of the ribavirin dose, which is the variable factor, unlike baseline factors, plays an important role in suppressing the virologic relapse in patients with CH-C genotype 1 treated by Peg-IFN plus ribavirin treatment. This suggests that maintaining the ribavirin dose should lower the relapse rate even in patients with advanced fibrosis who are liable to relapse. In fact, among patients with advanced fibrosis (METAVIR score 3–4), the relapse rate in those given ≥10 mg/kg/day of the average ribavirin dose was significantly lower than that in patients given <10 mg/kg/day of ribavirin (36% vs. 71%). However, the sample size was too small for subsequent analysis with stratification. Further study is needed to clarify the impact of ribavirin dose on viral relapse in patients with progression of fibrosis.

The relapse rate among patients with c-EVR showed a decline according to the increase in ribavirin dose during treatment week 0–48 and was not affected by the Peg-IFN α-2b dose when the patients were given more than 0.9 μg/kg/week of Peg-IFN α-2b. Among the patients with c-EVR, none with RVR had a relapse and all attained SVR irrespective of the dose of Peg-IFN α-2b or ribavirin. Examination of the impact of dose reduction after week 12 on relapse among patients with c-EVR showed that the ribavirin dose reduction after week 12 tended to affect the relapse rate in patients given ≥10 mg/kg/day of the ribavirin dose during the first 12 weeks, while the Peg-IFN α-2b dose after week 12 could be reduced without any increase in relapse rate in patients given more than 0.6 μg/kg/week of the average dose of Peg-IFN α-2b. On the other hand, maintaining the ribavirin did not lead to reduce the relapse rate in patients with LVR. About half relapsed even when given ≥12 mg/kg/day of the average ribavirin dose. This suggested that the relapse rate could not be reduced by management of the ribavirin dose in patients with LVR. Extended therapy should be chosen in LVR patients as shown in the previous studies [20–23].

Shiffman *et al.* [24] recently reported that maintaining the Hb level with epoetin alpha did not enhance SVR if ribavirin was started at the standard dose (800–1400 mg/day, mean dose 13.3 mg/kg/day), although discontinuance and the reduction rates of ribavirin were decreased and a higher mean dose of ribavirin was administered in comparison with those treated with Peg-IFN plus ribavirin without epoetin. If these findings apply to patients with CH-C genotype 1, this would suggest that the ribavirin dose does not need to be maintained during treatment with Peg-IFN plus ribavirin, which would not agree with our findings. However, closer examination of the Shiffman *et al.* study shows that Peg-IFN plus a higher dose of ribavirin (1000–1600 mg/day, mean dose 15.2 mg/kg/day) with epoetin was found to suppress the relapse rate and enhance SVR. These data agree with ours with respect to the point that higher doses of ribavirin are associated with a lower relapse rate. What differs is the ribavirin dose needed to suppress the relapse. This is likely to be due to ethnic differences between the subjects. In Shiffman's study, approximately 40% were African-Americans in whom the virologic response is well established as being significantly lower than those of other ethnic groups [25,26], while in our study, all subjects were Japanese. In the African-Americans treated with Peg-IFN plus standard-dose ribavirin, the relapse rate (calculated from 48% of ETR and 19% of SVR) was 60%, while 18% relapse (from 38% of ETR and 31% of SVR) occurred in those given Peg-IFN plus high-dose ribavirin. The relapse rate of patients with c-EVR in our study was 19%, which was very close to that for those with Peg-IFN plus high-dose ribavirin in Shiffman's study. Ribavirin does not have a direct antiviral action against HCV [27,28], and is considered to play an important role in accelerating HCV-infected cell clearance [29] and eradicating them completely when an immune response against infected cells is induced by IFN or Peg-IFN [30,31]. Therefore, the difference between patients who are easy or difficult to treat due to ethnic differences or differences in response to Peg-IFN can result in the need for different doses of ribavirin to suppress the relapse rate in patients with CH-C genotype 1.

In conclusion, our results have demonstrated that ribavirin is dose-dependently correlated with a relapse in patients with CH-C genotype 1 responding to Peg-IFN plus ribavirin. Maintaining a high dose (≥12 mg/kg/day) of ribavirin during the full treatment period could strongly suppress the relapse in such patients, while Peg-IFN α-2b could be reduced without affecting relapse in patients with c-EVR. This possibility should be explored in a prospective study.

## ACKNOWLEDGEMENTS AND DISCLOSURES

Other institutions and participants in the Osaka Liver Forum are: Osaka General Medical Center, A Inoue; Toyonaka Municipal Hospital, M Inada; Sumitomo Hospital, A Yamada; Kinki Central Hospital of Mutual Aid Association of Public School Teachers, E Hayashi; Yao Municipal Hospital, H Fukui; Otemae Hospital, Y Doi; Itami City Hospital, T Kashihara; Ashiya Municipal Hospital, K Kiriyama; National Hospital Organization Minami Wakayama Medical Center, K Fujimoto; Saiseikai Senri Hospital, K Suzuki; Nishinomiya Municipal Central Hospital, H Ogawa; Kano General Hospital, S Kubota; Saso Hospital, M Nishiuchi; and Osaka Kaisei Hospital, N Imaizumi.

This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from Ministry of Health Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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J Gastroenterol (2009) 44:601–607
DOI 10.1007/s00535-009-0038-6

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Factors contributing to antiviral effect of adefovir dipivoxil therapy added to ongoing lamivudine treatment in patients with lamivudine-resistant chronic hepatitis B

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Received: 25 November 2008 / Accepted: 9 January 2009 / Published online: 22 April 2009


**Abstract**
*Purpose* The antiviral effect of adefovir dipivoxil (ADV) added to ongoing lamivudine (LAM) treatment for LAM-resistant chronic hepatitis B (CHB) differs among patients. We investigated clinical factors affecting the response to ADV therapy in LAM-resistant CHB.
*Methods* The subjects were 75 LAM-resistant CHB patients treated with ADV in addition to LAM. Virological response (VR) was defined as HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the start of ADV therapy. Clinical factors contributing to VR were examined by univariate and multivariate analyses.
*Results* Lower HBV DNA at baseline and negative hepatitis B e antigen (HBeAg) were significant factors affecting VR in univariate analysis. In multivariate analysis, lower HBV DNA at baseline ($P = 0.005$), negative HBeAg ($P = 0.009$), and higher ALT ($P = 0.036$) were significant independent factors contributing to VR. In HBeAg-positive patients, HBV DNA clearance was more frequently observed during ADV therapy in patients with baseline HBV DNA ≤7.0 logcopies/ml than in those with baseline HBV DNA >7.0 logcopies/ml. By contrast, the link of lower HBV DNA at baseline to better therapeutic response was not evident in HBeAg-negative patients.

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*Conclusion* In ADV therapy added to ongoing LAM treatment for LAM-resistant CHB, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. Addition of ADV should be done promptly before marked increase in HBV DNA, especially in CHB patients showing LAM resistance positive for HBeAg.

**Keywords** Adefovir dipivoxil · Lamivudine resistance · Chronic hepatitis B

## Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) [1]. Chronic HBV infection can cause liver cirrhosis and hepatocellular carcinoma (HCC), resulting in hepatic disease-related deaths of 500,000 to 1.2 million persons [2, 3]. To prevent disease progression and improve the prognosis of patients with chronic HBV infection, HBV DNA replication must be continuously suppressed as much as possible by antiviral therapy. For this purpose, nucleos(t)ide analogs are currently used for a wide range of patients with chronic HBV infection because of their strong antiviral activities and fewer side effects.

Lamivudine (LAM) is the first approved nucleos(t)ide analog for chronic hepatitis B (CHB) patients, but the increasing incidence of LAM resistance during long-term LAM therapy is a serious problem. The emergence rate of the LAM-resistant virus has been reported to be 24% at 1 year and 70% at 4 years of treatment [4]. Almost all LAM resistance is caused by rtM204V/I mutation occurring in the reverse transcriptase domain of the HBV polymerase gene [5].

To counteract this resistance, adefovir dipivoxil (ADV) was considered as it exerts antiviral effects not only on nucleos(t)ide analog-naïve CHB patients but also on LAM-resistant ones [6–9]. ADV-resistant mutation has been reported to be detected in 11% of patients at 3 years and 29% at 5 years for nucleos(t)ide analog-naïve CHB patients [10]. ADV resistance results from rtA181V/T and/or rtN236T mutation [10]. Either switching from LAM to ADV or adding ADV to LAM has been shown to be effective for LAM-resistant CHB patients. In the case of switching from LAM to ADV, ADV resistance has been reported to appear in 18% of patients at 1 year, which is more frequent than in the case of ADV monotherapy for nucleos(t)ide analog-naïve patients [11]. On the other hand, in the case of ADV administration in addition to LAM, the emergence of resistant virus for both LAM and ADV has been reported to be rare for at least 3 years of treatment [12]. Therefore, ADV therapy added to ongoing LAM treatment is currently accepted as the main therapeutic regimen for LAM-resistant CHB patients rather than a switch from LAM to ADV. However, the antiviral effect of ADV therapy in addition to LAM treatment differs among patients with LAM-resistant CHB.

In this study, we investigated clinical factors influencing the therapeutic efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients.

## Patients and methods

### Patients

The participating centers were 12 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, NTT Nishinihon Osaka Hospital, Higashiosaka City General Hospital, Suita Municipal Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital, and Osaka University Hospital). The subjects were 75 consecutive CHB patients showing LAM resistance. Before the preceding LAM therapy, they all had had hepatitis B surface antigen (HBsAg) for more than 6 months and levels of HBV DNA detectable by the polymerase chain reaction (PCR) method [13]. None of them tested positive for hepatitis C virus antibody or human immunodeficiency virus antibody, nor was there evidence of other forms of liver diseases, such as alcoholic liver disease, drug-induced liver disease, or autoimmune hepatitis.

### Anti-HBV treatment

All patients were administered 100 mg of LAM daily. Thirteen (17%) patients had had a history of interferon (IFN) therapy. LAM resistance was judged by detection of rtM204V/I mutation (for 37 patients) or by the existence of virological breakthrough (for 38 patients). Virological breakthrough was defined as the reappearance of detectable HBV DNA of more than 1 log increase in HBV DNA from the nadir on repeated occasions. The median duration of the preceding LAM therapy was 38 (range, 11–83) months. After the emergence of LAM resistance, all patients received 10 mg of ADV daily in addition to ongoing LAM therapy. After the commencement of ADV therapy, liver function and HBV DNA tests were conducted monthly for the first 6 months and every 2 months thereafter. Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were checked every 2 months. The median follow-up duration of ADV therapy was 22 (range 12–51) months. HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the beginning of ADV therapy was defined as a virological response (VR).

### Baseline characteristics of the patients

The baseline characteristics of the patients at the commencement of ADV therapy were as follows. They were 59 males and 16 females, with a median age of 54 (range 27–79) years. Forty-one (55%) tested positive for HBeAg, and anti-HBe developed in 34 patients. The virus was genotyped for 13 patients, all of whom were infected with HBV of genotype C. The HBV DNA ranged from 3.1 to >7.6 (median 7.1) logcopies/ml, and the median ALT level ranged from 15 to 500 (median 105) IU/L. The median levels of total bilirubin and albumin were 0.8 (range 0.4–3.9) mg/dl and 3.9 (range 2.1–4.8) g/dl, respectively. The median platelet counts were 11.7 (range 3.5–25.5) $\times$ $10^4/mm^3$. Of the 75 patients, 27 (36%) showed features of cirrhosis by liver biopsy and/or imaging procedures. Five patients (7%) developed HCC as detected by imaging modalities.

### HBV testings

HBsAg, HBeAg, and anti-HBe were examined by chemiluminescent immunoassay. HBV DNA was measured by the PCR-based method (Amplicor HBV monitor, Roche Diagnostics, Tokyo, Japan) [13], with a lower detection limit of 2.6 logcopies/ml. The LAM-resistant rtM204V/I mutation was examined by PCR-enzyme-linked minisequence assay [14]. HBV genotype was determined based on PCR-direct sequencing of portions of core and polymerase genes. The primers used for this study were BF1s (5′-TTT TTC ACC TCT GCC TAA TCA-3′, nt 1821–1841), BR3 (5′-TTC CCG AGA TTG AGA TCT TC-3′, nt 2440–2421), BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369), and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195–1176) [15, 16].

### Statistical analysis

Group comparisons were carried out by the chi-square test, Student's $t$ test and Mann–Whitney's $U$ test. Independent factors contributing to VR during ADV therapy added to ongoing LAM treatment were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A $P$-value of less than 0.05 (two-tailed) was considered to indicate a significant difference. All statistical analyses were performed using the SPSS version 15.0J software (SPSS, Chicago, IL).

## Results

### Virological and biochemical response to ADV therapy added to ongoing LAM in CHB patients showing LAM resistance

Of the 75 CHB patients showing LAM resistance who underwent ADV therapy added to ongoing LAM treatment, HBV DNA clearance was achieved in 29 (39%) of 75 at 6 months, 35 (47%) of 75 at 12 months, and 34 (72%) of 47 at 24 months. Among the HBeAg-positive patients, HBeAg loss was observed in 8 (20%) of 41 at 6 months, 7 (18%) of 39 at 12 months, and 6 (22%) of 27 at 24 months. As for the biochemical response, ALT normalization (≤40 IU/l) was seen in 57 (76%) of 75 at 6 months, 56 (75%) of 75 at 12 months, and 40 (85%) of 47 at 24 months of treatment.

### Pretreatment clinical factors associated with therapeutic response to ADV in addition to LAM treatment

We first investigated pretreatment clinical factors associated with the therapeutic efficacy of ADV added to ongoing LAM treatment by univariate analysis. The baseline characteristics of patients at the beginning of ADV therapy in addition to LAM in the presence or absence of VR are shown in Table 1. Patients showing VR had significantly lower HBV DNA at baseline than patients who did not achieve VR [median 6.3 (range 3.1 to >7.6) vs. 7.3

**Table 1** Patient clinical characteristics at the beginning of ADV therapy in addition to LAM in LAM-resistant CHB patients in the presence or absence of virological response (VR)

| Clinical characteristics | VR ($n$ = 35) | Non-VR ($n$ = 40) | $P$ value |
|---|---|---|---|
| Gender (male/female) | 26/9 | 33/7 | 0.386 |
| Age (years) | 52 (28–67) | 55 (27–79) | 0.896 |
| Duration of prior LAM therapy (months) | 38 (12–83) | 37 (13–64) | 0.856 |
| Positive HBeAg | 12 (34%) | 29 (73%) | 0.001 |
| HBV DNA (logcopies/ml) | 6.3 (3.1 to >7.6) | 7.3 (3.9 to >7.6) | 0.002 |
| ALT (IU/l) | 106 (16–500) | 75 (15–455) | 0.136 |
| Total bilirubin (mg/dl) | 0.9 (0.4–3.9) | 0.7 (0.4–3.9) | 0.664 |
| Albumin (g/dl) | 4.0 (2.4–4.8) | 3.8 (2.1–4.6) | 0.351 |
| Platelet count ($\times 10^4/mm^3$) | 12.2 (4.8–24.1) | 11.5 (3.5–25.5) | 0.854 |
| Liver disease (chronic hepatitis/cirrhosis) | 20/15 | 28/12 | 0.247 |
| Presence of HCC (%) | 2 (6%) | 3 (8%) | 0.757 |

Continuous variables are expressed as median (range)

**Table 2** Baseline factors affecting virological response (logistic regression analysis, stepwise method)

| Factors | Category | Odds ratio | 95% CI | *P* |
|---|---|---|---|---|
| Gender | Male/female | | | NS |
| Age (years) | By 1 year | | | NS |
| Duration of prior LAM therapy (months) | By 1 month | | | NS |
| HBeAg | Negative/positive | 5.766 | 1.855–36.62 | 0.009 |
| HBV DNA (logcopies/ml) | By 1 logcopy/ml | 2.362 | 1.335–5.178 | 0.005 |
| ALT (IU/l) | By 1 IU/l | 1.006 | 1.000–1.011 | 0.036 |
| Total bilirubin (mg/dl) | By 1 mg/dl | | | NS |
| Albumin (g/dl) | By 1 g/dl | | | NS |
| Platelet count ($\times 10^4/mm^3$) | By $1 \times 10^4/mm^3$ | | | NS |
| Liver disease | Chronic hepatitis/cirrhosis | | | NS |
| Presense of HCC (%) | No/yes | | | NS |

*CI* Confidence interval, *NS* not significant

(range 3.9 to >7.6), $P = 0.002$]. HBeAg was detected in only 12 (34%) of 35 patients with VR, compared with 29 (73%) of 40 patients without VR ($P = 0.001$). Gender ratio, age, duration of preceding LAM therapy, ALT, total bilirubin, albumin, platelet counts, disease severity, and presence of HCC did not differ between VR and non-VR patients.

Factors affecting the therapeutic response to ADV therapy in addition to ongoing LAM were also evaluated by multivariate analysis (Table 2). Eleven pretreatment clinical factors were applied to the analysis as variables. Two factors, lower baseline HBV DNA ($P = 0.005$, odds ratio: 2.362, 95% confidence interval: 1.335–5.178) and negative HBeAg ($P = 0.009$, odds ratio: 5.766, 95% confidence interval: 1.855–36.62), were selected as significant independent factors affecting VR, as was the case for univariate analysis. In addition, higher baseline ALT was also chosen as a significant independent factor ($P = 0.036$, odds ratio 1.006, 95% confidence interval: 1.000–1.011). As for the biochemical response to ADV therapy added to LAM, no pretreatment clinical factors showed a significant relationship with the occurrence of ALT normalization in our 75 LAM-resistant CHB patients.

## HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment according to HBeAg status

Next, we investigated HBV DNA clearance during ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients positive or negative for HBeAg (Fig. 1). In HBeAg-positive patients, HBV DNA was cleared in 8 (20%) of 41 at 6 months, 12 (29%) of 41 at 12 months, and 16 (59%) of 27 at 24 months. On the other hand, HBV DNA clearance was seen in 21 (62%) of 34 at 6 months, 23 (68%) of 34 at 12 months, and 18 (90%) of 20 at 24 months in HBeAg-negative patients. A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was

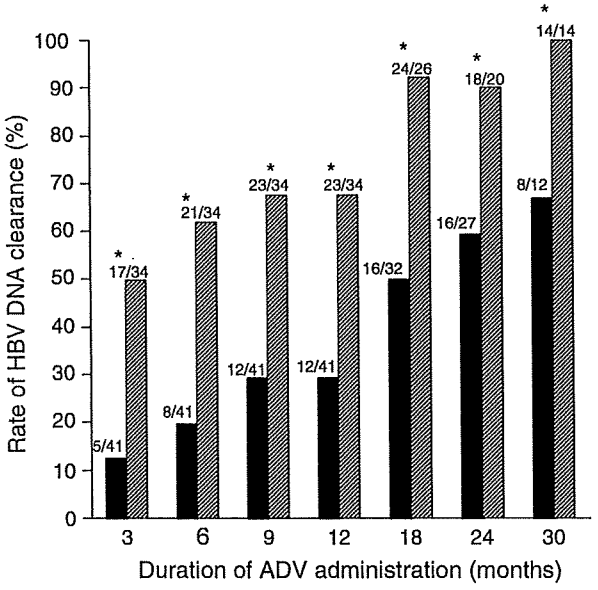


**Fig. 1** Rates of HBV DNA clearance in CHB patients positive or negative for HBeAg during ADV therapy in addition to LAM. $*P < 0.05$ between HBeAg-positive and HBeAg-negative patients. *Solid bars* HBeAg-positive patients, *hatched bars* HBeAg-negative patients

observed between HBeAg-positive and HBeAg-negative patients at 3, 6, 9, 12, 18, 24, and 30 months of treatment. Thus, patients negative for HBeAg tended to respond to ADV therapy added to ongoing LAM treatment better than those positive for it in LAM-resistant CHB.

## HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in relation to HBeAg status and baseline HBV DNA

We examined HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in HBeAg-positive and

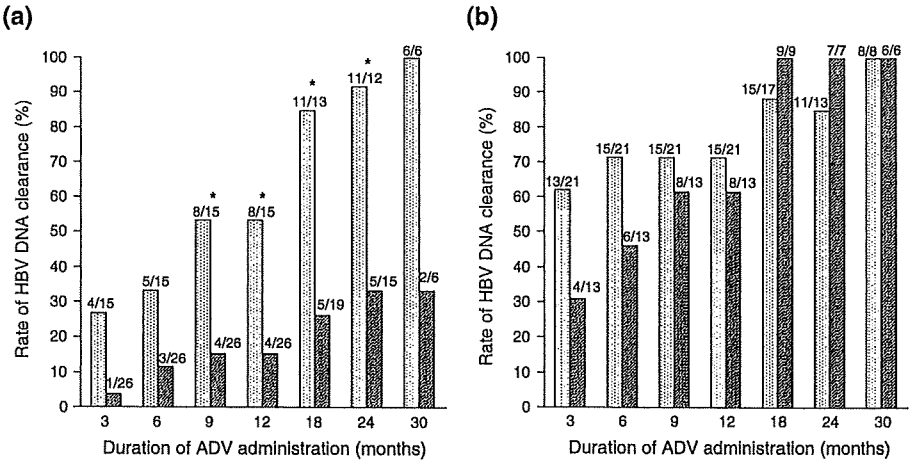


**Fig. 2** Rates of HBV DNA clearance during ADV therapy in addition to LAM according to HBV DNA at baseline in **a** HBeAg-positive CHB patients and **b** HBeAg-negative CHB patients. *$P < 0.05$ between patients with low (≤7.0 logcopies/ml) and high (>7.0 logcopies/ml) HBV DNA. *Dotted bars* Patients with HBV DNA ≤7.0 logcopies/ml at baseline, *hatched bars* patients with HBV DNA >7.0 logcopies/ml at baseline

HBeAg-negative CHB patients in relation to baseline HBV DNA. In the case of HBeAg-positive CHB patients (Fig. 2a), the rates of HBV DNA clearance were 33% (5/15) at 6 months, 53% (8/15) at 12 months, and 92% (11/12) at 24 months in patients with low viremia (baseline HBV DNA ≤7.0 logcopies/ml). By contrast, the frequencies of HBV DNA clearance were only in 12% (3/26) at 6 months, 15% (4/26) at 12 months, and 33% (5/15) at 24 months in patients with high viremia (baseline HBV DNA >7.0 logcopies/ml). A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was observed between patients with low and high viremia at 9, 12, 18, and 24 months of treatment. In the case of HBeAg-negative patients (Fig. 2b), the rates of HBV DNA clearance were 71% (15/21) at 6 months, 71% (15/21) at 12 months, and 85% (11/13) at 24 months in patients with low viremia (baseline HBV DNA ≤7.0 logcopies/ml). The frequencies of HBV DNA clearance were 46% (6/13) at 6 months, 62% (8/13) at 12 months, and 100% (7/7) at 24 months in patients with high viremia (baseline HBV DNA >7.0 logcopies/ml). No significant differences were observed in the frequency of HBV DNA clearance between patients with low and high viremia. According to these findings, the relevance of lower baseline HBV DNA for achieving a better antiviral effect was evident only in HBeAg-positive patients, but not in HBeAg-negative ones in ADV therapy added to LAM treatment for LAM-resistant CHB.

## Discussion

This study investigated factors affecting the antiviral efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients. Therapeutic efficacy was assessed as the presence or absence of VR. Both univariate and multivariate analyses revealed that lower baseline HBV DNA and negative HBeAg were strong factors associated with a better therapeutic response. Another significant factor revealed by multivariate analysis was high ALT, although it was weaker than the other two factors. In previous investigations, female gender, lower baseline HBV DNA, negative HBeAg, higher ALT, and genotype D rather than A have been reported to contribute to better VRs to ADV therapy in nucleos(t)ide-naïve and LAM-resistant CHB patients [17–21]. Our results agreed partially with them. The present study, as well as previous studies [18, 19], also revealed that a high baseline ALT may be a determining factor for a better response to ADV therapy in addition to LAM treatment in LAM-resistant CHB. This may be because the host immune response against viral antigens induced by active breakthrough hepatitis has a favorable antiviral effect during ADV therapy. In this study, however, a low baseline viremic level was shown to be a stronger factor than high baseline ALT. The baseline ALT level was the third factor contributing to VR. Therefore, in LAM-resistant CHB, ADV administration should be started before the flare-up of ALT elevation, especially in patients with severe liver disease such as cirrhosis.

In LAM-resistant patients, the HBV DNA level is low during the initial phase, but increases with time, leading to the onset of breakthrough hepatitis. Thus, in ADV therapy added to LAM treatment for LAM-resistant-CHB, the baseline HBV DNA level varies with the observation period after the emergence of LAM resistance. A previous report on Italian HBeAg-negative CHB patients showing LAM resistance revealed that patients with low viremia and normal ALT tended to respond to ADV therapy in addition to LAM treatment better than those with high viremia and abnormal ALT [17]. In the present study conducted in Japan, a genotype C-endemic area, such a close relationship between lower baseline HBV DNA and better therapeutic response was remarkable in

HBeAg-positive patients but not in HBeAg-negative ones. Our finding suggests that, in LAM-resistant CHB, ADV should be added before the HBV DNA begins to increase markedly, especially in HBeAg-positive patients.

In this study, none of the 75 patients showed virological breakthrough after the beginning of ADV administration. All displayed more than 1 log reduction of HBV DNA at 12 months of ADV treatment. This indicates that our patients may not have produced viruses resistant to both LAM and ADV. The emergence of resistant viruses has been reported to be rare in combination therapy using LAM and ADV for LAM-resistant CHB patients, although recent studies have found the existence of a virus resistant to both drugs [22, 23]. The rtA181V/T/S mutation has been reported to confer cross resistance to LAM and ADV [22, 23]. In ADV monotherapy for nucleos(t)ide analog-naïve CHB patients, the absence of HBV DNA reduction to <4 logcopies/ml at 24 weeks of treatment has been reported to be related to the higher emergence of a ADV-resistant virus [24], as is the case in LAM monotherapy [25]. In ADV therapy added to LAM treatment in LAM-resistant CHB patients, the poor response during the initial phase may lead to the development of virus resistance to LAM and ADV as well. From this point of view, the addition of ADV to ongoing LAM treatment before the elevation of HBV DNA may be beneficial in LAM-resistant CHB patients to avoid the development of a multi-drug-resistant virus. Recently, some investigators have reported that tenofovir disoproxil fumarate is effective against a virus resistant to both LAM and ADV [22, 23], but it has not yet been approved for clinical use.

Our results conclusively showed that, with ADV therapy added to LAM treatment for LAM-resistant CHB patients, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. After the emergence of LAM resistance, ADV should be added before the marked elevation of HBV DNA in order to attain better antiviral efficacy, especially in HBeAg-positive patients.

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J Gastroenterol (2009) 44:864–870
DOI 10.1007/s00535-009-0076-0

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Lamivudine-to-entecavir switching treatment in type B chronic hepatitis patients without evidence of lamivudine resistance

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Received: 17 February 2009 / Accepted: 15 April 2009 / Published online: 28 May 2009


**Abstract**
*Purpose* A considerable number of chronic hepatitis B (CH-B) patients remain under continuous lamivudine treatment, although switching treatment to entecavir could be beneficial. We investigated the antiviral efficacy of switching treatment to entecavir in CH-B patients without apparent evidence of lamivudine resistance during the preceding lamivudine treatment.
*Methods* Forty-four CH-B patients, who underwent lamivudine treatment for more than 6 months and showed no evidence of lamivudine resistance, switched to entecavir. Serial changes in hepatitis B virus (HBV) DNA were correlated with the patients' baseline HBV DNA at the commencement of entecavir administration. The entecavir-resistant substitution was examined by PCR-direct sequencing. The median follow-up period of entecavir treatment was 20 (10–23) months.
*Results* All 31 patients with baseline HBV DNA <2.6 logcopies/ml maintained HBV DNA-negative status during entecavir treatment. Of seven patients having HBV DNA of 2.6–<4.0 logcopies/ml, all achieved undetectable HBV DNA at the end of follow-up. As for six patients having HBV DNA ≥4.0 logcopies/ml, three patients achieved undetectable HBV DNA, whereas virological breakthrough was observed in one patient at month 15. An entecavir-resistant virus having rtM204V, rtL180M and rtS202G substitutions was detected in this patient.
*Conclusions* The lamivudine-to-entecavir switching treatment may be generally recommendable in CH-B patients without evidence of lamivudine resistance during

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the preceding lamivudine treatment. However, great care should be taken with respect to the emergence of entecavir-resistance, especially in patients who do not respond well to the preceding lamivudine treatment.

**Keywords** Chronic hepatitis B · Lamivudine resistance · Entecavir-resistance

## Introduction

Nucleos(t)ide analogs have been accepted as useful agents for suppressing hepatitis B virus (HBV) replication and disease progression in patients with type B chronic hepatitis (CH-B). Lamivudine, the first approved nucleoside analog, has been shown to provide short-term benefit for CH-B patients with respect to the reduction of HBV DNA, normalization of alanine aminotransferase (ALT) and improvement of liver histology [1, 2]. However, a serious shortcoming of lamivudine is the high incidence of drug resistance during long-term treatment. The detection rate of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of treatment [3]. Lamivudine resistance is caused by an rtM204V/I substitution within the reverse transcriptase domain of HBV polymerase gene [4–6]. An rtL180M substitution frequently emerges as a "replication-compensatory" one with the "resistance-causative" rtM204V/I substitution [4–7]. The emergence of lamivudine-resistant mutant HBV leads to the elevation of HBV DNA ("virological breakthrough") and the subsequent increase of ALT ("breakthrough hepatitis"), resulting in disease progression. Adefovir dipivoxil and tenofovir disoproxil fumarate have been shown to be effective in both nucleos(t)ide analog-naïve and lamivudine-resistant CH-B patients [8–13].

Recently, entecavir has been demonstrated to exert antiviral efficacy in both nucleos(t)ide analog-naïve and lamivudine-refractory CH-B patients [14–16]. The frequency of entecavir-resistance has been reported to be less than 1% at 4 years of treatment in nucleos(t)ide analog-naïve CH-B patients [17]. On the other hand, in switching treatment to entecavir for lamivudine-refractory CH-B patients, most of whom developed lamivudine resistance during the preceding lamivudine therapy, the cumulative probability of entecavir-resistance has been reported to be no less than 40% at 4 years of treatment [17]. Entecavir-resistance has been shown to be established by amino acid substitution(s) at rt184, rt202 and/or rt250 along with the lamivudine-resistant rtM204V and rtL180M substitutions [18]. In the case of nucleos(t)ide analog-naïve patients, the requirement of at least three amino acid substitutions serves as a high genetic barrier to entecavir-resistance. By contrast, in the case of lamivudine-resistant patients, a lower genetic barrier results in higher incidence of entecavir-resistance because two amino acid substitutions, rtM204V and rtL180M, already exist from the preceding lamivudine treatment. The reduced susceptibility to entecavir of the lamivudine-resistant virus compared with the wild-type virus is also a reason for the higher emergence rate of entecavir-resistance in lamivudine-resistant patients than in nucleos(t)ide analog-naïve ones [19].

Although lamivudine is not currently recommended as a first-line drug for nucleos(t)ide analog-naïve CH-B, a considerable number of CH-B patients are under continuous treatment with lamivudine. In these patients, the switch to entecavir treatment could be advantageous over continuation of lamivudine treatment by offering stronger antiviral efficacy and less chance of drug resistance. With respect to the manner of emergence of entecavir-resistance, switching a patient's treatment may be more appropriate before the appearance of lamivudine resistance than after its development. However, the usefulness of lamivudine-to-entecavir switching treatment has not been assessed in CH-B patients without apparent evidence of lamivudine resistance.

This led us to investigate the antiviral efficacy and emergence of entecavir-resistance in CH-B patients who showed no evidence of lamivudine resistance during the preceding lamivudine treatment and underwent the switching treatment to entecavir.

## Patients and methods

### Patients

This study included 44 consecutive CH-B patients from 10 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, Suita Municipal Hospital, Yao Municipal Hospital, Osaka Rousai Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital and Osaka University Hospital) who underwent continuous lamivudine treatment (100 mg/day) for more than 6 months and showed no apparent evidence of lamivudine resistance. Before starting the preceding lamivudine treatment, all patients had abnormal ALT, positive hepatitis B surface antigen (HBsAg) and a detectable level of HBV DNA according to PCR-based assay (Amplicor HB Monitor, Roche Diagnostics) or branched DNA assay (Quantiplex HBV DNA, Chiron). None of them showed evidence of dual infection with hepatitis C virus or human immunodeficiency virus, or other forms of liver diseases such as alcoholic liver disorder, autoimmune hepatitis and drug-induced liver injury. The total duration of the preceding lamivudine treatment ranged from 6 to 73 (median, 14)

months. The absence of lamivudine resistance was defined by no detection of the rtM204V/I substitution as measured by the PCR–enzyme linked minisequence assay (ELMA) (Sumitomo Metal Industries) [20] for 33 patients, or by the lack of virological breakthrough as judged by more than 1 log increment in HBV DNA from the nadir for the remaining 11 patients. All of the 44 patients switched to 0.5 mg/day of entecavir administration. After the beginning of entecavir treatment, liver function tests and HBV markers were measured at 1- to 2-month intervals. When virological breakthrough was observed during follow-up, entecavir-resistance-associated mutations were examined by means of a PCR-direct sequencing method. The follow-up period of entecavir treatment ranged from 10 to 23 (median 20) months.

### Baseline characteristics of the patients

At the commencement of switching treatment to entecavir, the 28 males and 16 females were aged 33–79 (median 59) years. Seventeen patients (39%) tested positive for hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) developed in all of the 27 HBeAg-negative patients. Among the 27 HBeAg-negative patients, four achieved HBeAg clearance during the preceding lamivudine treatment. HBV DNA at baseline varied among patients from <2.6 to 5.2 logcopies/ml. The baseline ALT ranged from 11 to 78 (median 25) IU/l. Regarding the liver diseases of the patients, 27 (61%) showed features of chronic hepatitis, 11 (25%) of liver cirrhosis and six (14%) of hepatocellular carcinoma (HCC) according to liver biopsy and/or abdominal imaging procedures. HBV genotype was examined for 14 patients, and all of them had HBV genotype C, the most predominant genotype in Japan. Informed consent was obtained from all patients.

### Serological and virological markers of HBV

HBsAg, HBeAg and anti-HBe were determined by chemiluminescent immunoassay. HBV DNA was measured by the PCR-based method (Amplicor HBV monitor, Roche Diagnostics) whose lower detection limit is 2.6 logcopies/ml. Lamivudine-resistant rtM204V/I substitution was examined by the PCR–ELMA method (Sumitomo Metal Industries) (20), which is capable of detecting the mutant virus in a mixed viral population if it is present at more than 10% of the total population. The entecavir-resistance-associated substitutions and HBV genotype were determined by a PCR-direct sequencing method. As for oligonucleotide primers for PCR reaction, the outer primer sets were BF5 (5′-AAG AGA CAG TCA TCC TCA GG-3′, nt 3183–3202) and BR1s (5′-AAA AAG TTG CAT GGT GCT GG-3′, nt 1825–1806), and the inner primer sets were BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195–1176). After DNA extraction, the DNA sample was subjected to the PCR reaction for 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min) using the inner primer set, followed by a final extension at 72°C for 10 min. If amplification was not successful by the single PCR reaction, the nested PCR was conducted; the first round PCR was done using the outer primer sets for 35 cycles, and the aliquot of the product was used for the second round PCR for 30 cycles using inner primer sets. All sequencing reactions of the PCR products were carried out using the BigDye Terminater Ver. 3.1 Cycle Sequencing Kit, and 3100 or 3730 Genetic Analyzer (Applied Biosystems), which allowed determination of the amino acid sequences of rt85-344. For determining the HBV genotype, nucleotide sequences obtained in each of the patients were aligned along with representative HBV strains of genotype A–H, and a phylogenetic tree was constructed in the homepage of DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

### Statistical analysis

Statistical analysis for group comparison was performed by Fisher's exact probability test and Mann–Whitney's non-parametric $U$ test using the SPSS version 15.0J software (SPSS Inc, Chicago, IL). A $p$ value of less than <.05 was considered to be significant.

## Results

### Classification of patients who underwent lamivudine-to-entecavir switching treatment according to baseline HBV DNA

The 44 CH-B patients who underwent the switching treatment from lamivudine to entecavir were first classified according to their baseline HBV DNA at the commencement of entecavir administration. HBV DNA was not detectable (<2.6 logcopies/ml) in 31 patients (70%) at baseline. Seven patients (16%) had baseline HBV DNA of 2.6–<4.0 logcopies/ml. In the remaining six patients (14%), the baseline HBV DNA was ≥4.0 logcopies/ml. When patient clinical characteristics were compared among the three patient groups (Table 1), nine (29%) of the 31 patients with baseline HBV DNA <2.6 copies/ml tested positive for HBeAg at the commencement of switching treatment to entecavir, compared with five of the six (83%) patients with baseline HBV DNA ≥4.0 copies/ml ($p < .05$). Gender ratio, age, ALT at baseline, liver disease, duration of the preceding lamivudine treatment and

**Table 1** Patient clinical characteristics and the therapeutic efficacy in 44 CH-B patients in relation to their baseline HBV DNA

| | Baseline HBV DNA | | |
|---|---|---|---|
| | <2.6 logcopies/ml ($n = 31$) | 2.6–<4.0 logcopies/ml ($n = 7$) | ≥4.0 logcopies/ml ($n = 6$) |
| At the commencement of switching treatment to entecavir | | | |
| Gender (male/female) | 19/12 | 5/2 | 4/2 |
| Age (years) | 60 (35–79)[a] | 65 (41–69) | 55 (33–65) |
| HBeAg (positive/negative) | 9/22 | 3/4 | 5/1[b] |
| HBV DNA (logcopies/ml) | <2.6 | 3.1 (2.6–3.6)[c] | 4.6 (4.0–5.2)[c,d] |
| rtM204V/I mutation (absence/NT) | 23/8 | 5/2 | 5/1 |
| ALT (IU/l) | 25 (11–64) | 31 (13–46) | 20 (17–78) |
| Chronic hepatitis/cirrhosis/HCC | 19/7/5 | 4/2/1 | 4/2/0 |
| Follow-up period of entecavir treatment (months) | 19 (10–23) | 19 (10–22) | 20 (16–22) |
| The rate of undetectable HBV DNA level during follow-up | 31 (100%) | 7 (100%) | 3 (50%)[c] |
| Emergence of entecavir-resistance during follow-up | 0 (0%) | 0 (0%) | 1 (17%) |
| At the commencement of preceding lamivudine treatment | | | |
| HBeAg (positive/negative) | 12/19 | 4/3 | 5/1 |
| HBV DNA (logcopies/ml) | 6.5 (4.3–7.6‹) | 6.6 (6.2–7.6‹) | 7.6‹ (5.9–7.6‹) |
| Duration of preceding lamivudine treatment (months) | 15 (6–73) | 10 (7–42) | 9 (8–32) |

*NT* not tested

[a] Values are expressed as median (range)

[b] $p < .05$ versus baseline HBV DNA <2.6 logcopies/ml group

[c] $p < .01$ versus baseline HBV DNA <2.6 logcopies/ml group

[d] $p < .01$ versus baseline HBV DNA of 2.6–<4.0 logcopies/ml group

follow-up period of entecavir treatment did not differ among the three groups. Also, there was no significant difference in HBV DNA and the frequency of positive HBeAg at the commencement of preceding lamivudine treatment among them.

## Antiviral efficacy and drug resistance in lamivudine-to-entecavir switching treatment in relation to baseline HBV DNA

Next, we investigated serial changes in HBV DNA after the switch from lamivudine to entecavir treatment in CH-B patients in relation to the baseline HBV DNA. All 31 patients with baseline HBV DNA <2.6 logcopies/ml maintained undetectable HBV DNA during the follow-up period of entecavir treatment. Figure 1 shows the longitudinal evaluation of HBV DNA during the switching treatment to entecavir in patients with a detectable level of baseline HBV DNA. In patients having baseline HBV DNA of 2.6–<4.0 logcopies/ml (Fig. 1a), all of the seven patients achieved sustained undetectable HBV DNA during follow-up, although HBV DNA was transiently detected in one patient. As for patients having baseline HBV DNA ≥4.0 logcopies/ml (Fig. 1b), three (50%) of the six patients achieved sustained undetectable HBV DNA during follow-up. In two patients, HBV DNA was not cleared entirely, but declined to 2.9 and 2.7 logcopies/ml at month 18, respectively. In sequencing analysis at that time, the former patient had the lamivudine-resistant rtM204I substitution, although it was not detected by the PCR–ELMA assay at the start of entecavir treatment. The latter patient had no drug resistance-associated substitutions. In the sixth patient, HBV DNA decreased initially, but virological breakthrough was seen at month 15. The entecavir-resistant virus was detected after virological breakthrough. The detailed disease course of the entecavir-resistant patient is described below. As for the relationship of baseline HBV DNA to the frequency of undetectable HBV DNA, HBV DNA was cleared more frequently in patients with baseline HBV DNA <2.6 logcopies/ml than in those with baseline HBV DNA ≥4.0 logcopies/ml (100 vs. 50%, $p < .01$) (Table 1).

Serial changes in ALT during lamivudine-to-entecavir switching treatment were further examined. Among the 31 patients with baseline HBV DNA <2.6 logcopies/ml, the baseline ALT was within the normal range (≤40 IU/l) in 27 patients, 24 of whom showed sustained ALT normalization during follow-up. In the remaining three patients, ALT became slightly abnormal (≤60 IU/l) during follow-up. As for four patients with abnormal baseline ALT, the level was normalized in three, whereas a slight elevation of ALT (≤60 IU/l) continued in one during follow-up.

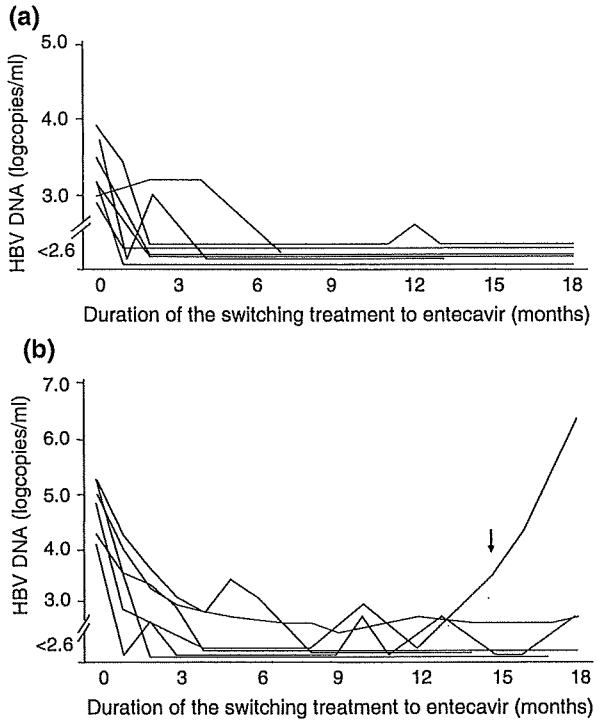


**Fig. 1** Changes in HBV DNA after commencement of switching treatment from lamivudine to entecavir in CH-B patients with baseline HBV of (**a**) 2.6–<4.0 logcopies/ml and (**b**) ≥4.0 logcopies/ml. The *black arrow* indicates the time point of virological breakthrough

Among the 13 patients having a detectable level of baseline HBV DNA, five patients (three with baseline HBV DNA of 2.6–<4.0 logcopies/ml and two with baseline HBV DNA ≥4.0 logcopies/ml) had abnormal ALT at baseline but showed ALT normalization during follow-up. In the remaining eight patients, ALT continued to be normal from the beginning of entecavir treatment.

### Disease course of the CH-B patients showing entecavir-resistance during lamivudine-to-entecavir switching treatment

The disease course of the entecavir-resistant patient is shown in Fig. 2. This patient was a 33-year-old HBeAg-positive male, whose liver biopsy showed features of chronic hepatitis. He underwent the preceding lamivudine treatment for 8 months. HBV DNA decreased from >7.6 to 4.6 logcopies/ml, and ALT was normalized during the lamivudine therapy. The rtM204V/I substitution was not detected before the switch to entecavir treatment by the PCR–ELMA analysis. After the commencement of entecavir treatment, HBV DNA was cleared at month 5. However, virological breakthrough was seen at month 15, and HBV DNA was further increased to 6.1 logcopies/ml at month 18. The sequencing analysis at month 18 revealed the rtM204V, rtL180M and rtS202G substitutions. Two additional substitutions, rtL267M and rtQ316H, were also found, when the amino acid sequences were compared with three representative genotype C HBV isolates (Genbank accession nos. V00867, X01587 and D00630) [21–23]. Breakthrough hepatitis was not evident after the emergence of entecavir-resistant mutant virus. The sequencing analysis also revealed that he was infected with HBV of genotype C.

## Discussion

Entecavir treatment has been shown to exhibit more powerful antiviral efficacy and less frequent drug resistance than lamivudine treatment in nucleos(t)ide analog-naïve CH-B patients [14, 15, 17]. Entecavir is also effective in patients showing lamivudine resistance during the preceding lamivudine treatment, but its efficacy is limited due to the higher incidence of entecavir-resistance, compared with nucleos(t)ide analog-naïve ones [16, 17]. This is because entecavir-resistance is established based on two lamivudine-resistant substitutions, rtM204V and rtL180M, and additional mutation(s) occurring at rt184, rt202 and/or rt250 [18]. A considerable number of CH-B patients remain under continuous lamivudine treatment, while the lamivudine-to-entecavir switching treatment could yield a practical benefit. The switching treatment may be more promising for patients before the appearance of lamivudine resistance than after its development. In the present study, we investigated the efficacy of lamivudine-to-entecavir switching treatment in CH-B patients without apparent evidence of lamivudine resistance during the preceding lamivudine treatment.

We evaluated the antiviral efficacy of the switching treatment to entecavir in relation to the baseline HBV DNA at the commencement of the entecavir administration. In all patients having baseline HBV DNA <2.6 logcopies/ml, who revealed a good response to the preceding lamivudine treatment, HBV DNA continued to be undetectable during the switching treatment to entecavir. Also, all patients having baseline HBV DNA of 2.6–<4.0 logcopies/ml achieved sustained undetectable HBV DNA during the follow-up period of entecavir treatment. Among six patients having baseline HBV DNA ≥4.0 logcopies/ml, who did not respond well to the preceding lamivudine treatment, HBV DNA was cleared in three during follow-up. Its reduction by up to 3.0 logcopies/ml was seen in two additional cases without emergence of the entecavir-resistant virus. Thus, the antiviral efficacy of the lamivudine-to-entecavir switching treatment was exhibited in almost all CH-B patients in parallel with that of the preceding

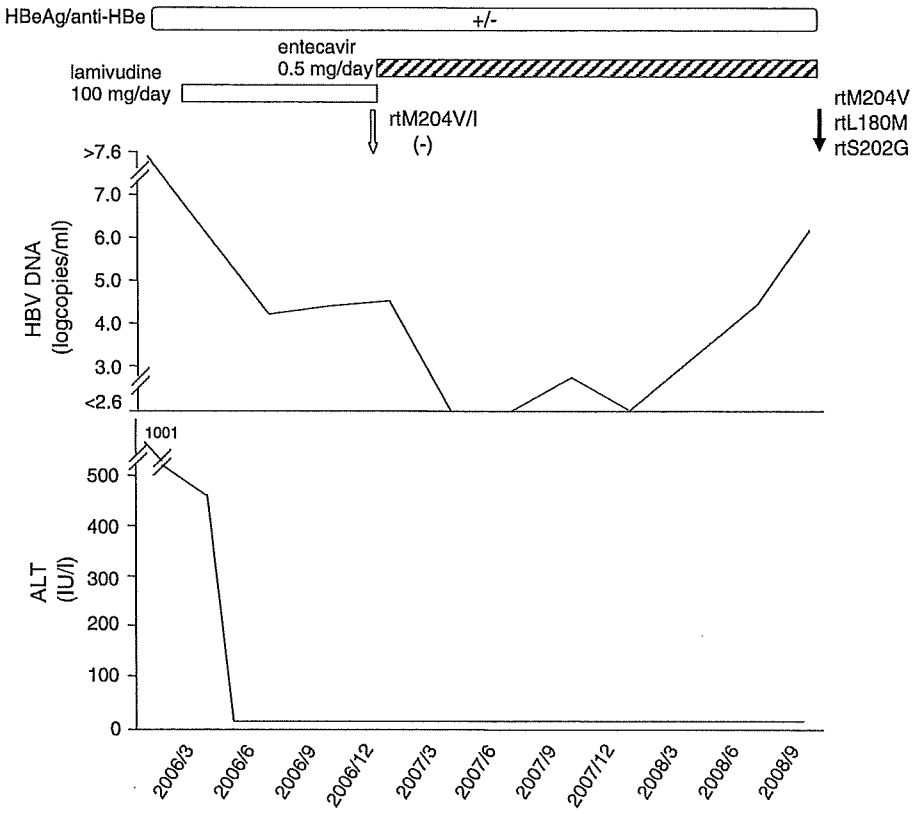


**Fig. 2** Disease course of the CH-B patient showing entecavir-resistance during switching treatment to entecavir. The *white arrow* indicates the time point of the PCR–ELMA assay to detect rtM204V/I mutation, whereas the *black arrow* indicates the time point of the PCR-direct sequencing analysis

lamivudine treatment. In addition, the switching treatment to entecavir tended to yield a greater decrease in HBV DNA than the preceding lamivudine treatment. These results indicate that the switch from lamivudine to entecavir may be generally recommendable compared with continuation of lamivudine administration in CH-B patients without evidence of lamivudine resistance.

In this study, one of the six patients having baseline HBV DNA ≥4.0 logcopies/ml showed entecavir-resistance during the switching treatment to entecavir. It was probably due to the existence of an extremely small amount of lamivudine-resistant virus mixed with a predominant wild-type virus, which could not be detected by the sensitive PCR–ELMA assay at the start of the switch to entecavir treatment. It is speculated that, during entecavir treatment, the lamivudine-resistant virus having rtM204V and rtL180M substitutions may become predominant with time, followed by the establishment of entecavir-resistant virus via the additional rtS202G substitution. Compared to the low incidence of drug resistance in entecavir treatment for nucleos(t)ide analog-naïve CH-B patients [17], the entecavir-resistance may occur more frequently in the lamivudine-to-entecavir switching treatment for patients without evidence of lamivudine resistance. In particular, patients who do not achieve a good response to the preceding lamivudine treatment are speculated to have a higher risk for the development of entecavir-resistance in the switching treatment to entecavir, although it should be verified by further studies.

In conclusion, in CH-B patients receiving the continuous lamivudine treatment, it may be recommendable to switch to entecavir treatment before the appearance of lamivudine resistance. It may contribute to reducing the subsequent emergence of drug resistance. However, great care should be taken with respect to the emergence of entecavir-resistant virus after the switch to entecavir treatment, especially in patients who do not respond well to the preceding lamivudine treatment. Our retrospective study with a small number of patients and a short duration of follow-up cannot draw a definite conclusion but still provides some information about the clinical possibilities of the lamivudine-to-entecavir switching treatment. Further detailed investigation with a larger number of patients and a longer follow-up period may offer better understanding.

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*Journal of Viral Hepatitis*, 2009, 16, 844–852 doi:10.1111/j.1365-2893.2009.01131.x

# Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis

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*Received December 2008; accepted for publication February 2009*

SUMMARY. In hepatitis C virus (HCV) infection, the Th1-type immune response is involved in liver injury. A predominance of immunosuppressive regulatory T cells (Treg) is hypothesized in patients with persistently normal alanine aminotransferase (PNALT). Our aim was to clarify the role of Treg in the pathogenesis of PNALT. Fifteen chronically HCV-infected patients with PNALT, 21 with elevated ALT (CH) and 19 healthy subjects (HS) were enrolled. We determined naturally-occurring Treg (N-Treg) as CD4+CD25high+ FOXP3+ T cells. The expression of FOXP3 and CTLA4 in CD4+CD25high+ cells was quantified by real-time reverse transcriptase-polymerase chain reaction. Bulk or CD25-depleted CD4+ T cells cultured with HCV-NS5 loaded dendritic cells were assayed for their proliferation and cytokine release. We examined CD127–CD25–FOXP3+ cells as distinct subsets other than CD25+ N-Treg. The frequencies of N-Treg in patients were significantly higher than those in HS. The FOXP3 and CTLA4 transcripts were higher in PNALT than those in CH. The depletion of CD25+ cells enhanced HCV-specific T cell responses, showing that co-existing CD25+ cells are suppressive. Such inhibitory capacity was more potent in PNALT. The frequency of CD4+CD127–CD25–FOXP3+ cells was higher in CH than those in PNALT. Treg are more abundant in HCV-infected patients, and their suppressor ability is more potent in patients with PNALT than in those with active hepatitis.

*Keywords*: HCV, PNALT, regulatory T cell.

## INTRODUCTION

Hepatitis C virus (HCV) causes a wide range of chronic liver diseases in infected hosts, including chronic hepatitis (CH), liver cirrhosis and hepatocellular carcinoma (HCC).

Abbreviations: ALT, alanine aminotransferase; CH, chronic hepatitis; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HS, healthy subjects; IFN, interferon; IL, interleukin; IU, international units; MoDC, monocyte-derived dendritic cell; N-Treg, naturally occurring regulatory T cell; PNALT, persistently normal ALT; RT-PCR, reverse transcriptase-polymerase chain reaction; SLE, systemic lupus erythematosus; TGF, transforming growth factor; Treg, regulatory T cell.

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One of the critical determinants promoting the development of HCV-induced liver disease is sustained liver inflammation, explaining the therapeutic rationale of alleviating this condition to help prevent liver cancer [1]. Among chronically infected individuals, approximately 20–30% display persistently normal serum alanine aminotransferase levels [2,3]. Although it is reported that 40–50% of them progress to the active stage of liver inflammation within 5 years of observation [4], the incidence of HCC in the remaining patients continues to be lower than in those with elevated serum ALT levels [5]. Cumulative studies have revealed that HCV is not directly cytopathic to hepatocytes. It has been demonstrated that a Th1-type or cytotoxic T lymphocyte (CTL) response is critically involved in HCV-mediated liver injury [6,7]. Therefore, it is conceivable that some suppressor mechanisms exist against Th1-type immune responses in patients with persistently normal ALT levels (PNALT), which may be distinct from those in patients with active liver inflammation.

Regulatory T cells (Treg) are a unique subset of T cells with inhibitory capacity against auto-reactive T cells [8]. Substantial data have been reported about the involvement of Treg in the pathogenesis of various diseases, including autoimmune, cancer or infectious diseases [9–13]. Currently, the existence of several types of Treg has been reported [14]. Naturally occurring Treg (N-Treg) are derived from the thymic stromal environment from progenitor cells and suppress auto-reactive T cells in antigen-specific and antigen-nonspecific manner. Forkhead/winged helix transcription factor (FOXP3) is one of the specific markers of N-Treg, the expression of which is well correlated with the gain of a suppressor function [15,16]. As cells with high expression of CD25 also display FOXP3, it is generally accepted that CD25+FOXP3+ is the most reliable marker for Treg. In HCV infection, several reports have described a higher frequency of N-Treg in the periphery and the liver [17–20], suggesting their active role in HCV persistence. It has also been demonstrated that CD25+FOXP3+ regulatory cells are inducible in the periphery [21]. Owing to the lack of a specific phenotypic marker of these induced regulatory cells, referred to as adaptive Treg, their role in the pathogenesis of HCV infection has not been clearly understood. A recent study has demonstrated that the expression of interleukin (IL)-7 receptor (CD127) is downregulated in Treg to a degree that is inversely correlated with FOXP3 expression [22]. These findings offer the possibility that adaptive Treg are traceable, not all but in part, by the combination of CD127 and FOXP3 independent of CD25 expression.

In this study, our aim was to elucidate whether or not Treg are involved in the pathogenesis of PNALT patients, by comparing the frequency and function of these cell subsets with those in active hepatitis patients or healthy subjects. A distinct equilibrium was found between N-Treg and CD127–CD25–FOXP3+ T cells according to differences in liver inflammation.

## MATERIALS AND METHODS

### *Subjects*

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, 15 patients with PNALT levels and 21 patients with elevated or fluctuating ALT levels (the CH group) were enrolled in this study. As controls, 19 healthy subjects (HS) who were negative for HCV and hepatitis B virus (HBV) markers were examined. The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each subject. In this study, PNALT patients were defined as those whose ALT levels remained within the normal range (<30 IU/mL) without any medications for more than 1 year. At enrolment, the patients were confirmed to be positive for both serum anti-HCV and HCV RNA, but were negative for other viral infections, including HBV and human immunodeficiency virus. The presence of other causes of liver disease, such as autoimmune, alcoholic and metabolic disorders was excluded by the use of laboratory and imaging analyses. Liver biopsy was carried out in some of the patients. Histological examination was performed according to the METAVIR scoring system. In all patients, a combination of repetitive biochemical tests, ultrasonography or computed tomography scans ruled out the presence of cirrhosis and liver tumours. The clinical background of the subjects are shown in Table 1.

**Table 1** Baseline clinical characteristics of the patients

| | Chronic hepatitis patients | Patients with PNALT | Healthy subjects* | |
|---|---|---|---|---|
| *n* | 21 | 15 | 19 | |
| Sex (M/F) | 8/13 | 5/10 | ND | NS |
| Age | 50.6 ± 11.6 | 47.8 ± 12.7 | ND | NS |
| ALT (IU/L) | 88.3 ± 41.4 | 20.9 ± 6.9 | ND | $P < 0.0001^{\dagger}$ |
| Plt ($10^4/\mu L$) | 13.5 ± 5.4 | 20.0 ± 3.9 | ND | $P < 0.01^{\dagger}$ |
| HCV RNA (Meq/mL) | 8.6 ± 11.3 | 9.7 ± 7.8 | ND | NS |

*The background data of healthy subjects (blood donors) were not accessible owing to the confidentiality regulations of the blood centre, but their serum ALT levels were confirmed to be within the normal range. †Statistical significance was analysed by Mann–Whitney *U* test between chronic hepatitis patients and patients with PNALT. The values are expressed as mean ± SD. PNALT, persistently normal alanine aminotransferase level; ND, not determined; NS, not significant; plt, platelet count.

### *Frequency analyses of Treg cells*

For the numerical analyses of Treg cells, heparinized venous blood was obtained from all subjects. Peripheral blood mononuclear cells were collected by density-gradient centrifugation on a Ficoll–Hypaque cushion. The cells were subsequently stained with a combination of various fluorescence-labelled anti-human mouse monoclonal antibodies for phenotypic markers. The antibodies for CD25 (clone B1.49.9) and CD4 (clone 13B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA), that for CD127 (clone 40131) from R&D Systems (Minneapolis, MN, USA) and that for FOXP3-PE (clone PCH101) from eBioscience (San Diego, CA, USA), respectively. The cells were stained in phosphate-buffered saline containing 1% fetal bovine serum (FBS) with various antibodies or isotype controls for 15 min at room temperature. Intracellular staining of FOXP3 was performed using a human FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. The cells were analysed by FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest software.

### *Functional analysis of CD4+CD25+ T cells in HCV-specific CD4+ T cell response*

We first examined the HCV-specific CD4+ T cell response in the presence or absence of CD4+CD25+ T cells. Monocyte-derived dendritic cells (MoDC) were generated from CD14+ cells as reported previously. In brief, CD14+ cells were cultured in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS, 50 IU/mL of penicillin, 50 mg/mL of streptomycin, 2 mM of L-glutamine, 10 mM of Hepes buffer, 10 mM of nonessential amino acids in the presence of 50 ng/mL of granulocyte/macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL of IL-4 (PeproTech) for 7 days at 37 °C and 5% $CO_2$. On day 6 of the culture, MoDC were pulsed with 10 $\mu$g/mL of recombinant HCV NS5 (amino acid position: NS5B 1-544; kindly provided by Japan Tobacco, Inc., Tokyo, Japan) and cultured for 24 h. The antigen-pulsed MoDC were then cultured with autologous bulk CD4+ T cells or CD4+CD25– T cells in 96-well flat-bottom plates (Corning, NY, USA) for 5 days. Enrichment of CD4+ T cells or CD4+CD25– T cells was performed using a CD4+CD25+ Regulatory T cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. On day 6 of the co-culture, the cells were pulsed with 1 $\mu$Ci of [3H]-thymidine during the last 16 h of incubation. The supernatants were collected before pulsing with [3H]-thymidine and subjected to cytokine enzyme-linked immunosorbent assay (ELISA). The incorporation of [3H]-thymidine in CD4+ T cells was measured using a $\beta$-counter (Wallac-Perkin-Elmer, Wallac, Finland).

### *Enzyme-linked immunosorbent assay*

The concentrations of IL-10, TGF-$\beta$1 and interferon (IFN)-$\gamma$ in the culture supernatants were determined by ELISA. We used matched pairs of relevant monoclonal antibodies (Endogen, Woburn, MA, USA) for IL-10 and IFN-$\gamma$, and the DuoSet ELISA development system (R&D Systems) for TGF-$\beta$1, according to the manufacturer's instructions. The detection thresholds of IL-10, TGF-$\beta$1 and IFN-$\gamma$ were 10, 10 and 16 pg/mL, respectively.

### *Real time reverse transcriptase-polymerase chain reaction (RT-PCR)*

In order to analyse the expression of FOXP3 and CTLA-4 in N-Treg, we collected CD4+CD25high T cells by using FACSAria. The purity of the isolated cells was more than 95% as determined by FACS. Total RNA was extracted from sorted CD4+CD25high T cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using the SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA, USA). Assays-on-demand primers and probes (PE Applied Biosystems, Foster City, CA, USA) were used to quantify FOXP3 and CTLA4 expression. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The thermal cycling conditions for all genes were as follows: the reaction was started with a 10-min denaturing cycle at 95 °C, followed by 40 cycles of PCR performed with 15 s of denaturing at 95 °C, then 1 minute at 60 °C for annealing and extension. We identified a calibrator sample from the healthy volunteers. The expressions of molecules were given as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, we quantified $\beta$-actin mRNA from each sample as a control of internal RNA and corrected all values with this.

### *Statistical analysis*

Statistical analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Mann–Whitney *U*-test was used to compare differences in unpaired samples. For all analyses, a *P*-value of less than 0.05 was considered to be statistically significant.

## RESULTS

### *Peripheral N-Treg are increased in HCV-infected patients*

We compared the frequency of Treg between HCV-infected patients and healthy donors. In HCV-positive individuals, they were further categorized into PNALT and CH groups according to the difference in their serum ALT levels. The clinical backgrounds of these groups were not different except for

serum ALT levels and platelet counts (Table 1). N-Treg were defined as the cells with CD4+CD25high+FOXP3+ cells. As the cut-off value between CD25high+ and CD25intermediate+ cells is a critical determinant for Treg analyses, we defined CD4+CD25high+ as the cells with CD25 levels higher than those of CD4–CD25+ cells (Fig. 1a). We first compared the frequency of CD4+FOXP3+ T cells. The frequency of FOXP3+ cells in the CD4+ T cell population in HCV-infected patients was significantly higher than those in the HS (Fig. 1b). However, no difference was observed in FOXP3+ cells between the PNALT and CH patients (Fig. 1b). The frequency of CD4+CD25high+FOXP3+ T cells in CH or PNALT patients were significantly higher than those in HS, whereas those in HCV-positive patients did not differ regardless of their ALT levels (Fig. 1c). Similar results were obtained for the frequency of CD4+CD25–FOXP3+ T cells (Fig. 1d).

Next, we examined whether or not the frequency of N-Treg is correlated with clinical parameters. Among all HCV-infected patients, no correlation was observed between the frequency of N-Treg (CD4+CD25high+ FOXP3+ T cells) and serum ALT, HCV RNA levels, age or platelet counts (data not shown). In the analyses of patients who had undergone liver biopsy, the frequency of N-Treg was not correlated with METAVIR grade/stage scores (data not shown).

### *The expressions of FOXP3 and CTLA4 are higher in N-Treg from PNALT patients compared with those from the CH group*

FOXP3 is the master gene of Treg in the development and gaining of suppressor functions. Alternatively, CTLA4 is one of the key molecules of Treg in exerting inhibitory function. We thus evaluated FOXP3 and CTLA4 mRNA expression in sorted N-Treg (CD4+CD25high+ T cells) by means of real-time RT-PCR. The expression of FOXP3 in PNALT or CH patients was significantly higher than those in HS (Fig. 2a). Of note is the higher expression of FOXP3 in N-Treg from the PNALT group than in those from the CH group (Fig. 2a). In contrast, the expression of CTLA4 in N-Treg from the PNALT was higher than those in the CH, while it did not differ between the CH and HS groups (Fig. 2b).

### *CD4+CD25+ T cells from PNALT patients have more suppressive capacity in the HCV-specific CD4+ T cell response than those from CH patients*

In order to compare the ability of N-Treg to inhibit the antigen-specific CD4+ T cell response, we used autologous MoDC pulsed with HCV proteins as antigen-presenting cells. We examined CD4+ T cell proliferation or cytokine

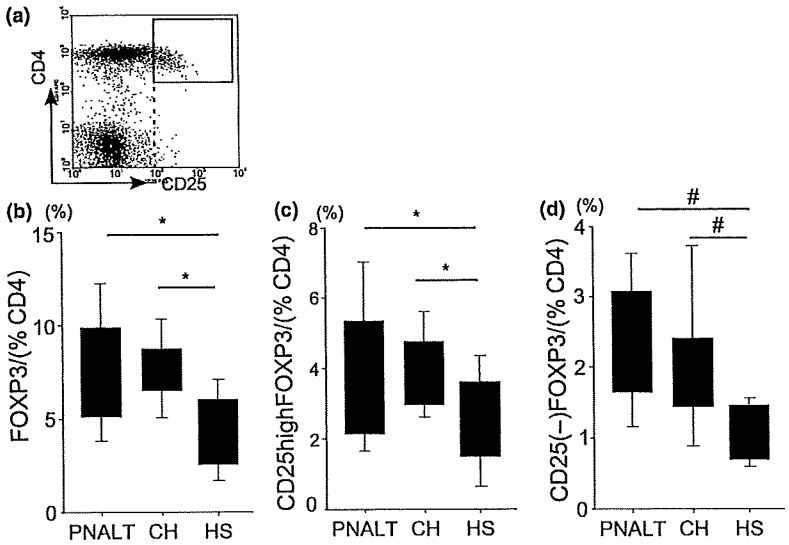


Fig. 1 Comparison of frequencies of naturally-occurring regulatory T cells (N-Treg) and FOXP3-positive cells among the groups. (a) Gating of CD4+CD25high+ T cells under FACS analysis. The cut-off value of CD25high expression is set at a level that is more than that of CD4–CD25+ cells (dotted line); CD4+CD25high+ T cells are shown in the rectangle drawn in the representative dot plot. (b) Frequencies of FOXP3+ cells, (c) N-Treg (CD25high+FOXP3+ cells) and (d) CD25–FOXP3+ cells in CD4+ T cells were compared among the groups. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; , $P < 0.0001$ by Mann–Whitney *U*-test. *Abbreviations:* PNALT, hepatitis C virus (HCV)-infected patients with persistently normal alanine aminotransferase (ALT) levels; CH, HCV-infected patients with elevated ALT levels; HS, healthy subjects.