3. Results

3.1. Acquired antitumor immunity was induced by α-GalCer treatment of CMS4 liver tumor

We examined whether α-GalCer treatment for CMS4 liver or spleen tumor would induce acquired antitumor immunity. Mice bearing liver or spleen CMS4 tumor were treated i.p. with α-GalCer. Fourteen days after α-GalCer treatment, spleen CD8+ T cells from treated mice were prepared and subjected to IFN-γ ELISPOT. The high numbers of IFN-γ spots were detected in the CMS4 liver tumor model, but not in the CMS4 spleen tumor model (Fig. 1A).

We next analyzed whether the α-GalCer treatment of CMS4-treated liver or spleen would impact the progression of subcutaneous rechallenged CMS4 tumors. Fourteen days later after α-GalCer treatment, 1×10^6 CMS4 cells were rechallenged subcutaneously in the right flank. As shown in Fig. 1B, CMS4 subcutaneous tumors in α-GalCer treated mice bearing CMS4 liver tumor were significantly inhibited compared with those in non-treated mice, but those in mice bearing CMS4 spleen tumor were not. Colon26, BALB/c syngeneic colon cancer cell, subcutaneous tumors were not inhibited in mice receiving α-GalCer treatment for CMS4 liver or spleen tumor (data not shown). Strong acquired immunity could also be generated after α-GalCer treatment of MC38 liver tumors in C57BL/6 mice, but not of MC38 spleen tumors (Fig. 1C). These findings suggested that tumor-specific acquired immunity could be generated efficiently by α-GalCer treatment in the liver, but not in the spleen.

3.2. Administration of α-GalCer activated DCs and increased CD8- conventional DC fraction in the liver

Recent research revealed that NKT cells-DC interactions by α-GalCer are critically important in the sequential activation of effector cells in both innate and acquired immunity [12,18]. However, details of the DC activation by α-GalCer in the liver have not yet been evaluated.

First, we investigated the increase of liver and spleen DCs after α-GalCer or vehicle treatment. As shown in Fig. 2A, liver DCs increased significantly after α-GalCer administration whereas spleen DCs from α-GalCer treated mice did not. The proportion of liver DCs in liver MNCs also significantly increased by α-GalCer administration, but that of spleen DCs did not (data not shown). Next, we examined the change of DC subtypes after α-GalCer treatment by analyzing the relative surface expressions of the CD8α and the CD11b molecules [19]. The proportion and the number of CD8- conventional DCs (CD11b+CD8-) significantly increased in the liver by α-GalCer treatment, but not in the spleen. In marked contrast, those of CD8+ conventional DCs (CD11b-CD8α+) exhibited no significant change in both the liver and spleen by α-GalCer treatment (Fig. 2B and C).

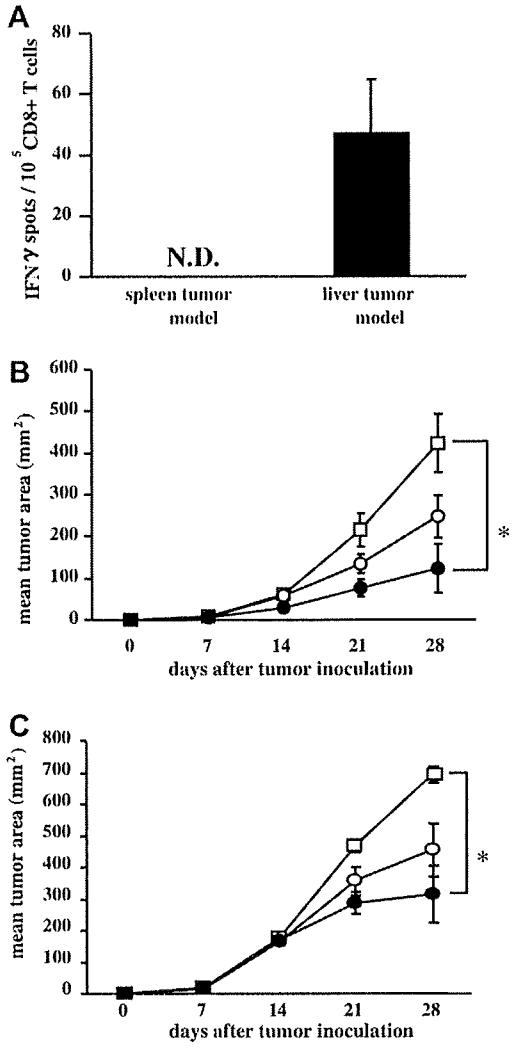

Fig. 1. Induction of local and systemic acquired antitumor immunity after α-GalCer treatment of CMS4 liver and spleen tumor. BALB/c mice were injected intrahepatically or intrasplenically with 5×10^5 CMS4 cells or MC38 cells. One day later, mice were injected i.p. with α-GarCer. (A) Fourteen days later, spleen CD8+ T cells were isolated from both the CMS4 liver and spleen tumor models and subjected to IFN-γ ELISPOT to analyze $p53_{232-240}$ peptide specific IFN-γ production. The results are shown as spots/100,0000 CD8+ T cells; mean ± SD of triplicate samples. CD8+ T cell reactivity against peptide-unpulsed BMDCs served as the negative control in all cases, and this value was subtracted from all experimental determination to determine p53-specific spot numbers. *$p < 0.05$. N.D., not detected. Similar results were obtained from two separate experiments. (B and C) Fourteen days later, mice were challenged subcutaneously with 1×10^6 CMS4 cells (B) or MC38 cells (C) in the right flank (all treatment groups $N = 8$). Tumor size was assessed every 7 days after subcutaneous injection of tumor cells (=on day 0). α-GalCer-treated CMS4 or MC38 liver tumor (•), α-GalCer-treated CMS4 or MC38 spleen tumor (○), non-treated mice (□). Each data point represents the mean tumor size ±SE. *$p < 0.05$.

We examined the CD80, CD86 and CD40 expressions of liver and spleen DCs after administration of α-GalCer. CD86 and CD40 molecules on both liver and spleen DCs from α-GalCer-treated mice were

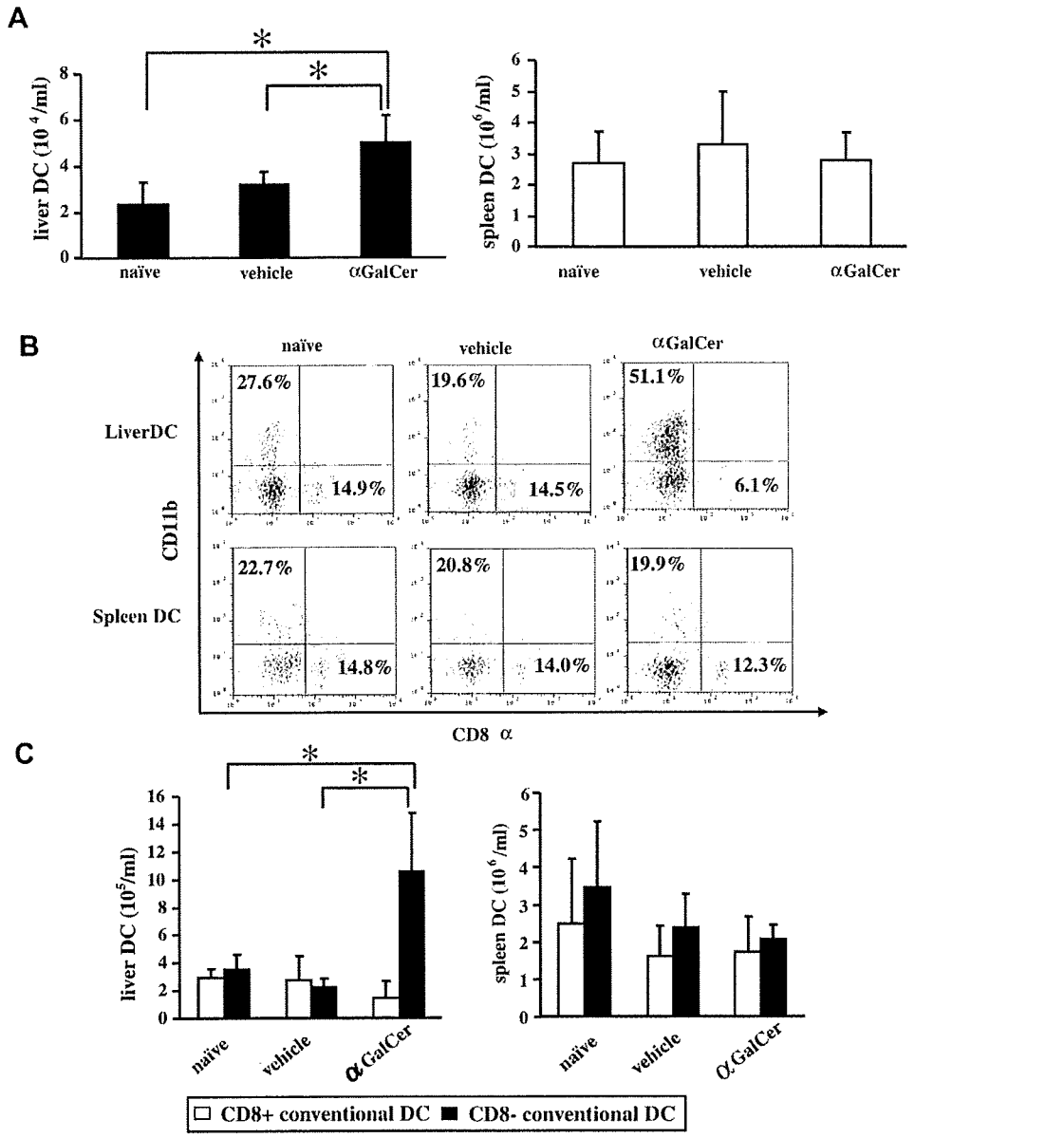

Fig. 2. α-GalCer treatment increased liver CD8- conventional DC. BALB/c mice were treated with α-GalCer or vehicle. Liver and spleen DCs were prepared on day 1 after α-GalCer treatment. (A) Liver DCs (left panel) increased significantly after α-GalCer treatment, but spleen DCs (right panel) did not. (B and C) The change of CD8+ or CD8- conventional DC subtypes after α-GalCer treatment was examined by flow cytometry. The data are represented as the average of numbers obtained from 5 separate experiments. *$p < 0.05$.

expressed more strongly than those from vehicle-treated mice and non-treated mice. CD80 molecules on liver DC from α-GalCer-treated mice were expressed significantly more strongly than those from vehicle-treated or non-treated mice, but those on spleen DC showed no significant change by α-GalCer treatment (Fig. 3). The expressions of CD80, CD86 and CD40 molecules on liver DCs tended to be lower than those on spleen DCs in non-treated mice. However, after α-GalCer treatment, their expressions on liver DCs tended to increase to levels similar to those on spleen DCs.

3.3. Liver DCs from α-GalCer-treated mice could produce more Th1 cytokines and present higher T cell immunostimulatory ability than spleen DCs

Th1-cytokines, such as IL-12, INF-γ and TNF-α, play key roles in determining the strength and/or the phenotypes of the antitumor immune responses [20,21]. We next examined the production of Th1 cytokines from DCs after α-GalCer treatment. The production of these cytokines from DCs derived from vehicle-treated and non-treated mice were not detected in the

liver or the spleen. In marked contrast, all IL-12, INF-γ and TNF-α production from liver DCs derived from α-GalCer-treated mice were significantly higher than those from spleen DCs (Fig. 4A–C). To investigate the difference of the antigen-presenting function between liver DCs and spleen DCs, we examined the allostimulatory capacity of liver and spleen DCs using a mixed lymphocyte reaction (MLR). Liver DCs from α-GalCer-treated mice showed higher T cell proliferation ability than those from vehicle-treated or non-treated mice and spleen DCs from all treatment groups. Spleen DCs from all treatment groups and liver DCs from vehicle-treated or non-treated mice showed little T cell proliferation ability (Fig. 4D). These results suggested that α-GalCer treatment increased the function of DCs in the liver more strongly than those in the spleen.

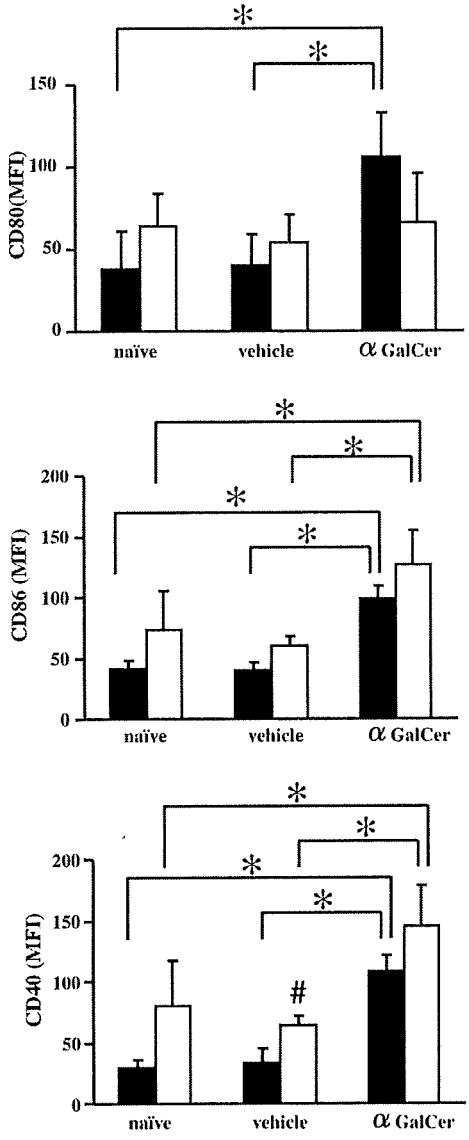

Fig. 3. α-GalCer treatment increased the expression of antigen presenting related molecules on both liver and spleen DCs. DCs were stained with PE- or FITC-conjugated monoclonal antibodies (CD11c, CD40, CD80, CD86 and MHC class II), and the expressions of these molecules were analyzed by flow cytometry. The data are represented as the average of MFI obtained from 5 separate experiments. $^{*}p < 0.05$ for each treatment group, $^{\#}p < 0.05$ between liver DCs (■ black bar) and spleen DCs (□ white bar). Naïve: DCs derived from non-treated mice; vehicle: DCs derived from vehicle-treated mice; α-GalCer: DCs derived from α-GalCer-treated mice.

3.4. *Vaccination of $p53_{232-240}$ peptide-pulsed liver DCs isolated from α-GalCer-treated mice resulted in generating $p53_{232-240}$ peptide specific CTLs more efficiently than that of spleen DCs*

Based on the above results, liver DCs had more antigen-presenting function than spleen DCs in α-GalCer-treated mice. We next evaluated the potential of tumor associated antigen specific CTL induction by vaccination of peptide-pulsed liver DCs or spleen DCs. We vaccinated normal mice i.p. with peptide-pulsed DC. Five days later, spleen CD8+ T cells were isolated and subjected to IFN-γ ELISPOT assay. As shown in Fig. 5, the numbers of IFN-γ spots observed for T cell responses against $p53_{232-240}$ peptide in mice vaccinated with α-GalCer-activated liver DCs were significantly higher than those in mice with vehicle- or non-treated-liver DCs. There were no detectable spots in mice vaccinated with spleen DCs from all treatment groups, suggesting that spleen DCs displayed no stimulatory activity for CTL induction regardless of the administration of α-GalCer *in vivo*. These results revealed that liver DCs in α-GalCer-treated mice have the highest potential for inducing tumor-associated antigen-specific CTLs, which might be associated with the *in vivo* generation of acquired immunity against liver tumor by α-GalCer treatment shown in Fig. 1.

4. Discussion

We and others previously reported that the early eradication of tumor cells in the liver mainly depended on NKT cells and NK cells [3,4]. In this study, we demonstrated that α-GalCer treatment resulted in generating stronger acquired immunity after eradication of primary CMS4 and MC38 liver tumor, but not after spleen tumor treatment. This suggests that liver, and not spleen, is an unique immunological organ that is favorable for generation of acquired immunity. We examined whether CTLs generated by immunization with peptide- and α-GalCer-pulsed BMDC could show equally antitumor effect in skin, liver and spleen in the normal mice. The generated CTLs in treated mice have equal access to all organs and are capable of killing tumor cells (Sasakawa, unpublished data). Thus, our data encour-

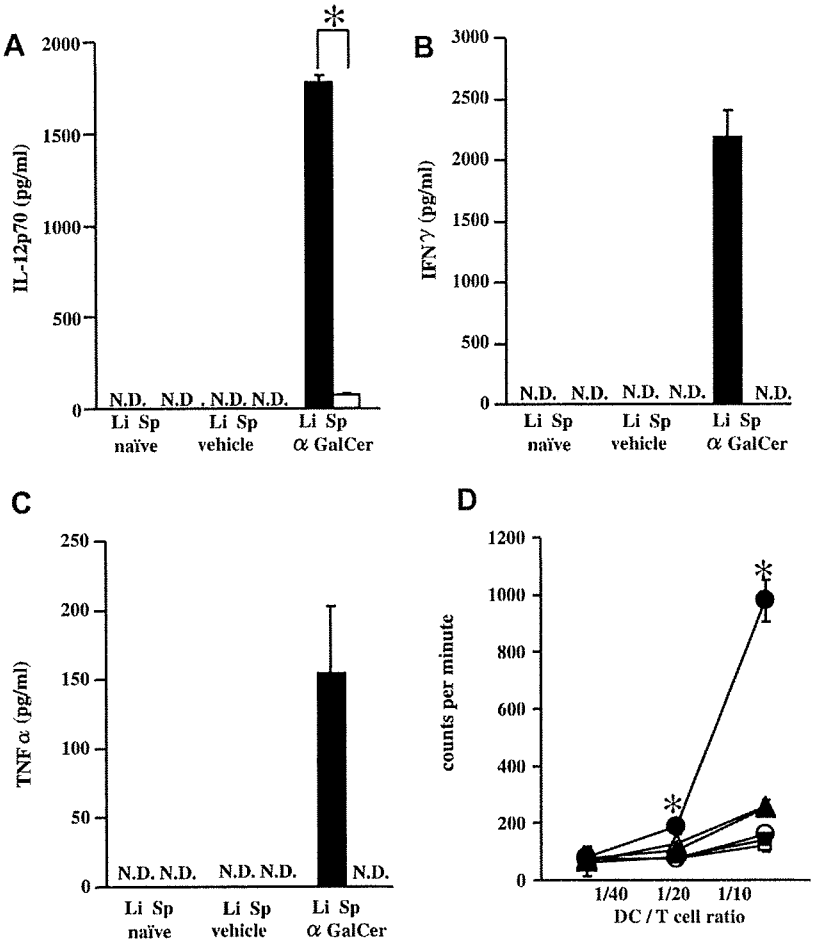

Fig. 4. Th1 type cytokine production of liver DCs from α-GalCer treated mice. Liver and spleen DCs were prepared 24 h after i.p. treatment of α-GalCer or vehicle. 2 × 10^5 DCs were stimulated with LPS (10 μg), and the supernatants of the DC cultures were subjected to specific ELISA. IL-12 (A), IFN-γ (B) and TNF-α (C). N.D., not detected. (D) We examined the allostimulatory capacity of liver and spleen DCs by MLR. Liver DC from non-treated mice (■), vehicle-treated mice (▲), and α-GalCer-treated mice (•). Spleen DC from non-treated mice (□), vehicle-treated mice (△), and α-GalCer-treated mice (○). Each data point represents the mean tumor size ± SD. *$p < 0.05$ counts per minute (CPM) of liver DCs vs CPM of spleen DCs from α-GalCer, vehicle or non-treated mice, respectively. Similar results were obtained from three separate experiments.

aged us to investigate the ability of liver DC to generate acquired antitumor immunity in comparison with spleen DCs.

In the current study, we investigated the activation of liver and spleen DC function after α-GalCer treatment. The expressions of antigen-presenting related molecules on liver DCs were weaker than those on spleen DCs in normal or vehicle treated mice. Pillarisetty et al. reported that liver DCs are generally weak activators of immunity in contrast to spleen DCs in normal mice and the expressions of MHC and costimulatory molecules on liver DCs were lower than those on spleen DCs in normal mice [22]. This is consistent with our results. In marked contrast, α-GalCer administration resulted in a significant increase of DCs in the liver and the expressions of antigen-presenting related molecules was more strongly upregulated in the liver than in the spleen. It has been reported that the expression of CD8α molecule is an activating marker of conventional DCs from progenitor cells [23]. We demonstrated that α-GalCer administration induced not only an increase of total DCs but also a significant increase of CD8- conventional DCs in the liver, which suggested that α-GalCer treatment resulted in developing progenitor DCs efficiently to matured conventional DCs. More strikingly, the production of Th1 type cytokine from α-GalCer-treated liver DCs were significantly more than from α-GalCer-treated spleen DCs. Previous reports demonstrated that the capacity of Th1 type cytokine to link between innate and adaptive immunity by interacting with DCs and T cells, is important for the induction of adaptive antitumor immune response and long-term therapeutic effect [24]. Furthermore, liver DCs showed higher T cell proliferation ability

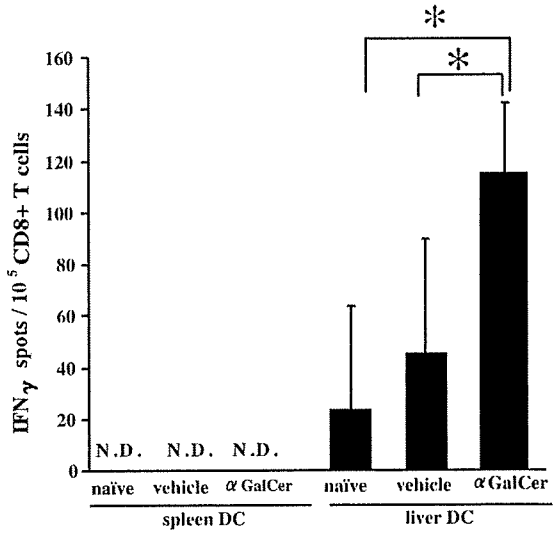

Fig. 5. Evaluation of $p53_{232-240}$ peptide specific CD8+ CTL induction after vaccination of p53 peptide-pulsed DCs from each treated mice. Normal BALB/c mice were immunized i.p. with 1×10^6 $p53_{232-240}$ peptide pulsed liver or spleen DCs isolated from α-GalCer or vehicle treated mice. Five days after vaccination, CD8+ T cells were isolated from the spleen of immunized mice. The frequency of $p53_{232-240}$ peptide specific CD8+ CTL was evaluated by IFN-γ ELSIPOT assay. The results are shown as spots/100,0000 CD8+ T cells; mean ± SD of triplicate samples. CD8+ T cell reactivity against peptide-unpulsed BMDCs served as the negative control in all cases, and this value was subtracted from all experimental determination to determine p53-specific spot numbers. $^*p < 0.05$. N.D., not detected. Similar results were obtained from three separate experiments.

than spleen DCs after α-GalCer treatment. Taken together, these results suggested that α-GalCer treatment resulted in the efficient activation of liver DCs more strongly than spleen DC, which might be associated with the induction of antitumor acquired immunity in the liver.

To examine whether the α-GalCer activated liver and spleen DCs could actually induce acquired immunity, we vaccinated $p53_{232-240}$ peptide-pulsed α-GalCer activated liver and spleen DCs. The frequencies of CD8+ T cells in response to $p53_{232-240}$ peptide were much higher in α-GalCer activated liver DCs vaccinated mice than those in vehicle-treated liver DCs vaccinated mice. Interestingly, the vaccination of $p53_{232-240}$ peptide-pulsed spleen DCs isolated from both α-GalCer and vehicle-treated mice did not generate $p53_{232-240}$ peptide-specific CTL responses. These data suggested that the immunological microenvironment in the spleen may support DCs to be potentially very tolerogenic resulting in inability of generating acquired immunity. In marked contrast, liver DCs potentially have the ability of generating antitumor acquired immunity and that α-GalCer could markedly enhance this ability. A normal mouse liver contains lymphocytes that are usually enriched with 10% NKT cells in contrast to mouse spleen that contains only 2% NKT cells [25]. α-GalCer presented by DCs activates NKT cells upregulating CD40 ligand on NKT cells, which in turn leads to the activation of DCs [17]. Actually we confirmed that i.p. injection of α-GalCer activated equally well in both liver and spleen NKT cells (Sasakawa, unpublished data). Thus, the higher population of NKT cells in the liver may be associated with efficient activation of liver DCs after α-GalCer treatment, which might characterize the unique immunological responses in the liver.

Despite recent progress and early success with various types of immunotherapy, there is still significant room for improvement in these regimens against liver cancer. We demonstrated that liver is an immunologically unique organ that is favorable for generation of acquired antitumor immunity. We propose that α-GalCer treatment may be an attractive strategy for suppressing tumor growth in the liver and promoting regression of metastatic lesions in other organs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.027.

References

[1] Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. CD1d-restricted and TCR-mediated activation of Vα14NKT cells by glycosylceramides. Science 1997;278:1626–1629.

[2] Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-γ-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nat Immunol 2002;3:867–874.

[3] Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, et al. CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 2003;106:81–89.

[4] Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, et al. Treatment of hepatic metastasis of the colon26 adenocacinoma with an α-galactosylceramide, KRN7000. Cancer Res 1998;58:1202–1207.

[5] Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Vα24+Vβ11+NKT cells in human

subjects results in highly coordinates secondary activation of acquired and innate immunity. Blood 2004;103:383–389.
[6] Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res 2002;8:3702–3709.
[7] Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991;9:271–296.
[8] Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 1997;90:3245–3287.
[9] Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 2003;198:267–279.
[10] Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med 2004;199:1607–1618.
[11] Shortman K, Liu TJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151–161.
[12] Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, Iijima R, et al. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. J Immunol 2001;166:6578–6584.
[13] Nakagawa R, Inui T, Nagafune I, Tazunoki Y, Motoki K, Yamauchi A, et al. Essential role of bystander cytotoxic CD122+CD8+ T cells for the antitumor immunity induced in the liver of mice by alpha-galactosylceramide. J Immunol 2004;172:6550–6557.
[14] Mayordomo JI, Loftus DJ, Sakamoto H, De Cesare CM, Appasamy PM, Lotze MT, et al. Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. J Exp Med 1996;183:1357–1365.
[15] Tatsumi T, Takehara T, Yamaguchi S, Sasakawa A, Sakamori R, Ohkawa K, et al. Intrahepatic delivery of α-galatosylceramide pulsed dendritic cells suppresses liver tumor. Hepatology 2007;45:22–30.
[16] Yamaguchi S, Tatsumi T, Takehara T, Sakamori R, Uemura A, Mizushima T, et al. Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines. Cancer 2007;110:1469–1477.
[17] Chaudhry UI, Katz SC, Kingham TP, Pillarisetty VG, Raab JR, Shah AB, et al. In vivo overexpression of Flt3 ligand expands and activates murine spleen natural killer dendritic cells. FASEB J 2006;20:982–984.
[18] Seino K, Motohashi S, Fujisawa T, Nakayama T, Taniguchi M. Natural killer T cell-mediated anti-tumor immune responses and their clinical application. Cancer Sci 2006;97:807–812.
[19] Vremec D, Shortman K. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. J Immunol 1997;159:565–573.
[20] Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, et al. Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. J Immunol 1994;153:1697–1706.
[21] Lasek W, Feleszko W, Golab J, Stoklosa T, Marczak TM, Dabrowska A, et al. Antitumor effects of the combination immunotherapy with interleukin-12 and tumor necrosis factor alpha in mice. Cancer Immunol Immunother 1997;45:100–108.
[22] Pillarisetty VG, Shah AB, Miller G, Bleier JI, DeMatteo RP. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. J Immunol 2004;172:1009–1017.
[23] Markus G, Traver D, Miyamoto T, Weissman IL, Akashi K. Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood 2001;97:3333–3341.
[24] Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. Trends Immunol 2002;23:201–208.
[25] Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in human liver. Immunol Rev 2000;174:5–20.

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Pegylated interferon alpha-2b (Peg-IFN α-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN α-2b plus ribavirin

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SUMMARY. Chronic hepatitis C (CH-C) genotype 1 patients who achieved early virologic response have a high probability of sustained virologic response (SVR) following pegylated interferon (Peg-IFN) plus ribavirin therapy. This study was conducted to evaluate how reducing drug doses affects complete early virologic response (c-EVR) defined as hepatitis C virus (HCV) RNA negativity at week 12. Nine hundred eighty-four patients with CH-C genotype 1 were enrolled. Drug doses were evaluated independently on a body weight base from doses actually taken. From multivariate analysis, the mean dose of Peg-IFN α-2b during the first 12 weeks was the independent factor for c-EVR ($P = 0.02$), not ribavirin. The c-EVR rate was 55% in patients receiving ≥1.2 μg/kg/week of Peg-IFN, and declined to 38% at 0.9–1.2 μg/kg/week, and 22% in patients given <0.9 μg/kg/week ($P < 0.0001$). Even with stratified analysis according to ribavirin dose, the dose-dependent effect of Peg-IFN on c-EVR was observed, and similar c-EVR rates were obtained if the dose categories of Peg-IFN were the same. Furthermore, the mean dose of Peg-IFN during the first 12 weeks affected HCV RNA negativity at week 24 ($P < 0.0001$) and SVR ($P < 0.0001$) in a dose-dependent manner. Our results suggest that Peg-IFN was dose-dependently correlated with c-EVR, independently of ribavirin dose. Thus, maintaining the Peg-IFN dose as high as possible during the first 12 weeks can yield HCV RNA negativity and higher c-EVR rates, leading to better SVR rates in patients with CH-C genotype 1.

Keywords: chronic hepatitis C, drug dose, early virologic response, HCV RNA negativity, pegylated interferon plus ribavirin, sustained virologic response.

INTRODUCTION

Pegylated interferon (Peg-IFN) plus ribavirin therapy can improve anti-viral efficacy for patients with chronic hepatitis C [1–5], and the prognosis of patients in whom hepatitis C virus (HCV) is successfully eradicated improves markedly [6–10]. However, HCV still persists in approximately half of genotype 1 patients treated with Peg-IFN plus ribavirin [2–4]. Therefore, the treatment method needs to be well managed in order to maximize the virologic response in these patients with HCV genotype 1.

Abbreviations: c-EVR, complete EVR; CH-C, chronic hepatitis C; EVR, early virologic response; G-CSF, granulocyte-macrophage colony stimulating factor; Hb, haemoglobin; HCV, hepatitis C virus; Peg-IFN, pegylated interferon; Plt, platelet; SVR, sustained virologic response; WBC, white blood cell.

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In order to achieve sustained virologic response (SVR), earlier virologic response is very important for patients with chronic hepatitis C (CH-C) genotype 1. A high SVR rate (65–72%) was found in patients who achieved early virologic response (EVR) defined as a 2-log decrease in HCV RNA level at week 12, but only 0–3% SVR was seen in patients without EVR [3,11]. Additionally, complete EVR (c-EVR), which means HCV RNA negativity at week 12, is more strongly related to SVR [3].

The relationship between drug exposure and anti-viral effect has been reported in several papers [2,11–15]. McHutchison *et al.* [12] demonstrated that the SVR rate in patients who received ≥80% of their total planned doses of Peg-IFN and ribavirin for ≥80% of the scheduled duration of therapy was significantly higher than that of patients who received <80% of one or both drugs (51% *vs* 34%) and also suggested that the impact of dose reduction was greatest in patients for whom the dose had to be decreased within the first 12 weeks of treatment. In a subsequent analysis, reducing the dose of Peg-IFN and ribavirin to <80% of the full planned dose within the first 12 weeks was reported to reduce EVR rate from 80 to 33% [11]. Thus, drug adherence during the first 12 weeks has been shown to be very important for attaining EVR and SVR, but it remains obscure whether either drug can be reduced to a certain degree without adversely affecting the treatment efficacy.

In the present study, we examined the correlation between c-EVR and drug doses which are evaluated on a body weight basis from drug doses actually taken, in order to clarify the necessary drug exposure of Peg-IFN and ribavirin for achieving a higher c-EVR rate in patients with CH-C genotype 1.

PATIENTS AND METHODS

Patients

The current study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 984 patients with CH-C treated with a combination of Peg-IFN α-2b plus ribavirin were enrolled in this study between December 2004 and September 2006. The baseline characteristics of the patients are summarized in Table 1. All patients were Japanese, their mean age was 56.3 ± 10.1 years, and 56% were males. The mean serum alanine aminotransferase level was 79 ± 61 IU/L.

Patients eligible for this study were those who were infected with HCV genotype 1 and had a viral load of more than 10^5 IU/mL, but were negative for hepatitis B surface antigen or anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

Table 1 Baseline characteristics of patients

Factor	Mean ± SD or number
n	984
Age (year)	56.3 ± 10.1
Sex: male/female	555/429
Body weight (kg)	61.8 ± 11.5
History of interferon treatment	
Naïve/experienced (relapser/nonresponder)*	575/409(160/182)
White blood cells (per mm^3)	5052 ± 1550
Neutrophils (per mm^3)	2577 ± 1092
Red blood cells ($\times 10^4/mm^3$)	442 ± 47
Haemoglobin (g/dL)	14.1 ± 1.4
Platelets ($\times 10^4/mm^3$)	15.9 ± 5.5
AST (IU/L)	66 ± 45
ALT (IU/L)	79 ± 61
Serum HCV RNA (kIU/mL)†	1600
Histology (METAVIR)‡	
Fibrosis; 0/1/2/3/4	49/314/197/105/18
Activity; 0/1/2/3	23/329/304/27

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.
*Viral response to previous treatment was unknown in 57 patients, and 10 patients had discontinued treatment. †Data shown are median values. ‡301 missing.

Treatment

All patients received Peg-IFN α-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough) for the duration of the study of 48 weeks. Peg-IFN α-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight ≤60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to a standard treatment protocol for Japanese patients.

Dose reduction

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the haematological adverse effects. The dose of Peg-IFN α-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to <1500/mm^3, the neutrophil count to <750/mm^3 or the platelet (Plt) count to <8 × $10^4/mm^3$, and was discontinued if the WBC count declined to <1000/

mm^3, the neutrophil count to $<500/mm^3$ or the Plt count to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg if the haemoglobin (Hb) level decreased to <10 g/dL, and was discontinued if the Hb level decreased to <8.5 g/dL. Both Peg-IFN α-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, ferric medicine or haematopoetic growth factors, such as erythropoietin alpha, or granulocyte-macrophage colony stimulating factor (G-CSF), were not administered.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnotics, Branchburg, NJ, USA) and qualitatively analysed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL). The c-EVR was defined as the absence of detectable serum HCV RNA at treatment week 12, and SVR was defined as the absence of detectable serum HCV RNA at week 72. Patients with less than a 2-log decrease in HCV RNA level at treatment week 12 compared with the baseline had to stop treatment and were regarded as nonresponders. All patients with detectable serum HCV RNA at treatment week 24 were also considered nonresponders and excluded from further treatment.

Assessment of drug exposure

The amounts of Peg-IFN α-2b and ribavirin actually taken by each patient during the first 12 weeks of the treatment were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline: Peg-IFN α-2b expressed as μg/kg/week, and ribavirin expressed as mg/kg/day.

Evaluation of impact of drug exposure on c-EVR

We evaluated the relationship between the drug exposure of both drugs and c-EVR by univariate and multivariate analysis for c-EVR, using the factors of mean administration doses of both drugs during the first 12 weeks and the factors at baseline. Furthermore, Peg-IFN α-2b dose (average dose per body weight and per week) was classified into five categories (up to 0.6 μg/kg; from 0.6 to <0.9 μg/kg; from 0.9 to <1.2 μg/kg; from 1.2 to <1.5 μg/kg; from 1.5 μg/kg and above). Ribavirin exposure was classified into four categories (up to 8 mg/kg; from 8 to <10 mg/kg; from 10 to <12 mg/kg; from 12 mg/kg and above), in order to examine the impact of Peg-IFN dose exposure on c-EVR. This impact was also evaluated based on the percentage of the total prescribed dose and compared with that based on the mean dose per body weight.

Statistical analysis

Baseline data for various demographic, biochemical and virologic characteristics of the patients are expressed as mean ± SD or median values. To analyse the relationship between baseline data including drug exposure and c-EVR, univariate analysis using the Mann–Whitney *U*-test or chi-squared test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. A two-tailed *P*-value < 0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL, USA).

RESULTS

Progress of patients treated with Peg-IFN α-2b and ribavirin

Of the 984 patients, 81 discontinued treatment because of adverse events (n = 74) or voluntary withdrawal (n = 7) by treatment week 12. The 903 patients who completed 12 weeks of treatment were assessed for c-EVR. During 12–48 weeks of treatment, 331 of the nonresponders and nine of breakthrough discontinued treatment, as did 91 patients (adverse events, n = 71; voluntary withdrawal, n = 20). A total of 472 patients completed 48 weeks of treatment.

Drug reduction and virologic response

Peg-IFN α-2b was reduced without discontinuation in 29% (n = 266) and ribavirin was reduced without discontinuation in 40% (n = 359) of the 903 patients who completed 12 weeks of treatment. The c-EVR rate was 49% (445/903) and HCV RNA was negative at week 24 in 60% (542/903) of patients who completed 12 weeks of treatment. Of the 445 patients with c-EVR, 327 patients achieved SVR (73%). Only 7% of the 458 patients without c-EVR did so.

Impact of dose exposure of Peg-IFN α-2b and ribavirin on c-EVR

The mean dose of Peg-IFN α-2b actually taken during the first 12 weeks by each patient was 1.33 μg/kg/week (range 0.41–2.16 μg/kg/week; median 1.40 μg/kg/week) and that of ribavirin was 10.4 mg/kg/day (range 2.9–16.2 mg/kg/day; median 10.6 mg/kg/day).

The mean doses of both drugs and the factors at baseline correlated with the c-EVR were assessed by univariate and multivariate logistic regression analyses. Univariate analysis showed that factors significantly associated with c-EVR were age, sex, WBC, neutrophils, red blood cells, Hb, Plt, aspartate aminotransferase, the degree of liver fibrosis and the mean doses of Peg-IFN α-2b and ribavirin during the first 12 weeks (Table 2). The factors selected as significant by the univari-

Table 2 Univariate analysis for c-EVR among patients who completed 12 weeks treatment

Factor	c-EVR (+)	c-EVR (−)	*P*-value
n	445	458	
Age (year)	54.4 ± 10.4	57.5 ± 9.6	<0.001
Sex: male/female	267/178	237/221	0.01
Serum HCV RNA (kIU/mL)*	1500	1600	0.28
White blood cells (per mm^3)	5336 ± 1536	4818 ± 1547	<0.001
Neutrophils (per mm^3)	2789 ± 1133	2398 ± 1038	<0.001
Red blood cells ($\times 10^4/mm^3$)	450 ± 46	435 ± 49	<0.001
Haemoglobin (g/dL)	14.3 ± 1.4	13.9 ± 1.4	<0.001
Platelets ($\times 10^4/mm^3$)	17.3 ± 5.2	15.0 ± 5.6	<0.001
AST (IU/L)	62 ± 44	69 ± 44	<0.001
ALT (IU/L)	77 ± 64	80 ± 57	0.07
Histology (METAVIR)†			
Fibrosis: 0–2/3–4	273/37	247/74	<0.001
Activity: 0–1/2–3	171/139	159/162	0.16
Peg-IFN dose (μg/kg/week)‡	1.39 ± 0.22	1.28 ± 0.30	<0.001
Ribavirin dose (mg/kg/day)‡	10.6 ± 1.7	10.1 ± 2.1	0.002

c-EVR, complete early virologic response; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Peg-IFN, pegylated interferon. *Data shown are median values. †272 missing. ‡Mean doses during 0–12 weeks.

Table 3 Multivariate analysis for c-EVR among patients who completed 12 weeks treatment

Factor	Category	Odds ratio	95% CI	*P*-value
Age	by 1 year	0.982	0.966–0.999	0.04
Sex	male/female	–	–	NS
Neutrophils	by $100/mm^3$	1.017	1.002–1.033	0.03
Red blood cells	by $1 \times 10^4/mm^3$	–	–	NS
Haemoglobin	by 1 g/dL	–	–	NS
Platelets	by $1 \times 10^4/mm^3$	1.051	1.014–1.088	<0.01
AST	by 1 IU/L	–	–	NS
Fibrosis*	0–2/3–4	–	–	NS
Peg-IFN dose†	by 0.1 μg/kg/week	1.079	1.011–1.151	0.02
Ribavirin dose†	by 1 mg/kg/day	–	–	NS

95% CI, 95% confidence interval; Peg-IFN, c-EVR, complete early virologic response; pegylated interferon; N.S., No Significant difference; AST, aspartate aminotransferase.
*METAVIR fibrosis score. †Mean doses during 0–12 weeks.

ate analysis were evaluated by multivariate logistic regression analysis. The mean dose of Peg-IFN α-2b during the first 12 weeks was the independent factor for c-EVR ($P = 0.02$), apart from the neutrophils ($P = 0.03$) and Plt value at baseline ($P < 0.01$) and age ($P = 0.04$) (Table 3). In contrast, the mean dose of ribavirin during the first 12 weeks showed no correlation with c-EVR.

The c-EVR rates were 54% (137/253) and 56% (246/443) for patients who received ≥1.5 and 1.2–1.5 μg/kg/week of Peg-IFN α-2b on average during the first 12 weeks, and declined to an average rate of 38% (40/105) in patients given 0.9–1.2 μg/kg/week of Peg-IFN α-2b, and an average rate of 22% (22/102) in patients given <0.9 μg/kg/week ($P < 0.0001$) (Table 4). The c-EVR rate among the patients with ≥1.2 μg/kg/week of Peg-IFN α-2b was significantly higher than that of the patients with <1.2 μg/kg/week [≥1.2 μg/kg/week, 55% (383/696) *vs* <1.2 μg/kg/week, 30% (62/207), $P < 0.0001$].

Next, we analysed the impact of Peg-IFN α-2b on c-EVR in stratified analysis according to ribavirin dose. Figure 1 shows the relationship of c-EVR and the degree of Peg-IFN α-2b exposure for two groups of ribavirin doses: the group with ≥10.6 mg/kg/day of ribavirin and that with <10.6 mg/kg/day (10.6 mg/kg/day was the median value). In either group, the mean dose of Peg-IFN α-2b was dose-dependently correlated with c-EVR ($P < 0.0001$), and c-EVR rates were very similar in both groups if the dose categories of Peg-IFN α-2b were the same.

Table 4 The c-EVR rate according to Peg-IFN and ribavirin doses during weeks 0–12 for patients who completed 12 weeks treatment

Ribavirin dose (mg/kg/day)**	Peg-IFN α-2b dose (μg/kg/week),*				
	≥1.5	1.2–1.5	0.9–1.2	<0.9	Total
≥12	57% (60/105)	61% (22/36)	38% (6/16)	22% (2/9)	54% (90/166)
10–12	54% (46/85)	58% (154/267)	36% (14/39)	23% (11/47)	51% (225/438)
8–10	50% (25/50)	53% (52/99)	52% (15/29)	18% (4/22)	48% (96/200)
<8	46% (6/13)	44% (18/41)	24% (5/21)	21% (5/24)	34% (34/99)
Total	54% (137/253)	56% (246/443)	38% (40/105)	22% (22/102)	49% (445/903)

c-EVR, complete early virologic response; Peg-IFN, pegylated interferon.
*$P < 0.0001$ for comparison of the four Peg-IFN groups. **$P = 0.05$ for comparison of the four ribavirin groups.

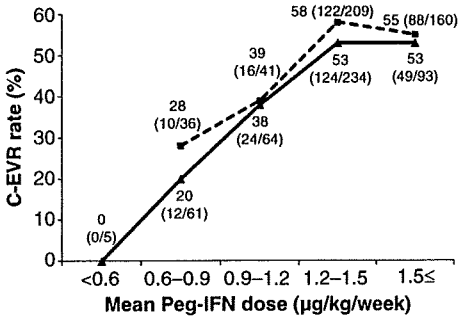

Fig. 1 Complete-EVR rate according to pegylated interferon alpha-2b (Peg-IFN α-2b) and ribavirin doses during weeks 0–12 for patients who completed 12 weeks of treatment. (-▲-) Group with the mean ribavirin dose <10.6 mg/kg/day. (··■··) Group with the mean ribavirin dose ≥10.6 mg/kg/day. The Peg-IFN α-2b dose was dose-dependently correlated with c-EVR in both groups ($P < 0.0001$). There was no significant difference between the two ribavirin-dose groups ($P = 0.19$).

c-EVR rates according to Peg-IFN α-2b drug exposure using a percentage cut off and mean dose cut off

Table 5 shows the c-EVR rates according to the category of Peg-IFN α-2b doses during the first 12 weeks based on the percentage of the total prescribed dose and the mean doses. The whole c-EVR rate was 54% (377/698) for patients who received more than 80% of the prescribed dose, and 43% (47/109) in patients given 60–80% of the prescribed dose, and 21% (21/96) in patients given <60% of the prescribed dose of Peg-IFN α-2b. Among patients given ≥80% of the prescribed dose of Peg-IFN α-2b, the c-EVR rate was significantly lower in patients given <1.2 μg/kg/week of Peg-IFN α-2b than those given ≥1.2 μg/kg/week (32% *vs* 55%, $P < 0.05$). On the other hand, even in patients given 60–80% of the prescribed dose of Peg-IFN α-2b, if they were given ≥1.2 μg/kg/week of Peg-IFN α-2b, a higher c-EVR rate was attained in comparison with those given <1.2 μg/kg/week (71% *vs* 38%, $P = 0.01$); the c-EVR rate in patients given 60–80% of the prescribed dose and ≥1.2 μg/kg/week of Peg-IFN α-2b was not inferior to that in patients given ≥80% of the prescribed dose and ≥1.2 μg/kg/week of Peg-IFN α-2b.

Impact of dose exposure of Peg-IFN α-2b during the first 12 weeks of the treatment on HCV RNA negativity at week 24 and SVR

Patients positive for HCV RNA at week 24 week during Peg-IFN α-2b and ribavirin treatment were regarded as non-responders and stopped treatment [11]. We analysed the

Table 5 The c-EVR rate according to Peg-IFN dose during weeks 0–12 based on the percentage of the planned dose and the mean doses

Peg-IFN α-2b dose (μg/kg/week)	≥80%	60–80%	<60%	Total
≥1.2	55%* (371/679)	71%** (12/17)	–	55% (383/696)
<1.2	32% (6/19)	38% (35/92)	22% (21/96)	30% (62/207)
Total	54% (377/698)	43% (47/109)	21% (21/96)	49% (445/903)

c-EVR, complete early virologic response; Peg-IFN, pegylated interferon.
*$P < 0.05$; patients with ≥1.2 μg/kg/week *vs* <1.2 μg/kg/week among the patients with more than 80% of the total prescribed dose of Peg-IFN α-2b. **$P = 0.01$; patients with ≥1.2 μg/kg/week *vs* <1.2 μg/kg/week among the patients with more than 60–80% of the total prescribed dose of Peg-IFN α-2b.

relationship between the dose exposure to Peg-IFN α-2b during the first 12 weeks and HCV RNA negative rates at week 24 or SVR in 903 patients completing 12 weeks of treatment. As a result, HCV RNA negative rates at week 24 and SVR rates declined according to the decrease in the dose of Peg-IFN α-2b during the 12 weeks of treatment; patients given ≥1.5, 1.2–1.5, 0.9–1.2 and <0.9 μg/kg/week of Peg-IFN α-2b during the first 12 weeks of the treatment showed HCV RNA negativity of 63%, 66%, 48% and 39%, respectively ($P < 0.0001$), and SVR of 46%, 43%, 30% and 20%, respectively ($P < 0.0001$).

DISCUSSION

Adherence to ribavirin was reported to be the important factor for EVR as well as that to Peg-IFN in most previous studies [2,11,12]. However, the drug exposure of Peg-IFN α-2b and ribavirin had not been analysed independently with respect to their individual influence on the anti-viral effect in these studies. Adherence to both drugs may be related factors, i.e. most patients who can tolerate a high dose of Peg-IFN are in good condition and thus can also receive a high dose of ribavirin. In the present study, the impact of the dose of Peg-IFN α-2b and ribavirin on the anti-viral effect was evaluated by multivariate logistic regression analysis, using the mean administration doses of both drugs during the first 12 weeks and baseline factors. As a result, the dose exposure of Peg-IFN α-2b was found to be the significant factor affecting c-EVR as well as baseline factors such as age, neutrophils and Plt values, but not ribavirin. This suggests that the c-EVR rate can be raised by maintaining the dose of Peg-IFN α-2b during the first 12 weeks in patients with disadvantageous factors at baseline. In fact, the c-EVR rate was higher in those who received ≥1.2 μg/kg of Peg-IFN α-2b than in those given <1.2 μg/kg of Peg-IFN α-2b for aged patients over 60 years of age (≥1.2 μg/kg; 46% *vs* <1.2 μg/kg; 28%, $P < 0.01$) or for patients with a low Plt value ($<12 \times 10^4/mm^3$)(≥1.2 μg/kg; 45% *vs* <1.2 μg/kg; 22%, $P < 0.001$). Therefore, a marked dose reduction of Peg-IFN α-2b should not be risked at the start even for aged patients or patients with lower Plt value, which is indicative of advanced fibrosis. The administration of ≥1.2 μg/kg/week of Peg-IFN α-2b is desirable as a starting dose for achieving c-EVR even in these patients: that of <1.2 μg/kg/week can lead to a non-viral response or a late viral response. Independent evaluation of the c-EVR rate according to the degree of the ribavirin dose showed a stepwise decline as the total cumulative dose of Peg-IFN α-2b decreased. Therefore, the dose of Peg-IFN α-2b should be maintained as high as possible even in patients who have to reduce Peg-IFN α-2b to <1.2 μg/kg/week. Using G-CSF for patients who develop severe neutropenia and are forced to decrease Peg-IFN can be beneficial, especially in the first 12 weeks.

The goal of 80% of the planned drug dosage for 80% of the assigned duration was derived from an adherence criterion that had been adopted previously for assessment of the efficacy of other pharmaceutical agents, such as drugs to treat cancer and human immunodeficiency virus [16]. However, in Peg-IFN plus ribavirin therapy for patients with CH-C, the planned administration dose [17,18] differs on a body weight basis by 27% for Peg-IFN α-2b and 40% for ribavirin among patients of 50–100 kg of body weight, which would be equivalent to the same rate differences for 80% of the planned drug dosage. In detail, the target dose of Peg-IFN α-2b scheduled to be administered is 1.5 μg/kg, but the usual dose for the individual patient is from 1.28 to 1.76 μg/kg/week based on body weight among patients weighing 50–100 kg according to the practice guidelines of the American Association for the Study of Liver Diseases and the manufacturer's drug information in the USA and Europe [17,18]. The range of ribavirin dose per kg of body weight is from 12 to 20 mg/kg/day. Therefore, in this study, the drug exposure was assessed from the average dose per kg of body weight.

In the evaluation of c-EVR rates according to Peg-IFN α-2b drug exposure using a percentage cut off and mean dose cut off in this study, the c-EVR rate of patients given <1.2 μg/kg/week of Peg-IFN α-2b was low (32%) even in those who received ≥80% of the total planned doses of Peg-IFN α-2b. If given ≥1.2 μg/kg/week of Peg-IFN α-2b, the c-EVR rate (71%) in patients who received 60–80% of the total doses was not inferior to that in patients given ≥80% of the total dose of Peg-IFN α-2b (54%). This means that patients whose starting dose of Peg-IFN α-2b is <1.5 μg/kg/week should not have their dosage reduced to 80% of the planned dose (<1.2 μg/kg/week) in order to have a higher probability of c-EVR, while those given ≥1.5 μg/kg/week of Peg-IFN α-2b at the start can have their dosage reduced to 80% (≥1.2 μg/kg/week) without lowering the c-EVR rate. Thus, the drug dose on a body weight basis itself should be examined as an index of the drug exposure in order to evaluate the anti-viral effect of both drugs accurately for patients with CH-C.

As for the impact of the drug exposure to ribavirin on c-EVR, the drug dose of ribavirin during the first 12 weeks was shown to have no relationship with the c-EVR rate, although it was precisely evaluated in this study, using doses actually taken on body weight. However, ribavirin can be more effective for decreasing the viral relapse after interferon or Peg-IFN α-2b and ribavirin combination therapy in patients with CH-C genotype 1 [2,3,19–24]. Recently, Shiffman *et al.* [15] have reported that a higher starting dose of ribavirin (1000–1600 mg/day) plus a regular dose of Peg-IFN α-2b with epoetin was associated with a lower relapse rate in treatment with CH-C genotype 1. Considering the viral relapse after treatment, it is thought that the ribavirin dose should not be reduced quickly in patients with mild side effects, even though it does not affect c-EVR. In fact, among the patients who attained c-EVR, a higher rate of viral relapse was found in the patients given <10 mg/kg/day of the mean ribavirin dose during 48 weeks in comparison

with those given ≥10 mg/kg/day of the mean ribavirin dose in this study [26.9% (49/182) *vs* 12.4% (26/209), $P < 0.001$] (data not shown). It seems possible to start ribavirin at a lower dose and increase it by degrees with monitoring of Hb level during treatment of patients with mild anaemia or ischemic heart disease, because the ribavirin dose appears to affect the viral relapse as the total dose over 48 weeks, not during the first 12 weeks.

In conclusion, our results have demonstrated that Peg-IFN α-2b is dose-dependently correlated with c-EVR and maintaining as high a drug dose of Peg-IFN α-2b as possible (≥1.2 μg/kg/week) during the first 12 weeks can yield higher c-EVR rates, leading to better treatment outcomes for patients with CH-C genotype 1.

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REFERENCES

1 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; 41: 17–27.
2 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–965.
3 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–982.
4 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140: 346–355.
5 Zeuzem S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; 40: 993–999.
6 Hiramatsu N, Hayashi N, Kasahara A *et al.* Improvement of liver fibrosis in chronic hepatitis C patients treated with natural interferon alpha. *J Hepatol* 1995; 22: 135–142.
7 Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; 27: 1394–1402.
8 Ikeda K, Saitoh S, Arase Y *et al.* Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999; 29: 1124–1130.
9 Kasahara A, Tanaka H, Okanoue T *et al.* Interferon treatment improves survival in chronic hepatitis C patients showing biochemical as well as virological responses by preventing liver-related death. *J Viral Hepatitis* 2004; 11: 148–156.
10 Imai Y, Kasahara A, Tanaka H *et al.* Interferon therapy for aged patients with chronic hepatitis C: improved survival in patients exhibiting a biochemical response. *J Gastroenterol* 2004; 39: 1069–1077.
11 Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; 38: 645–652.
12 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; 123: 1061–1069.
13 Shiffman ML, Ghany MG, Morgan TR *et al.* Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. *Gastroenterology* 2007; 132: 103–112.
14 Reddy KR, Shiffman ML, Morgan TR *et al.* Impact of ribavirin dose reductions in hepatitis C virus genotype 1 patients completing peginterferon alfa-2a/ribavirin treatment. *Clin Gastroenterol Hepatol* 2007; 5: 124–129.
15 Shiffman ML, Salvatore J, Hubbard S *et al.* Treatment of chronic hepatitis C virus genotype 1 with peginterferon, ribavirin, and epoetin alpha. *Hepatology* 2007; 46: 371–379.
16 Paterson DL, Swindells S, Mohr J *et al.* Adherence to protease inhibitor therapy and outcomes in patients with HIV infection. *Ann Intern Med* 2000; 133: 21–30.
17 Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; 39: 1147–1171.
18 Dienstag JL, McHutchison JG. American Gastroenterological Association medical position statement on the management of hepatitis C. *Gastroenterology* 2006; 130: 225–230.
19 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 352: 1426–1432.
20 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1485–1492.
21 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1493–1499.

22 Hiramatsu N, Kasahara A, Nakanishi F *et al.* The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan. *Hepatol Res* 2004; 29: 142–147.
23 Bronowicki JP, Ouzan D, Asselah T *et al.* Effect of ribavirin in genotype 1 patients with hepatitis C responding to pegylated interferon alfa-2a plus ribavirin. *Gastroenterology* 2006; 131: 1040–1048.
24 Hiramatsu N, Oze T, Yakushijin T, *et al.* Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alfa-2b plus ribavirin. *J Viral Hepat* 2009; In press.

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Mutations Associated With the Therapeutic Efficacy of Adefovir Dipivoxil Added to Lamivudine in Patients Resistant to Lamivudine With Type B Chronic Hepatitis

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Factors influencing the therapeutic efficacy of adefovir dipivoxil added to continuing lamivudine have not been elucidated in lamivudine-resistant patients with type B chronic hepatitis. The viral mutations influencing the efficacy of treatment with adefovir dipivoxil were investigated by sequencing analysis of the whole virus genome. Thirty patients resistant to lamivudine receiving adefovir dipivoxil therapy added to lamivudine were studied. From serum samples obtained before the administration of adefovir dipivoxil, full-length viral DNA sequences were determined by PCR-direct sequencing. Susceptibility of the virus to adefovir was examined further using in vitro transfection analysis. By screening the whole viral genome, the presence of two mutations, a T-to-C/G/A mutation at nt1753 (V1753) and an A-to-C mutation at nt2189 (C2189), correlated with the higher incidence of sustained viral DNA clearance during therapy ($P < 0.005$ and $P < 0.05$). In multivariate analysis, the V1753 ($P = 0.001$) and the C2189 ($P = 0.007$) mutations, and elevated transaminase ($P = 0.011$) and low viral load ($P = 0.008$) at the baseline were selected as significant independent factors associated with improved antiviral efficacy. In vitro transfection analysis showed no differences in susceptibility to adefovir among wild-type virus and C1753 and C2189 mutant viruses, suggesting that the virus possessing these mutations may be eradicated more efficiently than the wild-type virus by treatment regardless of a direct antiviral effect of adefovir. ***J. Med. Virol. 81:798–806, 2009.***

KEY WORDS: antiviral therapy; hepatitis B virus; mutation

INTRODUCTION

Treatment of patients with chronic hepatitis B virus (HBV) infection is aimed at suppressing continuously viral replication thereby preventing progression of liver disease. Lamivudine has been shown to achieve reduction of HBV DNA and histological improvement in the liver in most patients with type B chronic hepatitis [Lai et al., 1998; Dienstag et al., 1999]. However, its long-term administration often causes the emergence of drug-resistant virus, resulting in loss of antiviral activity. The frequency of lamivudine resistance has been reported to be 24% after 1 year and 70% after 4 years of therapy [Lai et al., 2003]. This resistance is conferred by an rtM204V/I mutation occurring within the reverse transcription domain of the HBV polymerase gene [Liaw et al., 2000; Leung et al., 2001; Lai et al., 2003]. The rtL180M mutation is also combined frequently with rtM204V/I [Lai et al., 2003].

Adefovir dipivoxil has been shown to result in significant virological and histological improvement in both nucleoside-naïve and lamivudine-resistant patients with type B chronic hepatitis [Hadziyannis et al., 2003; Marcellin et al., 2003; Perrillo et al., 2004; Peters et al., 2004]. The incidence of adefovir dipivoxil resistance in nucleoside-naïve patients has been reported to be 6% after 3 years and 29% after 5 years of therapy [Hadziyannis et al., 2005, 2006], which is less frequent than that occurring with lamivudine therapy

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alone. As for lamivudine-resistant patients, adefovir dipivoxil resistance has been observed in 18% of patients after 1 year after the change from lamivudine to adefovir dipivoxil [Lee et al., 2006]. Two mutations, rtA181V/T and rtN236T, have been shown to confer resistance to adefovir dipivoxil [Hadziyannis et al., 2005, 2006; Lee et al., 2006]. In the case of adefovir dipivoxil administration added to lamivudine therapy, mutant strains resistant to both drugs develop rarely, although there have been reports of a few patients with resistance to both [Villet et al., 2006; Karatayli et al., 2007].

Thus far, factors affecting the efficacy of adefovir dipivoxil added to lamivudine treatment have not been clarified fully in lamivudine-resistant patients with type B chronic hepatitis. In the present study, sequencing analysis of full-length HBV DNA was undertaken in lamivudine-resistant patients with type B chronic hepatitis, who received adefovir dipivoxil added to the continuing lamivudine treatment, and attempts were made to identify the viral mutations associated significantly with therapeutic efficacy.

PATIENTS AND METHODS

Patients and Treatment

Thirty consecutive patients with type B chronic hepatitis at Osaka University Hospital or the National Hospital Organization Osaka National Hospital participated in this study. These patients were also subjects in a previous study that investigated the correlation of viral genomic changes with occurrences of lamivudine-resistant rtM204V/I and rtL180M mutations [Ohkawa et al., 2008]. All 30 patients had been treated with 100 mg/day of lamivudine and had shown lamivudine resistance with the detection of lamivudine-resistant mutant virus. The total duration of the preceding lamivudine therapy ranged from 20 to 60 (median 35) months. All the patients received 10 mg/day of adefovir dipivoxil continuously in addition to lamivudine. Liver function tests and HBV markers were measured every month for the initial 6 months and every 2 months thereafter. The follow-up period of adefovir dipivoxil therapy ranged from 6 to 40 (median 19) months.

Clinical Features of the Patients

The clinical and virological features of the patients at the commencement of adefovir dipivoxil administration are shown in Table I. The 26 males and 4 females, aged 25–71 (median 49) years, all had hepatitis B surface antigen (HBsAg). Hepatitis B e antigen (HBeAg) was found in 21 (70%) patients. HBV DNA ranged from 4.9 to >7.6 (median 7.2) $\log_{10}$ copies/ml, and alanine aminotransferase (ALT) ranged from 16 to 455 (median 108) IU/L. With respect to liver diseases, 23 (77%) were diagnosed with chronic hepatitis, 3 (10%) with cirrhosis and 4 (13%) with hepatocellular carcinoma (HCC) according to a liver biopsy and/or imaging procedures. None of the patients had evidences of hepatitis C virus- or human immunodeficiency virus-related diseases, alcoholic liver disease, autoimmune hepatitis and drug-induced liver injury. Serum samples for sequencing analysis were collected within 2 months before the commencement of adefovir dipivoxil administration and stored at −80°C. Pairwise serum samples obtained before lamivudine therapy were also collected and used for analysis in 14 of the 30 patients with type B chronic hepatitis. Informed consent was obtained from all patients.

Measurement of HBV Markers

HBsAg, HBeAg and antibody to HBeAg (anti-HBe) were measured by enzyme immunoassay. Serum HBV DNA was quantitated by a PCR-based assay (Amplicor HB Monitor, Roche Diagnostics Co. Ltd, Tokyo, Japan) having a lower detection limit of 2.6 $\log_{10}$ copies/ml.

Sequencing of Full-Length HBV DNA

The full-length HBV DNA was amplified by PCR and subjected to direct sequencing analysis as described elsewhere [Kanada et al., 2007]. In 25 of the 30 patients with type B chronic hepatitis, sequence data of full-length HBV DNA, that had been determined in a previous study [Ohkawa et al., 2008], were also used in this study. In the remaining five patients, HBV DNA sequences were updated using serum samples obtained just before the commencement of adefovir dipivoxil administration. The Genbank accession numbers of the nucleotide sequences in HBV strains determined in this study are shown in Table I.

Plasmid and Transfection

The HBV-expressing plasmid pHBC carried approximately 1.2 times the genomic length of HBV adr4 strain of genotype C (Genbank accession no. X01587) [Fujiyama et al., 1983]. pHBC-C1753 and pHBC-C2189, which had the C1753 and C2189 mutations in their inserted HBV sequences, were generated by site-directed mutagenesis. pCMV-SEAP was the expression plasmid of a secreted alkaline phosphatase.

Huh7 cells (3×10^5 cells) were seeded on a 35-mm-diameter culture dish and transfected with 1 μg of HBV-expressing plasmid and 0.06 μg of pCMV-SEAP using the FuGENE6 reagent (Roche Diagnostics Co. Ltd). After overnight culture, the cells were treated with 1 or 10 μM of lamivudine (GlaxoSmithKline Co. Ltd, Tokyo, Japan), 1 or 10 μM of adefovir (Toronto Research Chemicals, Inc., North York, Canada), 10 μM of lamivudine plus 10 μM of adefovir, or left untreated. The cells were harvested 3 days after transfection. The culture supernatant was used for measurement of alkaline phosphatase activity to evaluate the efficiency of transfection.

Detection of HBV DNA Replicative Intermediate

For detection of the HBV DNA replicative intermediate, the cells were lysed with buffer containing

TABLE I. Clinical and Virological Features in 30 Patients With Type B Chronic Hepatitis Treated With Adefovir Dipivoxil Added to Continuing Lamivudine

Patient no.	Age (years)	Gender (M/F)	Liver disease	Duration of preceding lamivudine therapy (months)	ALT (Iμ/l)	HBeAg/ anti-HBe	HBV DNA (log_{10} copies μl)	rtM204V/I mutation (V/I)	rtL180M mutation	V1753 mutation	C2189 mutation	Follow-up period of lamivudine plus adefovir dipivoxil therapy (months)	Sustained HBV DNA clearance	Genbank accession no.
1	51	M	Chronic hepatitis	33	64	+/−	7.2	I	+	−	+	40	+	AB367415
2	54	M	Chronic hepatitis	20	87	+/−	>7.6	V	+	−	+	33	+	AB367393
3	38	M	Chronic hepatitis	30	429	+/−	>7.6	I	+	+ (C)	+	32	+	AB367414
4	33	M	Chronic hepatitis	33	331	+/−	7.5	I	+	+ (C)	+	31	+	AB367413
5	71	F	Cirrhosis	21	148	−/+	7.1	I	−	−	+	31	+	AB367430
6	51	M	Cirrhosis	38	272	−/+	6.9	I	−	+ (G)	+	29	+	AB367804
7	51	M	Chronic hepatitis	36	327	−/+	5.5	V	+	+ (C)	−	28	+	AB367406
8	25	M	Chronic hepatitis	41	455	+/−	6.6	V	+	−	−	27	+	AB367394
9	55	M	HCC	35	96	+/+	5.2	V	+	−	+	26	+	AB367401
10	62	M	Chronic hepatitis	49	401	+/+	7.4	I	−	−	−	15	+	AB367428
11	38	M	Chronic hepatitis	41	122	+/+	7.1	V	+	+ (G)	−	14	+	AB367407
12	27	M	Chronic hepatitis	21	28	+/−	4.9	I	−	+ (A)	−	11	+	AB367422
13	37	F	Chronic hepatitis	54	16	−/+	5.3	I	+	+ (C)	+	6	+	AB367803
14	47	M	Chronic hepatitis	47	59	+/−	>7.6	I	+	−	−	32	−	AB367408
15	36	M	Chronic hepatitis	28	340	+/−	7.1	I	−	−	−	28	−	AB367425
16	39	M	HCC	28	47	+/−	5.0	I	−	−	+	25	−	AB367802
17	64	M	Cirrhosis	45	32	+/−	>7.6	V	+	+ (C)	−	20	−	AB367403
18	60	F	Chronic hepatitis	25	41	−/+	6.6	I	−	−	−	19	−	AB367427
19	57	F	Chronic hepatitis	55	140	+/−	>7.6	I	+	−	−	19	−	AB367410
20	38	M	Chronic hepatitis	28	112	+/−	7.5	V	+	−	+	18	−	AB367398
21	39	M	Chronic hepatitis	35	193	+/−	>7.6	I	+	−	−	17	−	AB367418
22	36	M	Chronic hepatitis	46	115	+/−	7.5	V	+	−	−	17	−	AB367404
23	56	M	HCC	26	35	+/−	>7.6	I	−	−	−	17	−	AB367435
24	46	M	Chronic hepatitis	48	78	−/+	>7.6	V	+	+ (G)	−	16	−	AB367800
25	54	M	Chronic hepatitis	40	104	−/+	7.2	V	+	−	−	13	−	AB367405
26	71	M	Chronic hepatitis	56	184	+/−	7.1	I	−	−	−	11	−	AB367434
27	53	M	HCC	27	120	−/+	6.7	I	−	−	+	10	−	AB367433
28	40	M	Chronic hepatitis	27	56	+/−	7.4	I	−	+ (C)	+	6	−	AB367423
29	63	M	Chronic hepatitis	26	20	+/−	>7.6	V	+	+ (C)	−	6	−	AB367399
30	36	M	Chronic hepatitis	60	66	−/+	6.8	I	−	−	+	6	−	AB367801

50 mM Tris–Cl (pH 7.5), 1 mM EDTA and 1% NP40, followed by the 15-min incubation on ice. Then, the sample was centrifuged to remove the nuclei pellet at 15,000 rpm at 4°C, and treated with 30 μg of DNase I at 37°C for 30 min. The sample was subjected to overnight incubation at 37°C in lysis buffer containing 1% SDS and 200 μg of proteinase K. After phenol/chloroform extraction and ethanol precipitation, the DNA sample was electrophoresed, transferred onto a nylon membrane and hybridized with an alkaline phosphatase-labeled HBV DNA probe. The signals were detected with the chemiluminescent substrate CDP-star (GE Healthcare Bio-Sciences Co. Ltd, Tokyo, Japan) and quantitated using an image analyzing software (ImageJ 1.38, supplied online by the National Institutes of Health, Bethesda, MD).

Statistical Analysis

Group comparisons of continuous and categorical variables were done using Fisher's exact probability test, χ^2 test with Yate's correction and Mann–Whitney's non-parametric *U*-test as appropriate. The group comparison of Kaplan–Meier curves for the cumulative probability of sustained HBV DNA clearance was performed by the log-rank test. The correlation of various clinical and virological factors with the cumulative probability of sustained HBV DNA clearance was evaluated by a Cox proportional-hazards model using univariate and stepwise multivariate procedures. The one-way analysis of variance and the Fisher's PLSD test were used for the in vitro transfection analysis.

RESULTS

Overall Therapeutic Efficacy of Adefovir Dipivoxil Added to Lamivudine in Lamivudine-Resistant Patients With Type B Chronic Hepatitis

Among the lamivudine-resistant patients with type B chronic hepatitis examined in this study, HBV DNA decreased to an undetectable level (<2.6 $\log_{10}$ copies/ml) in 6 (20%) of 30 patients at 6 months, 6 (26%) of 23 patients at 12 months and 9 (56%) of 16 patients at 18 months after the beginning of adefovir dipivoxil administration. Thirteen (43%) of the 30 patients achieved sustained HBV DNA clearance during follow-up. ALT normalization was observed in 21 (70%) of 30 patients at 6 months, 14 (61%) of 23 patients at 12 months and 11 (69%) of 16 patients at 18 months of therapy.

Viral Mutations Associated With Efficacy of Adefovir Dipivoxil Added to Lamivudine Treatment

The lengths of the 30 HBV DNA sequences obtained from the lamivudine-resistant patients with type B chronic hepatitis ranged from 3,161 to 3,230 nucleotides. All 30 patients were infected with HBV of genotype C as determined by phylogenetic tree analysis of the HBV isolates obtained in this study and the representative HBV isolates of major genotypes (data not shown). Viral mutations were sought that showed a relationship with the therapeutic efficacy of adefovir dipivoxil added to lamivudine over the whole HBV genome. As a result, there were only two mutations; one was a T-to-C/G/A mutation at nt1753 (V1753 mutation) located in the basic core promoter (BCP) [Yuh et al., 1992], and the other was an A-to-C mutation at nt2189 (C2189 mutation) in the core gene.

The V1753 mutation was detected in 11 (37%) of the 30 patients studied; the C1753 mutation was found in 7 patients, G1753 in three patients, and A1753 in 1 patient. Figure 1A shows the serial change in HBV DNA before and after the commencement of adefovir dipivoxil administration added to lamivudine treatment in patients with type B chronic hepatitis with and without the V1753 mutation. In patients with V1753, HBV DNA clearance was observed in 5 (45%) of 11 patients at 6 months, 5 (71%) of 7 patients at 12 months and 4 (80%) of 5 patients at 18 months of therapy. By contrast, in patients having T1753, HBV DNA clearance was seen in only 1 (5%) of 19 patients at 6 months, 1 (6%) of 16 patients at 12 months and 5 (45%) of 11 patients at 18 months. Significant differences in the frequency of HBV DNA clearance were observed at 6 and 12 months of therapy between patients with and without V1753 ($P < 0.02$ and $P < 0.005$). Thus, patients with V1753 tended to achieve HBV DNA clearance more frequently by adefovir dipivoxil added to lamivudine treatment than those with T1753.

The C2189 mutation was found in 13 (43%) of the 30 lamivudine-resistant patients with type B chronic hepatitis. Figure 1B shows the serial change in HBV DNA during adefovir dipivoxil administration added to lamivudine treatment in patients with type B chronic hepatitis with and without the C2189 mutation. In patients with C2189, HBV DNA was cleared in 5 (38%) of 13 patients at 6 months, 4 (44%) of 9 patients at 12 months and 8 (89%) of 9 patients at 18 months of therapy. On the other hand, in patients having A2189, HBV DNA was undetectable in 1 (6%) of 17 patients at 6 months, 2 (14%) of 14 patients at 12 months and 1 (14%) of 7 patients at 18 months of therapy. There was a significant difference in the frequency of HBV DNA clearance after 18 months of therapy between patients with and without C2189 ($P < 0.01$). HBV DNA tended to be cleared more frequently by adefovir dipivoxil administration added to lamivudine treatment in patients with C2189 than in those with A2189.

A group comparison of the Kaplan–Meier curves was undertaken for the cumulative probability of sustained HBV DNA clearance with respect to the occurrence of these two viral mutations. Patients with V1753 had a significantly higher cumulative probability of sustained HBV DNA clearance than those with T1753 ($P < 0.005$) (Fig. 2A). The cumulative probability of sustained HBV DNA clearance was also higher in patients with C2189 than in those with A2189 ($P < 0.05$) (Fig. 2B). The presence of the V1753 and C2189 mutations showed no

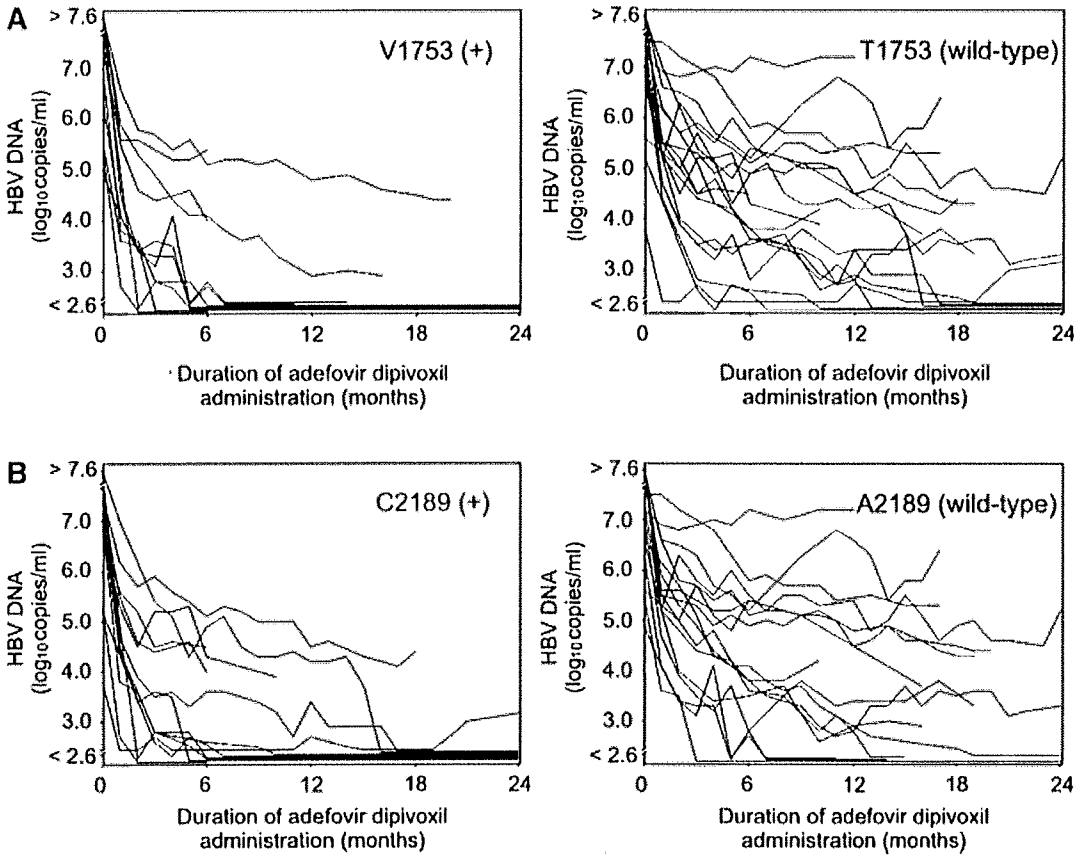

Fig. 1. Serial changes in the HBV DNA level up to 24 months of adefovir dipivoxil added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis in relation to the occurrences of (A) V1753 mutation and (B) C2189 mutation.

relationship with ALT normalization during adefovir dipivoxil added to lamivudine treatment.

Factors Associated With the Efficacy of Adefovir Dipivoxil Added to Lamivudine Treatment Determined by Univariate and Multivariate Analyses

Next, the clinical and virological factors affecting the therapeutic effect of adefovir dipivoxil added to lamivudine were investigated in the 30 patients with type B chronic hepatitis. Six clinical factors (age, gender, liver disease, ALT, HBeAg positivity, and HBV DNA), two lamivudine resistance-associated viral mutations (rtM204V/I and rtL180M) [3], two major naturally occurring viral mutations (A1896 and T1762/A1764) [Carman et al., 1989; Okamoto et al., 1994], and V1753 and C2189 mutations were investigated. As shown in Table II, only the V1753 and C2189 mutations were significant factors contributing to sustained clearance of HBV DNA ($P = 0.006$ and $P = 0.047$) by univariate analysis. High ALT and low HBV DNA (<7.5 $\log_{10}$ copies/ml) at baseline were selected as significant independent factors contributing to sustained clearance of HBV DNA ($P = 0.011$ and $P = 0.008$) in addition to the V1753 and C2189 mutations ($P = 0.001$ and $P = 0.007$) in multivariate analysis.

Serial Changes in the V1753 and C2189 Mutations During Antiviral Therapy

The V1753 and C2189 mutations were also examined using serum samples obtained before lamivudine therapy, which were available in 14 of the 30 type B chronic hepatitis patients. These mutations were assayed by PCR-direct sequencing. Of the 14 patients, the V1753 mutation was found in only 1 patient before lamivudine therapy and detected in additional 4 patients before adefovir dipivoxil administration. The C2189 mutation was found in three patients before lamivudine therapy, one of whom lost the mutation before adefovir dipivoxil administration. The additional three patients acquired this mutation before adefovir dipivoxil administration. Thus, both the V1753 and C2189 mutations tended to appear during lamivudine therapy accompanied by the lamivudine resistance in these patients.

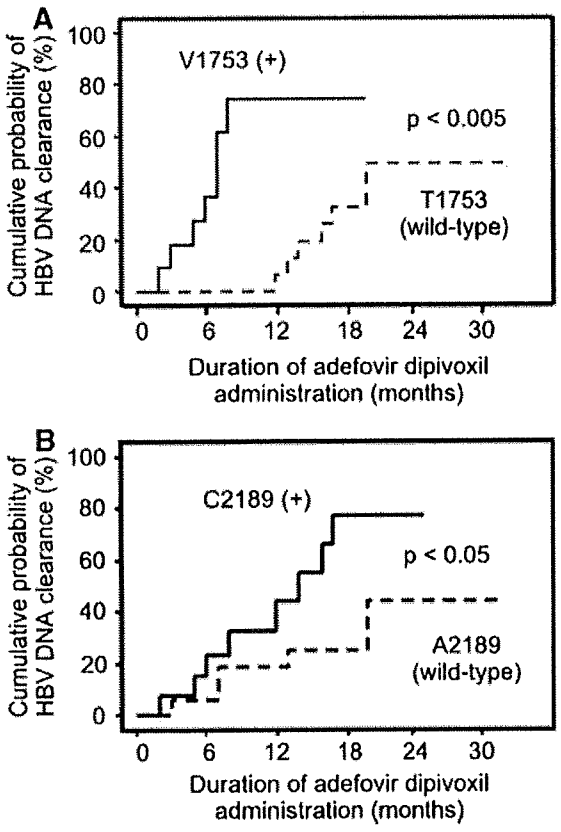

Fig. 2. Influence of the presence of the V1753 and C2189 mutant viruses on the efficacy of adefovir dipivoxil therapy added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis. The Kaplan–Meier estimates of the cumulative probability of sustained HBV DNA clearance correlated with the presence or absence of (A) V1753 mutation and (B) C2189 mutation.

Susceptibility to Lamivudine and/or Adefovir of Wild-Type and Mutant Viruses In Vitro

Finally, susceptibility to lamivudine and/or adefovir of the wild-type and C1753 and C2189 mutant viruses was examined in vitro. Cultured cells with forced expression of the wild-type or mutant virus were treated with lamivudine alone, adefovir alone, lamivudine plus adefovir, or left untreated, and the cellular HBV DNA replicative intermediate was examined. In the HBV-expressing cells without treatment (Fig. 3A,B), the replicative competence of the C1753 mutant virus was the same as that of the wild-type virus, whereas the C2189 mutant virus showed an approximately fivefold lower replicative competence than the wild-type virus. As for susceptibility to nucleos(t)ide analogs, the degree of reduction in viral replication by treatment with lamivudine alone, adefovir alone or lamivudine plus adefovir did not differ significantly among the wild-type virus and the C1753 and C2189 mutant viruses (Fig. 3A,C).

DISCUSSION

Adefovir dipivoxil added to ongoing lamivudine treatment has been accepted as a reliable therapeutic

TABLE II. Univariate and Multivariate Analyses to Investigate Factors Associated With Sustained HBV DNA Clearance in Patients With Type B Chronic Hepatitis Treated With Adefovir Dipivoxil Added to Continuing Lamivudine

Factors	Univariate analysis				Multivariate analysis			
	Hazard ratio	95% confidence interval	χ^2-value	*P*-value	Hazard ratio	95% confidence interval	χ^2-value	*P*-value
Clinical factors								
Age (/1 year increment)	0.987	0.843–1.033	0.302	0.583	—	—	—	—
Gender (female)	1.315	0.287–6.020	0.124	0.725	—	—	—	—
Liver disease (cirrhosis and HCC)	1.199	0.328–4.384	0.076	0.783	—	—	—	—
ALT (/1 Iu/l increment)	1.003	1.000–1.007	3.405	0.065	1.005	1.001–1.010	6.443	0.011
HBeAg (negative)	1.919	0.566–6.503	1.096	0.295	—	—	—	—
HBV DNA (<7.5 $\log_{10}$ copies/ml)	3.175	0.862–11.765	3.017	0.082	14.706	1.996–111.111	6.956	0.008
Virological factors								
rtM204V/I mutation (rtM204I)	1.060	0.346–3.254	0.011	0.918	—	—	—	—
rtL180M mutation (+)	0.766	0.234–2.505	0.195	0.659	—	—	—	—
A1896 mutation (+)	1.138	0.371–3.493	0.051	0.821	—	—	—	—
T1762/A1764 mutation (+)	0.500	0.062–4.013	0.426	0.514	—	—	—	—
V1753 mutation (+)	4.986	1.603–15.506	7.705	0.006	58.322	5.054–673.002	10.619	0.001
C2189 mutation (+)	3.155	1.017–9.804	3.957	0.047	7.042	1.704–29.412	7.270	0.007