72% at 1 year, 40% at 3 years, 16% at 5 years in without embolization groups. No significant difference in overall survival was seen between patients with and without embolization ($p = 0.20$)

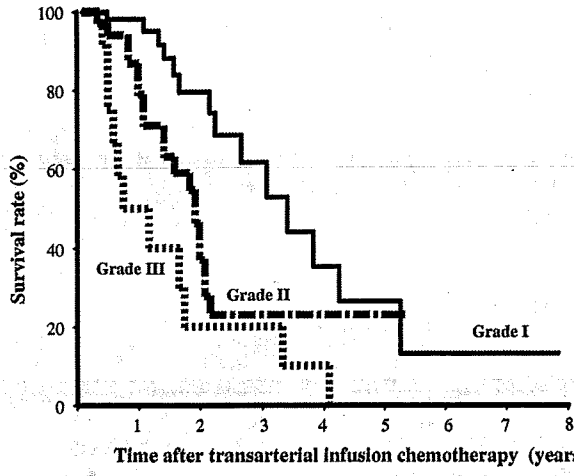

Fig. 2 Cumulative survival curves according to the degree of lipiodol (LPD) accumulation in the tumor. Survival rates of patients assessed as grade I were 98% at 1 year, 60% at 3 years, and 22% at 5 years. By comparison, rates in patients assessed as grade II were 68% at 1 year, 52% at 3 years, 22% at 5 years, and those in patients assessed as grade III were 48% at 1 year, 20% at 3 years, and 0% at 5 years. Survival probability correlated with degree of LPD accumulation between grade I and grade III ($p < 0.05$)

($p = 0.012$) as significant and independent determinants of survival.

Adverse Reactions and Complications

The total number of transarterial infusion chemotherapy procedures was 274. The most common adverse reactions were fever, nausea, and loss of appetite. Among patients with various NCI-CTC grade 2 adverse reactions, nausea and/or vomiting was the most common (96 patients; 35%), followed by grade 1 fever (71 patients; 26%), grade 3 thrombocytopenia (60; 22%), grade 2 abdominal pain (26; 9%), grade 2 liver dysfunction (26; 9%), grade 3 liver dysfunction (8; 3%), grade 3 renal dysfunction (2; 0.7%), grade 4 liver dysfunction (2; 0.7%), and anaphylactic shock (2; 0.7%). No intrahepatic biloma or liver abscess formation was seen. One patient received nine courses of transarterial infusion chemotherapy, with a total dose of CDDP of 310 mg. On injection of 15 mg/1.5 ml of CDDP/LPD suspension into the catheter on the ninth transarterial infusion chemotherapy, the patient experienced a decrease in systolic blood pressure from 110 to 78 mmHg and shortness of breath. He was successfully treated with oxygen and intravenous epinephrine and corticosteroid and was moved to the intensive care unit; he improved after 24 h and was transferred back to the general ward. Another patient received five courses of transarterial infusion chemotherapy, with a total dose of CDDP of 95 mg. Injection of 20 mg/2 ml of CDDP/LPD suspension into the catheter on the fifth transarterial infusion chemotherapy resulted in anaphylactic shock, but this patient also subsequently improved within 24 h.

Causes of Death

Forty-five of the 107 patients died during the study period. Causes of death were HCC-related (rupture of HCC) in 23 (51%), hepatic failure in 8 (18%), rupture of esophageal varices in 3 (7%), and other diseases in 11 (24%). No immediate or procedure-related death was seen within 30 days of infusion.

Discussion

The prognosis of patients with small HCC has improved markedly in recent years following the introduction of locoregional therapies. However, these therapies are not indicated in many patients due to large tumor size, multiple tumors, and poor underlying liver status. TACE has been widely used for these patients. Although various anticancer agents have been used as TACE agents for unresectable HCC, including ADM, epirubicin hydrochloride, MMC, SMANCS, and CDDP, the most effective anticancer drug for HCC remains to be defined. In vitro testing has indicated the efficacy of CDDP as suitable for TACE [38], but only a few reports have described the determinants of survival after initiation of TACE with CDDP/LPD suspension [39]. The purpose of the present study was to investigate the long-term prognosis of patients undergoing transarterial infusion chemotherapy with CDDP/LPD suspension for unresectable HCC and factors predictive of prognosis.

Overall survival rates in the 107 enrolled patients were 86% at 1 year, 40% at 3 years, 20% at 5 years, and 16% at 7 years. Ono et al. [39] reported survival rates of patients with unresectable HCC of 30% at 3 years with CDDP/LPD compared with 14% at 3 years with ADM. In other studies, survival rates at 3 years for unresectable HCC were 56% with ADM [40] and 32% with epirubicin hydrochloride [41]. Thus, the survival rate at 3 years achieved in the present study is very similar to those reported for ADM and epirubicin hydrochloride. The determinants of survival in the present study were grade I (>90% LPD accumulation in the first transarterial infusion chemotherapy), Child-Pugh grade A, and absence of PVTT; indeed, for patients with unresectable HCC free of PVTT who are rated as Child-Pugh grade A, a comparatively excellent long-term prognosis is expected for those who show >90% LPD accumulation after the first transarterial infusion chemotherapy.

CDDP is a potent anticancer drug against HCC in vitro. Using the 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MMT) assay, Furukawa et al. [38]

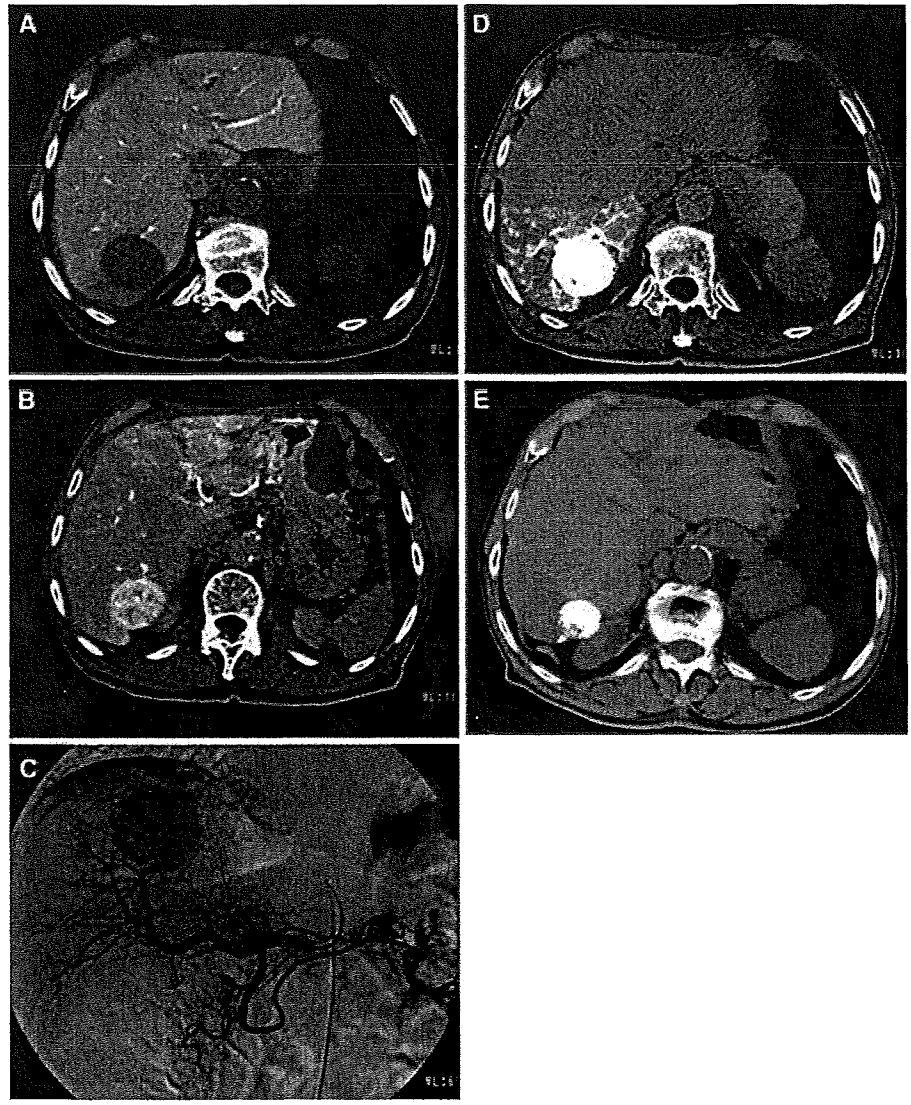

Fig. 3 Imaging studies in an 88-year-old man treated for unresectable HCC with TACE conducted between April 2006 and April 2008. Gelatin sponge embolization was conducted. **A** CTAP in April 2006. The HCC tumor (largest diameter, 4 cm) in S7 showed hypoperfusion on CTAP. **B** CTHA in April 2006 shows hyperenhancement of the same lesion. **C** DSA in April 2006 showing the same lesion. **D** CT taken 3 months after the first TACE. The lesion shows accumulation of LPD evaluated as grade I. **E** CT in April 2008 shows no recurrence 2 years later. Des-γ-carboxy prothrombin was decreased from 1100 to 10 mAU/ml. The patient remains alive and cancer-free at the time of writing

Table 3 Univariate and multivariate analyses of predictors of survival

	Univariate analysis (log-rank test): *p*-Value	Multivariate analysis (Cox proportional hazard model)		
		Hazard ratio	95% CI	*p*-Value
Grade I	0.001	0.335	0.172–0.654	0.001
Absence of PVTT	0.050	0.052	0.012–0.218	<0.001
Child-Pugh class A	0.083	0.436	0.228–0.834	0.012
No. of tumors = 1	0.095			
α-Fetoprotein <200 ng/ml	0.013			
Age <70	0.40			
Gender	0.80			
HBV/HCV/non-B non-C	0.33			
TAE (with/without)	0.20			
Tumor size <20 mm	0.42			

Note: *PVTT* portal vein tumor thrombosis, *TAE* transcatheter arterial embolization, *HBV* hepatitis B virus, *HCV* hepatitis C virus

reported the in vitro chemosensitivity of HCC to seven anticancer drugs as follows: ADM, 30%; CDDP, 20%; MMC, 17.5%; 5-fluorouracil, 12.5%; methotrexate, 5.4%; etoposide, 0%; and CPT-11, 0%. This indicates that ADM and CDDP are the most effective anticancer drugs for HCC in vitro. In their study, however, Kamada et al. [35] reported that the survival rate was significantly better for the CDDP/LPD group than for the ADM/LPD group. Comparison of the effects of and long-term prognosis for these anticancer drugs when used as TACE agents in randomized control trial studies is required.

LPD accumulation >90% after the first transarterial infusion chemotherapy was an independent determinant of survival. The proportion of patients who achieved this after the first transarterial infusion chemotherapy (55%) in the present study was higher than the 15% reported in our previous study [35]. This difference might be due to our present use of angiography combined with CT during arterial portography and hepatic arteriography, which provides better evaluation of drug accumulation in real time and, hence, allows the addition of an additional dose or drug when needed. In addition, CDDP/LPD was not injected into the right hepatic artery, left hepatic artery, or proper hepatic artery.

It seems that grading LPD uptake serves instead to represent a method to assess underlying tumor biology. Favorable tumor biology manifests with tumor necrosis and a high degree of LPD uptake, such as the case shown in Fig. 3, while unfavorable tumor biology results in lesser degrees of tumor necrosis and secondarily lower LPD uptake. It is doubtless that the effects of TACE are affected mainly by embolization with LPD and gelatin sponge. However, no significant difference in overall survival was seen between patients with and those without embolization in our study.

Ikeda et al. also reported that although transcatheter arterial infusion chemotherapy had a stronger antitumor effect than transarterial infusion chemotherapy, it did not significantly improve survival [42]. In contrast, Yamamoto et al. reported that complete embolization after injection of cisplatin-lipiodol suspension resulted in higher survival than incomplete embolization [32]. We consider that gelatin sponge embolization was locally effective in the tumor, but because survival rates were also related to liver function, gelatin sponge embolization was not a significant prognostic factor in this study.

Although we used a CDDP/LPD suspension in the present study, Takaki et al. recently reported that LPD retention was better with the emulsion than with the suspension [43]. Evaluation of the best mixing method for CDDP and LPD requires long-term investigation.

Analysis of adverse reactions and complications with transarterial infusion chemotherapy-CDDP/LPD showed minimal renal or liver dysfunction. This favorable finding may be due to selective infusion of the drug under CTAP and CTHA: because the injected area can be viewed directly under CTHA, the amount of injected drug that can cause damage to noncancer tissue is minimal [44], and the mean dose of CDDP per single session of transarterial infusion chemotherapy was a relatively low 30 mg. Nevertheless, anaphylactic shock was observed in two (0.7%) patients. A recent review reported five patients with gynecological malignancies who experienced anaphylaxis to CDDP after receiving previously uncomplicated courses of this agent, with the hypersensitivity reaction following a median of seven courses [45, 46]. In our study, two patients experienced hypersensitivity, at the fifth and ninth courses, respectively, suggesting the need for caution when administering platinum agents to patients previously treated with the agent. Monitoring during CDDP/LPD injection is therefore warranted, and injection should be stopped at the first sign of symptoms.

In conclusion, transarterial infusion chemotherapy with CDDP/LPD appears to be a useful treatment option for patients with unresectable HCC without PVTT and in Child-Pugh class A. LPD accumulation after the first transarterial infusion chemotherapy is an important prognostic factor. Careful consideration should be given to the possibility of anaphylactic shock upon repeat infusion with CDDP/LPD.

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MAJOR ARTICLE

G-to-A Hypermutation in Hepatitis B Virus (HBV) and Clinical Course of Patients with Chronic HBV Infection

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Background. The apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like family of cytidine deaminases induce G-to-A hypermutation in hepatitis B virus (HBV) genomes and play a role in innate antiviral immunity. The clinical relevance of this protein family is unknown.

Methods. We analyzed 33 instances in which 17 patients with chronic HBV infection experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level; we used a quantitative differential DNA denaturation polymerase chain reaction assay to quantify the hypermutated HBV genomes observed during 21 of these 33 increases in ALT level.

Results. Of the 9 increases in ALT level that involved a >5-fold increase (relative to basal levels) in the number of hypermutated genomes observed, 8 were associated with a >2-log reduction in plasma HBV DNA level. In contrast, a corresponding decrease in plasma HBV DNA level was observed for only 1 of the 12 increases in ALT level that did not involve an increase in the number of hypermutated genomes ($P < .001$). Hepatitis B e antigen clearance was often observed in patients who experienced an increase in the number of hypermutated genomes. Interferon treatment induced hypermutation in HBV genomes in an animal model. However, there was no apparent increase in the number of hypermutated genomes among the majority of patients who received interferon therapy, probably because the number of hypermutated genomes had already increased prior to the initiation of therapy.

Conclusion. Our results suggest that a marked increase in the number of hypermutated genomes represents a strong immunological host response against the virus and is predictive of hepatitis B e antigen clearance and plasma HBV DNA level reduction.

Despite the availability of safe and effective vaccines for >2 decades, hepatitis B virus (HBV) infection is still a global health problem. Worldwide, >2 billion people are infected with HBV, and chronic HBV infection affects ~400 million people [1, 2]. It is estimated that >500,000 people die annually because of cirrhosis and/or hepatocellular carcinoma due to HBV infection [3].

Recent reports have shown that cellular cytosine deaminase (apolipoprotein B messenger RNA [mRNA] editing enzyme, catalytic polypeptide–like 3G [APOBEC3G]), packaged in human immunodeficiency virus type 1 (HIV-1), induces G-to-A hypermutation to a nascent reverse transcript of HIV-1 and reduces the infectivity of HIV, thus contributing in part to innate antiviral activity [4–8]. HIV-1 overcomes this innate defense barrier in T cells with HIV virion infectivity factor, a protein that specifically targets APOBEC3G to proteasomal degradation [9–12]. HIV-1 can infect resting CD4 T cells in lymphoid tissues but not those circulating in peripheral blood [13–16]. Resting CD4 T cells in peripheral blood are protected from HIV infection through the action of the deaminase-active

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Table 1. Clinical profiles of 17 patients with chronic hepatitis B virus (HBV) infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level.

Patient	Sex	Age, years	ALT level, IU/L		Plasma HBV DNA level, log copies/mL	HBV serum marker status[a]		HBV subtype	Histologic result[b]	Receipt of IFN treatment
			Minimum	Maximum		HBeAg	HBeAb			
1	M	50	26	2000	8.1	+	−	C	F2, A2	Yes
2	M	31	22	230	8.2	+	−	C	F3, A2	Yes
3	F	23	14	313	8.7	+	−	C	F2, A2	Yes
4	M	22	16	846	6.9	+	−	C	F2, A1	Yes
5	F	42	10	100	7.8	+	−	C	L	No
6	F	33	21	748	8.8	+	−	C	F2, A3	Yes
7	M	23	22	339	8.4	+	−	C	L	Yes
8	F	54	22	108	6.7	−	+	C	F2, A2	No
9	M	44	17	512	9.5	+	−	C	F2, A3	No
10	M	27	39	115	8.8	+	−	C	F1, A1	Yes
11	M	36	16	452	3.8	+	−	C	F4, A3	Yes
12	M	20	21	1295	7.2	+	−	C	F2, A2	No
13	M	36	24	481	5.7	−	+	C	F2, A2	Yes
14	M	22	20	696	5.9	+	−	C	F1, A1	Yes
15	F	24	14	1544	7.7	+	−	C	F2, A2	Yes
16	M	35	10	1618	4.7	+	−	C	F2, A1	Yes
17	M	30	21	1655	6.7	+	−	C	L	Yes

NOTE. HBeAg, HBV e antigen; HBeAb, antibody against HBV e antigen; IFN, interferon; L, liver cirrhosis.

[a] Before increase in ALT level.

[b] Histologic evaluation of chronic hepatitis by use of the scoring system of Desmet et al. [29].

APOBEC3G [17]. Recent reports have shown that interferon (IFN)–α is a potent inducer of APOBEC3G [18–21]. It has also been reported that some of the HIV restriction exerted by APOBEC3G may be independent of its cytidine deaminase activity [17, 22–24].

We and others have reported the presence of small numbers of hypermutated genomes in serum samples obtained from HBV-infected patients [25–27]. Studies using HepG2 cell lines and primary human hepatocytes showed that such hypermutation is induced by the cytidine deaminase activity of the APOBEC family of proteins [27]. In our previous study, IFN induced little hypermutation in the HBV genome [27]. However, after extensive investigation supported by development of a quantitative analysis of hypermutation, we showed that both IFN-α and IFN-γ actually increase transcription of APOBEC3G mRNA in HepG2 cell lines and induce an increase in the number of hypermutated genomes [28]. We also showed that APOBEC3G induces hypermutation in HBV and reduces HBV replication levels in the absence of the deaminase activity. Thus, APOBEC3G has dual antiviral actions against HBV and is thought to be part of the host defense mechanisms, as has been shown for HIV infection. Although it is assumed that APOBEC3G is important in the host anti-HBV defense system, little is known about the clinical importance of this enzyme, because there are no methods available for the precise quantification of small amounts of hypermutated genomes.

Using a method that can measure small amounts of hypermutated genomes (differential DNA denaturation polymerase chain reaction [3D-PCR] combined with TaqMan PCR [28]), we analyzed fluctuations in the number of hypermutated genomes observed in patients with chronic HBV infection who experienced increased alanine aminotransferase (ALT) levels. The study group included patients who received IFN treatment and patients who did not.

METHODS

Patients. From 2002 through 2006 at Hiroshima University Hospital (Hiroshima, Japan), there were 17 consecutive patients with chronic hepatitis B who experienced >2 increases of >100 IU/L in ALT level and for whom stored serum samples were available. These 17 patients were enrolled in this study, among whom 33 such increases in ALT level were observed. Thirteen of 17 patients received IFN treatment, usually during an increase in ALT level. The clinical profiles of these 17 patients are shown in table 1. Written informed consent was obtained from all patients, and the study was approved by the Hiroshima University Ethics Committee.

HBV markers. Hepatitis B e antigen and antibody against e antigen were quantified by use of enzyme immunoassay kits (Abbott Diagnostics). HBV DNA was measured by use of real-time PCR performed with the 7300 Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer's instructions. The primers used for amplification were 5'-TT-

TGGGCATGGACATTGAC-3' (nt 1893–1912; nucleotide numbers are those of HBV subtype C as reported by Norder et al. [30]) and 5'-GGTGAACAATGTTCCGGAGAC-3' (nt 2029–2049). For real-time PCR, we used 25 μL of SYBR Green PCR Master Mix (Applied Biosystems) with 1 μL of the DNA solution and 200 nmol/L of each primer. The amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of amplification (denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min). The lower detection limit of this assay was 10^3 copies/mL.

Extraction of HBV DNA and quantitative analysis of hypermutated genomes. HBV DNA was extracted from 100-μL serum samples by use of the SMITEST DNA Extraction Kit (Genome Science Laboratories) and dissolved in 20 μL of water. Hypermutated genomes were quantified by use of TaqMan 3D-PCR performed with the 7300 Real-Time PCR System (Applied Biosystems); we used a procedure described elsewhere [28], with slight modifications. In brief, the HBV DNA fragments were amplified by use of 3D-PCR in which the denaturation temperature was set lower than usual so that only G-to-A hypermutated genomes would be amplified. The amplification conditions were as follows: activation at 95°C for 10 min; followed by initial denaturation at 89°C for 20 min, to allow nonhypermutated genomes reanneal; and 45 cycles of amplification (denaturation at 89°C for 20 s, annealing at 50°C for 30 s, and extension at 62°C for 90 s). TaqMan PCR was performed using the following primers: 5'-ACTTCAACCCCAACAMRRATCA-3' (nt 2978–2999) and 5'-AGAGYTTGKTGGAATGTKGTGGA-3' (nt 24–1), where M is A or C, R is G or A, Y is T or C, and K is G or T. The probe was a 6-carboxyfluorescein (FAM)–labeled MGB probe, 5'-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3' (nt 3184–3167). The detection limit of hypermutated genomes was 10^2 copies/mL, and nonhypermutated genomes were not amplified by 3D-PCR [28]. The reproducibility of the assay was quite high (as indicated by the small standard deviation relative to the results of the quantitative PCR control reaction), as reported in our previous study [28].

Cell culture and transfection. HepG2 cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and transfected with the plasmid pTRE-HB-wt, which contained 1.4–genome length wild-type HBV genomes [31], by calcium phosphate precipitation. Seventy-two hours after transfection, the supernatant was collected for HBV DNA quantification by real-time PCR and for quantitative analysis of G-to-A hypermutated genomes [28]. The remaining supernatant was stored at −80°C for infection experiments using human hepatocyte–chimeric mice.

Quantitative analysis of G-to-A hypermutated genomes with human hepatocyte–chimeric mice. A human hepatocyte–chimeric mouse model was developed, as described previously [32], and used in infection and IFN-treatment experiments. The human hepatocytes progressively repopulated the murine host liver and were susceptible to HBV produced in cultured cell lines [31]. All animal protocols were in accordance with the guidelines of the local animal experimentation committee. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Hepatocyte-chimeric mice were inoculated with 500 μL of the supernatant produced by transiently transfected cell lines. After confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days (the IFN-α was a gift from Hayashibara Biochemical Labs in Okayama, Japan). Human serum albumin in mouse serum was measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories), used in accordance with the manufacturer's instructions.

Statistical analysis. Differences between clinical groups with respect to HBV DNA and e antigen levels were examined for statistical significance, using the Mann-Whitney *U* test. A *P* value $<.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with StatView (version 5.0; SAS Institute).

RESULTS

Clinical course of disease in patients with increased ALT levels and fluctuations in the number of hypermutated genomes. Figure 1*A*–1*D* shows clinical courses for 4 representative patients (patients 1–4 in Table 1) with chronic HBV infection who experienced increases in ALT level. We observed marked decreases in HBV DNA level in association with marked increases in hypermutated genomes (figure 1*A*–1*C*, black arrows). In contrast, there was no apparent reduction in HBV level in the absence of an increase in hypermutated genomes (1*A*–1*D*, white arrows). We also analyzed the effect of IFN therapy on the number of hypermutated genomes. In some patients, we observed an increase in the number of hypermutated genomes during IFN therapy (figure 1*B* and 1*C*) as well as a marked increase in the number of hypermutated genomes and a reduction of the virus accompanied by an increase in ALT level just after cessation of IFN therapy (1*A*–1*C*, black arrows). However, in some patients, such as patient 1 (figure 1*A*), we observed no apparent increase in the number of hypermutated genomes in response to IFN therapy. However, the number of hypermutated genomes observed in samples from this patient obtained just before the initiation of IFN therapy (996/10^6 genomes) was already higher than the baseline level (157/10^6 genomes). Samples from patient 4 (figure 1*D*) showed an increase in the number of hypermutated genomes during IFN therapy (1907/10^6 genomes), though this is less than the increase observed during natural exacerbation (12,404/10^6 genomes). In fact, there was no significant difference between IFN-treated patients and untreated patients with respect to the number of hypermutated genomes observed (data not shown). These results suggest that the host's antiviral immunity level was higher at baseline than it was after

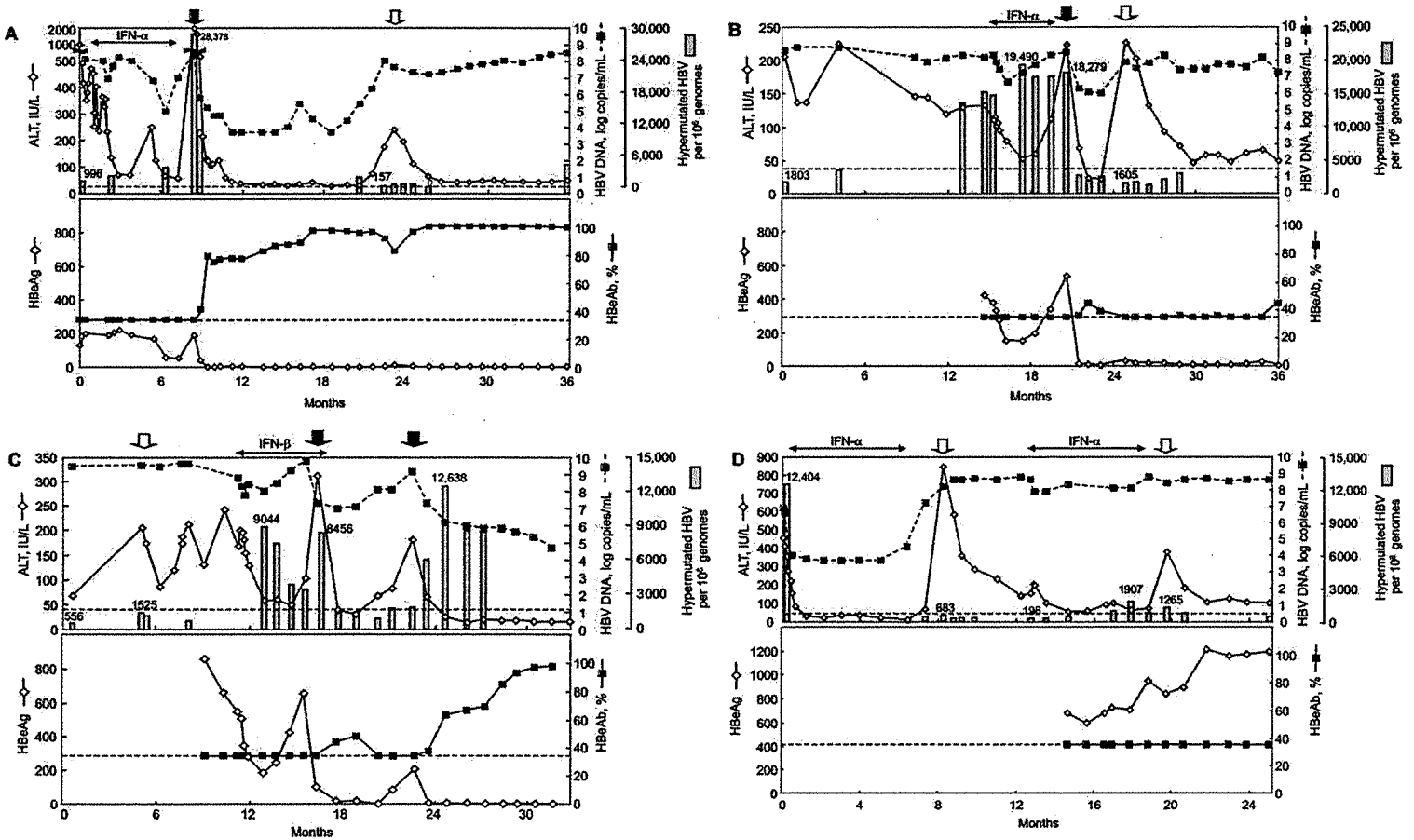

Figure 1. Clinical courses for 4 patients *(A–D)* with chronic hepatitis B virus (HBV) infection who experienced exacerbation of infection. *Black arrows,* exacerbation associated with an increase in the number of hypermutated genomes (>5 times basal levels); *white arrows,* exacerbation not associated with an increase in the number of hypermutated genomes; *horizontal dotted lines,* upper normal limit of alanine aminotransferase (ALT) (40 IU/mL; *upper panel, A–D*) and the detection limit for antibody against e antigen (HBeAb) (35%; *lower panel, A–D*). HBeAb, antibody against HBV e antigen; HBeAg, HBV e antigen; IFN, interferon.

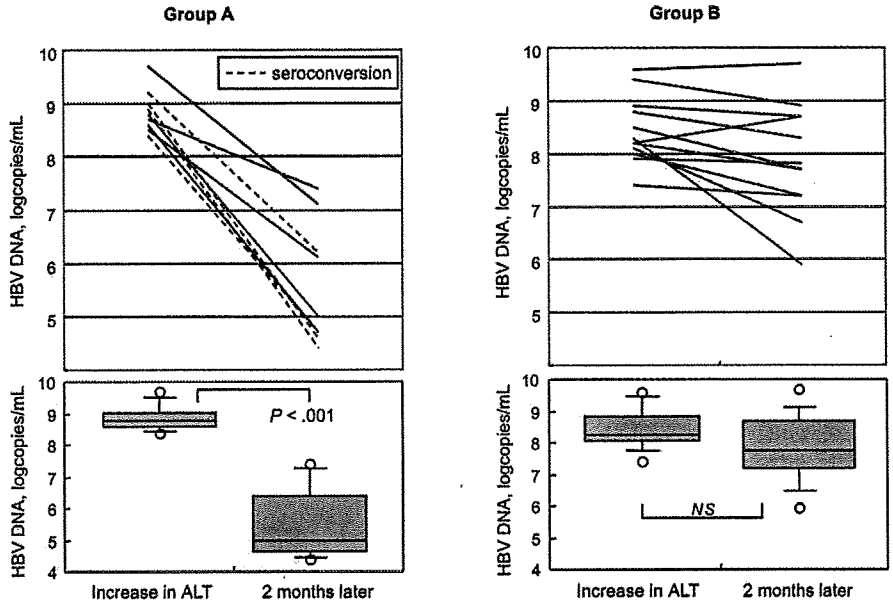

Figure 2. Relationship between increase in the number of hypermutated genomes and plasma levels of hepatitis B virus (HBV) DNA in 17 patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a >5-fold increase in the number of hypermutated genomes; group B included 12 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual HBV DNA levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for HBV DNA levels at same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

IFN or that the feedback system for IFN signaling was already active before initiation of therapy.

We also compared the degree of reduction in the plasma HBV DNA level for exacerbations (i.e., increases in ALT level) associated with a marked increase in the number of hypermutated genomes (i.e., those in which the peak number was >5 times the number observed prior to exacerbation) and for exacerbations not associated with such an increase. As shown in figure 2, 8 of 9 exacerbations that were coupled with a marked increase in the number of hypermutated genomes (group A) were associated with a >2-log reduction in the HBV DNA level. In contrast, only 1 of the 12 exacerbations not associated with a marked increase in the number of hypermutated genomes (group B) was associated with a >2-log reduction in plasma HBV DNA level. The median serum HBV DNA level decreased from 8.8 to 5.0 log copies/mL among the patients in group A ($P < .001$) but did not decrease for patients in group B (figure 2).

In addition, we compared the reduction in e antigen level for these 2 groups. Levels were reduced in both groups, but the median reduction was more prominent for patients in group A than for those in group B (figure 3). All 4 exacerbations coupled with e antigen seroconversion (from positive to negative) were associated with marked increase in hypermutated genomes (figure 3).

Effect of IFN treatment on the rate of HBV hypermutation in chimeric mice. Next, we examined the effect of IFN treatment on G-to-A hypermutation in HBV genomes in human hepatocyte–chimeric mice. Two mice were intravenously injected with supernatant produced by HepG2 cells transiently transfected with a plasmid containing 1.4–genome length wild-type HBV genomes. Ten weeks later, after confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days. We observed an ~1.5-log reduction in plasma HBV DNA level accompanied by an increase in the number of hypermutated genomes in both mice (figure 4*A*). In a mouse inoculated with HBV but treated with phosphate-buffered saline, no increase of hypermutated genomes was observed (figure 4*B*). We also observed a 36-fold increase in the level of APOBEC3G mRNA, as determined by human oligonucleotide microarray (data not shown).

Infectivity of hypermutated genomes. To study the biological significance of hypermutated genomes, culture supernatant from HepG2 cells transfected with both HBV and APOBEC3G (5 μg each) was injected into a chimeric mouse. As shown in figure 5, the culture supernatant contained a large number of hypermutated genomes. In contrast, we could not detect hypermutated genomes in the chimeric mouse inoculated with this

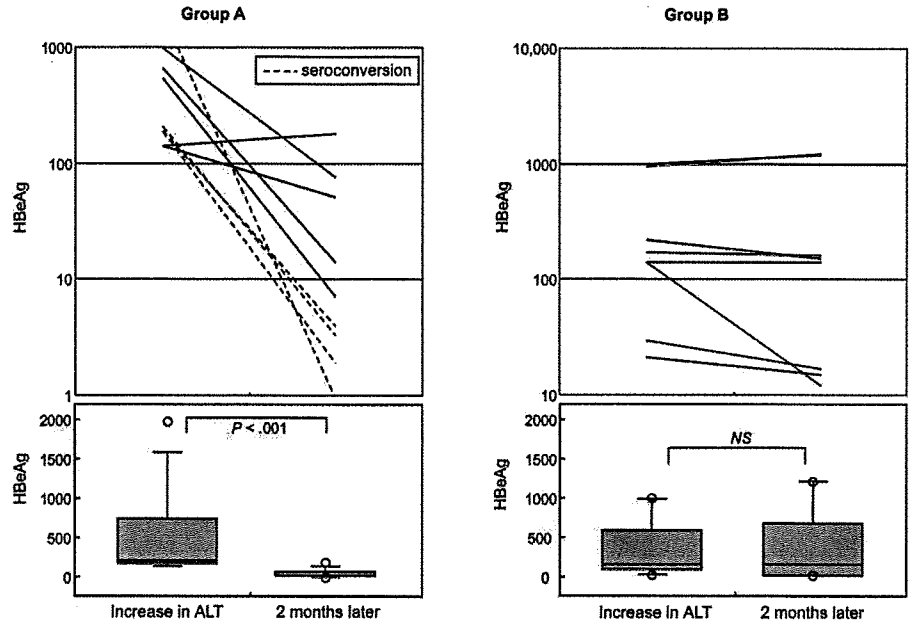

Figure 3. Relationship between increase in the number of hypermutated genomes and hepatitis B virus (HBV) e antigen (HBeAg) levels in 15 HBeAg-positive patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a >5-fold increase in the number of hypermutated genomes; group B included 8 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual e antigen levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for e antigen levels at these same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

supernatant (figure 5*A* and 5*B*). These results suggest that the infectivity (or replication ability) of HBV with hypermutated genomes is very poor. It is possible that the inoculum contained less abundantly mutated genomes. To test this, we cloned and sequenced 72 clones of 217-bp DNA fragments amplified at a denaturation temperature of 95°C. Of 72 clones obtained from the inoculum, we found 1 clone with 8 G-to-A substitutions, 1 clone with 5 substitutions, 2 clones with 3 substitutions, and 1 clone with 1 substitution (figure 5*C*). In contrast, 1 of the 72 clones obtained from the mouse serum had 1 G-to-A substitution. If G-to-A substitutions were excluded, the only other nucleotide substitution observed in the 144 clones sequenced was a single C-to-T substitution.

DISCUSSION

In a previous study, we found that the majority of serum samples obtained from HBV-infected patients contained a small number of hypermutated genomes [27]. Recently, we developed a method (TaqMan 3D-PCR) to measure small numbers of hypermutated genomes [28]. Using this method, we reported dual antiviral effects for APOBEC3G, namely induction of hypermutation and reduction of viral replication. We also reported that IFN increased the transcription of APOBEC3G and enhanced the effect of the protein in vitro [28]. Other investigators also showed that IFN enhances the action of APOBEC proteins against HIV [18–21]. It is thus assumed that the antiviral effect of APOBEC proteins should be enhanced by IFN and other cytokines in vivo.

In the present study, we showed that an increase in ALT level accompanied by an increase in the number of hypermutated genomes was associated with reduction in the plasma HBV DNA level. In contrast, no decrease in HBV DNA level was observed if the increase in ALT level occurred in the absence of an increase in the number of hypermutated genomes. It is difficult to know which of the dual antiviral effects of APOBEC3G (or other APOBEC proteins) reduced the viral level. It is also impossible to estimate the importance of APOBEC proteins in this reduction. However, it is clear that the increase in the number of hypermutated genomes of HBV correlates with activation of the host antiviral defense against HBV.

We also demonstrated that exacerbations of HBV infection associated with a marked increase in the number of hypermutated genomes were associated not only with a decrease in the plasma HBV DNA level but also with clearance of e antigen.

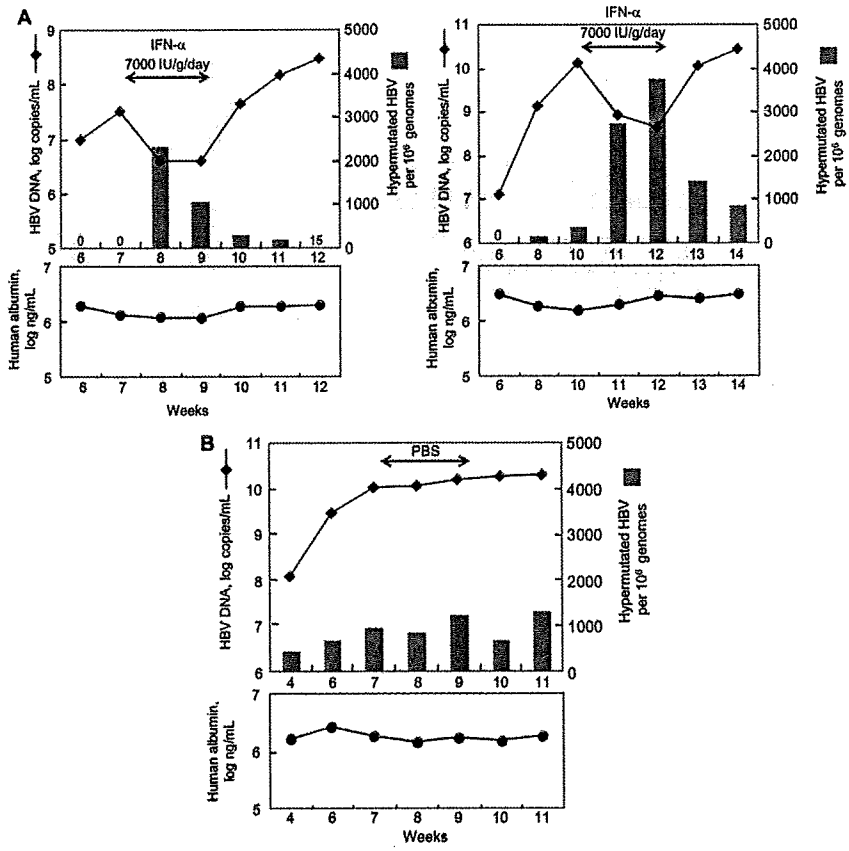

Figure 4. Effect of interferon (IFN)–α therapy on hepatitis B virus (HBV) hypermutation in HBV-infected, human hepatocyte–chimeric mice. Two chimeric mice *(A)* were inoculated with recombinant wild-type HBV produced by transfected HepG2 cells; 10 weeks later, after confirmation of high-level HBV viremia, they were treated with IFN-α at 7000 IU/g/day for 14 days, by intramuscular injection. *Upper panels* in both parts of *A*, serum HBV DNA levels and the number of hypermutated genomes; *lower panels* in both parts of *A*, human serum albumin concentrations. Note that the albumin levels are stable during IFN-α therapy. A control mouse *(B)* was inoculated with recombinant wild-type HBV produced by transfected HepG2 cells and treated with phosphate-buffered saline (PBS). Upper and lower panels of *B* show the same information as in *A*.

Furthermore, all exacerbations followed by seroconversion to positivity for antibody against e antigen were associated with a marked increase in the number of hypermutated genomes. Clearance of e antigen often results from a G-to-A nucleotide substitution at the first position of a 5'-GGGG stretch in the precore coding sequence (the G1896A mutation). Because this substitution (changing TGGGG to TAGGG) is in agreement with the dinucleotide pattern preferentially edited by APOBEC3G, one might assume that G-to-A substitution in this region could be caused by this enzyme and is related to the clearance of e antigen. However, we observed that hypermutation was induced in only some genomes, whereas the majority of genomes were unaffected. Thus, it seems unlikely that APOBEC proteins play a role in seroconversion to positivity for antibody against e antigen, although it is still possible that the 5'-GGGG stretch in the precore region is the preferred editing site for the enzyme. Importantly, such substitution of the 5'-GGGG stretch should result in the occurrence of multiple stop codons (TAG, TGA, and TAA) in HBV genomes, as we observed and reported in our previous study [28], which makes the replication of mutated genomes impossible.

In the present study, we did not observe any increase in the number of hypermutated genomes during IFN therapy in some patients. This finding is discrepant from the results of previous in vitro experiments that showed increased numbers of hypermutated genomes after the application of IFN [28]. Interestingly, our experimental results also showed the induction of APOBEC3G gene expression, an increase in the number of hypermutated genomes, and a reduction of plasma HBV DNA level in 2 human hepatocyte–chimeric mice treated with IFN (figure 4). What is the reason for the lack of increase in hypermutation in some IFN-treated patients? We usually administer IFN to patients who have high ALT levels. The patients in this study had abnormal ALT levels prior to treatment with IFN—that is, their livers were inflamed, and the levels of many cytokines produced by the immune cells in the liver were already high. We presume that the effect of these elevated cytokine levels masked the effect of the IFN we administered. It could also be argued that the effect

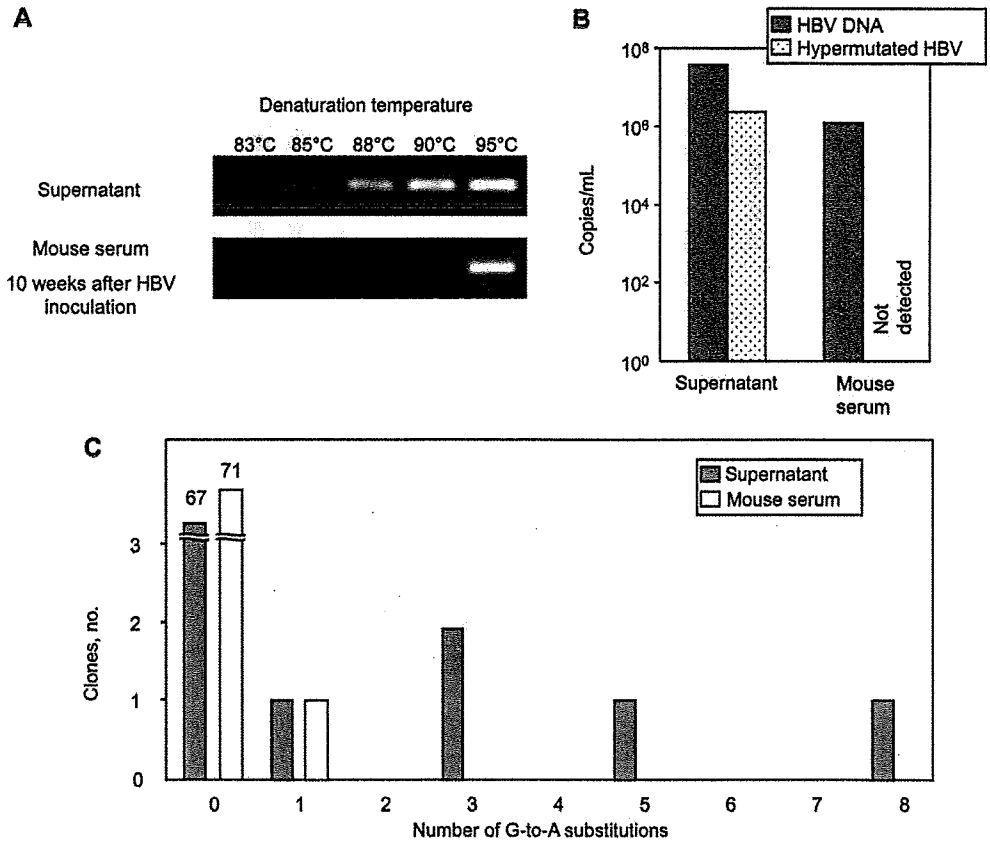

Figure 5. Results for a human hepatocyte–chimeric mouse inoculated with hepatitis B virus (HBV) produced by HepG2 cells transfected with an equal amount (5 μg each) of HBV and apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like 3G plasmids. The inoculum contained ~6.25% hypermutated genomes. A serum sample was obtained 10 weeks after the inoculation. *A*, HBV DNA was amplified by polymerase chain reaction (PCR) that used different denaturation temperatures and run on 2% agarose gel. *B*, Quantitative measurement of HBV DNA and hypermutated DNA in the inoculum and mouse serum. *C*, Number of G-to-A substitutions found in each of 72 clones obtained from products of PCR of culture supernatant or mouse serum.

observed in mice represents the absence of the immune response in mice, whereas the lack of a clear response to IFN in the study patients was the result of the complex immune response in human beings. Alternatively, the concentrations of IFN in treated patients might be lower than those used for the cell culture or the chimeric mice. Although we did not perform this analysis in the present study, it would be interesting to determine the expression levels of APOBEC proteins and IFN-stimulated genes in the liver of IFN-treated patients.

The present study showed that the number of hypermutated genomes increased during some increases in ALT level, probably as a result of IFN-activated APOBEC proteins and other cytokines in patients with chronic hepatitis B. However, the number of hypermutated genomes was very small, only 28,378 in 10^6 HBV genomes at most (figure 1*A*). Because it was possible that the less abundantly hypermutated genomes were not detected (i.e., that genomes with only 1 or 2 G-to-A substitutions were not amplified by 3D-PCR), cloning and sequencing were performed to detect such genomes. However, the number of genomes containing G-to-A substitutions was still low (5 [6.9%] of 72 clones), even in the culture medium of HepG2 cells cotransfected with APOBEC3G and HBV (figure 5*C*). This means that the number of genomes with only a small number of G-to-A substitution was not high, suggesting that only selected DNA molecules were heavily mutated while the remaining DNA was not. Does this mean that the effect of APOBEC proteins in antiviral defense is trivial in patients with chronic HBV infection? It is a possible that the heavily deaminated genomes are an easy target for uracil DNA glycocylase. Although the dual antiviral effects of APOBEC proteins are currently known to reduce the amount of HBV, the importance and magnitude of APOBEC proteins with respect to in vivo virus reduction should be investigated further.

Treatment of patients with chronic HBV infection has improved with the advent of new nucleoside and nucleotide analogues. However, reactivation of HBV and flare-ups of hepatitis are often seen in patients who stop such therapy. Furthermore, hepatitis B surface antigen clearance is rare in patients treated

with these antiviral drugs. On the other hand, most patients with chronic HBV infection achieve sufficient viral suppression and disease quiescence through immunological suppression of the virus. As we showed in this study, the immunological suppression of HBV is much stronger than that achieved with IFN therapy, but it is often transient. It is thus necessary to clarify the mechanism of transient immune response and to develop treatment that produces persistent suppression of HBV. Quantitative measurement of hypermutated genomes should be useful in monitoring the immune response in this context.

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