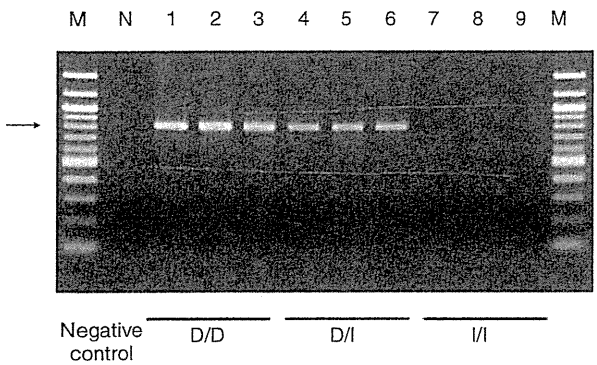

Figure 3 Detection of A3A-A3B fusion mRNA by PCR. A3A-A3B fusion mRNA was amplified by PCR using primers specific to A3A and A3B (see Materials and Methods) and detected by agarose gel electrophoresis. Lanes 1–3 are those from deletion homozygous patients, lanes 4–6 are from heterozygous patients and 7–9 are from insertion homozygous patients. M, molecular weight size marker (1 kb DNA Ladder; New England BioLabs, Ipswich, MA), N, negative control.

The deletion of APOBEC gene spans from 3′ end of the *A3A* gene to the 3′ portion of the *A3B* gene. This deletion results in the formation of a fusion gene that has *A3A* amino acid sequence and *A3B* 3′ untranslated region. The expression level of this protein may be different from the undeleted *A3A* due to different stability of RNA or different transcription levels. The results of context analysis also indicated that the effect of this alteration of expression levels of *A3A* is almost negligible compared with A3G. This is consistent with our results that showed lower levels of induction of hypermutation on HBV genome by *A3A* compared with *A3G*.

Our results indicated that the effect of both *A3A* and *A3B* is not significant in the development and progression of chronic hepatitis B. The two proteins also have only little influence on the hypermutation state of HBV in chronic HBV carriers. The results also showed higher induction levels of hypermutation by *A3G* than by *A3A*.

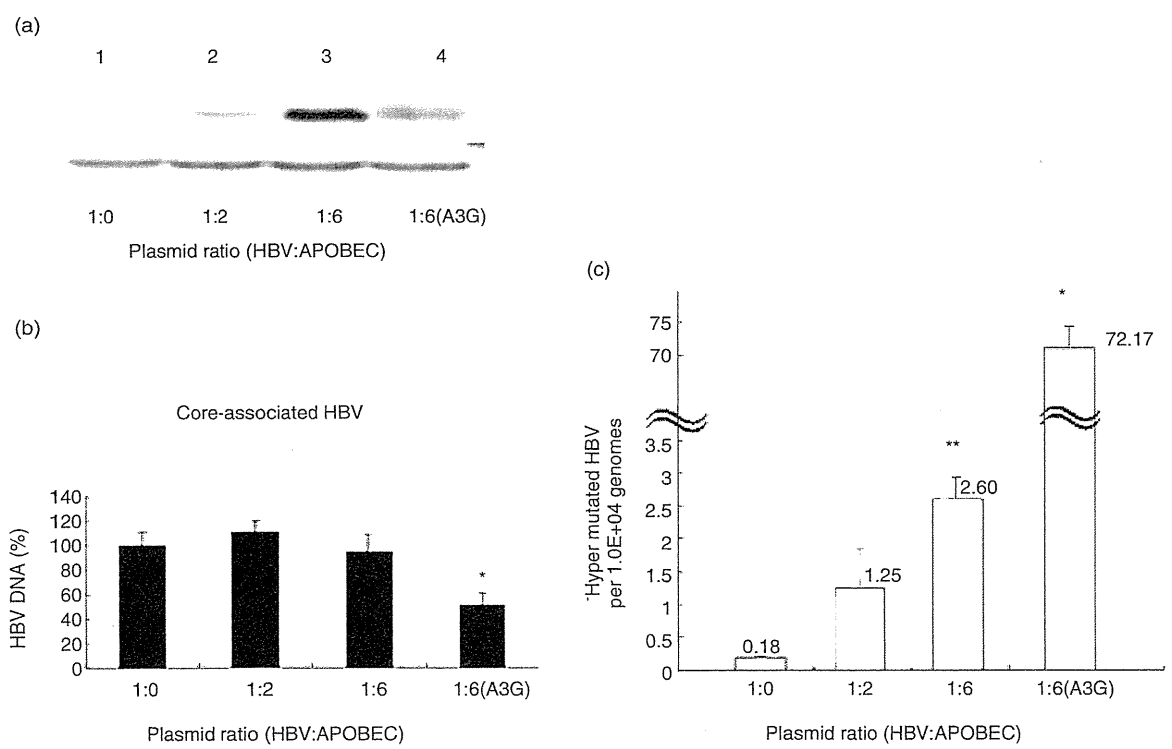

Figure 4 Analysis of inhibition of HBV replication and induction of hypermutation by A3A. HepG2 cells were transiently transfected with expression plasmid of A3A together with 1.4 genome length hepatitis B virus (HBV) expression vector. The indicated amounts of HBV and A3A (1:0, 1:2, 1:6) or A3G (1:6) expression plasmids were transfected into HepG2 cells. All experiments were performed more than twice with similar results. (a) APBEC3 gene expression levels were detected by western blot analysis. (b) The amounts of core associated replicative intermediates of HBV were measured by RT-PCR. (c) Quantitative measurement of hypermutated genomes by 3D real-time PCR. Data in (B) and (C) are mean ± SD. $*P < 0.05$; $**P < 0.01$

Sufficient evidence indicates that *A3G* has anti-viral effects on HBV,[14–17] suggesting that some but not all APOBEC3 proteins operate as part of the anti-viral immune system against HBV infection. Further study is needed to clarify the functional role of each APOBEC3 protein for innate anti-viral immunity in chronic HBV infection.

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Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice☆

Nobuhiko Hiraga[1,2], Michio Imamura[1,2], Tsuyoshi Hatakeyama[1], Shosuke Kitamura[1], Fukiko Mitsui[1,b], Shinji Tanaka[1], Masataka Tsuge[1,2], Shoichi Takahashi[1,2], Hiromi Abe[2,3], Toshiro Maekawa[2,3], Hidenori Ochi[2,3], Chise Tateno[2,4], Katsutoshi Yoshizato[2,4,5], Takaji Wakita[6], Kazuaki Chayama[1,2,3,*]

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[3]*Laboratory for Liver Diseases, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan*
[4]*PhoenixBio Co., Ltd., Higashihiroshima, Japan*
[5]*Osaka City University Graduate School of Medicine, Osaka, Japan*
[6]*Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan*

***Background/Aims*: Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.**

***Methods*: We performed infection experiments with either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and messenger RNA levels of IFN-stimulated genes were measured.**

***Results*: No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment, regardless of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.**

***Conclusions*: No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.**

***Keywords*: Superinfection; JFH-1; IFN-stimulated genes**

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* Corresponding author. Tel.: +81 82 2575190; fax: +81 82 2556220.
E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).

Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; OAS, 2′,5′-oligoadenylate synthetase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

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1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 million people are infected with HBV, and more than 170 million people are infected with HCV [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as mixovirus resistance protein A (MxA), RNA-dependent protein kinase (PKR), and 2′,5′-oligoadenylate synthetase (OAS) [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and severe progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate about whether or not there is interference between the two hepatitis viruses, with some favoring such interference [7] and others arguing against such a concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent replication of the second virus; however, viruses develop their own strategies to resist the effect of IFN. In clinical practice, practitioners often perceive that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of pegylated IFN (PEG-IFN) in combination with ribavirin has improved the eradication rate [9]. Eradication of HBV by IFN, however, is usually difficult, even when using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity, including IFN signaling, are well established in some viruses. Such mechanisms involve interruption of IFN signaling by interacting molecules that transduce the signal from the IFN receptor through the Janus kinase (Jak) signal transducer and activator of transcription (STAT) pathway [4]. Viral proteins of paramixoviruses, for example, inhibit IFN signaling [11]. Several studies have also examined the mechanisms by which HCV resists the host immune system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12]. Generally, expression of HCV protein is associated with inhibition of STAT1 function independent of STAT tyrosine phosphorylation [13]. Additionally, expression of the HCV core protein in cultured cells is associated with increased expression levels of the suppressor of cytokine signaling 3 (SOCS-3) [14]. The NS5A and E2 proteins are both inhibitors of PKR [15]. These strong actions of HCV against innate immunity are consistent with the high chronicity rate of the virus. IFN, however, effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly understood. To date, only a few studies have reported the molecular mechanisms of HBV resistance against the actions of IFN. The HBV-related resistance to IFN, for example, involves upregulation of protein phosphatase 2A (PP2A) as the primary event, which subsequently leads to inhibition of protein arginine methyltransferase 1 (PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular mechanisms, microarray analyses of serial liver biopsies of experimentally infected chimpanzees showed striking differences in the early immune responses to HBV and HCV. HCV, for example, induced early changes in the expression of many intrahepatic genes, including genes involved in type 1 IFN response [18], whereas HBV did not induce any detectable changes in the expression of intrahepatic genes in the first weeks of infection [19].

HBV–HCV double infection is a good model to use for assessment of the mechanism of IFN resistance by these two viruses because one can test the effect of IFN on one virus in the presence of the other virus. Recently, Bellecave et al. [20] established a novel *in vitro* model system in Huh7 cells that allowed the analysis of both viruses in a replicating context and reported the absence of direct viral interference. To this end, we used human hepatocyte chimeric mice and cell culture systems in the present study. The results showed that the presence of HBV does not affect the actions of IFN on HCV and vice versa. These results suggest the lack of interference between the two viruses in liver cells and indicate that the reported interference between the two viruses might be via inflammation including death of infected cells by cytotoxic T cells, cytokines including IFN-α and IFN-β, and interleukins produced by hepatocytes and infiltrating T cells.

2. Materials and methods

2.1. Transfection of Huh7 cells with HBV DNA and HCV RNA

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C and under 5% CO_2. Cloning of HBV DNA and the plasmid construction were performed as described previously [21]. For production of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were split and cultured in Hygromycin B-DMEM selection medium (300 μg/ml; Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured for identification of virus-producing cell lines. Clones positive

for both hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further analyzed for production of HBV particles. Finally, three cell lines that produced more than 10^5 copies per milliliter of HBV DNA in supernatant were selected and used for further experiments.

For transfection with HCV RNA, we used pJFH1, which contains the complementary DNA of full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In vitro* synthesis of HCV RNA and electroporation into Huh7 cells were performed as described previously [22,23]. Briefly, cells were treated with trypsin, washed twice with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) at a final concentration of 7.5×10^6 cells per milliliter. Then, 10 μg of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and subjected to an electric pulse (950 μF and 260 V) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish.

2.2. Generation of human hepatocyte chimeric mice

Generation of the urokinase-type plasminogen activator $(uPA)^{+/+}$ and severe combined immunodeficiency $(SCID)^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [21,23,24]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. The concentration of serum human serum albumin, which correlates with the repopulation index [24], was measured in mice as described previously [21]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

2.3. Human serum samples

Human serum samples containing high titers of either HBV DNA (5.3×10^6 copies per milliliter) or genotype 1b HCV (2.2×10^6 copies per milliliter) were obtained from patients with chronic hepatitis with a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use. Chimeric mice were injected intravenously with 50 μL of either HBV- or HCV-positive human serum. Some mice were injected with HBV-positive human serum at 6 weeks after injection of HCV-positive human serum.

2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μL RNase-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20-μL reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

2.5. RNA extraction and measurement of mRNAs of interferon-induced genes by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.6. Statistical analysis

Changes in HBV DNA and HCV RNA in mice sera were compared by Mann–Whitney test and unpaired t test. Differences in HBV DNA and HCV core antigen in mice sera and culture supernatants were analyzed by one-way analysis of variance followed by Scheffe's test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Infection of chimeric mouse with HBV and HCV and susceptibility to interferon

To investigate the interference between HBV and HCV and to examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50 μL of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was 1.4×10^8 copies per milliliter (range: 5.3×10^6–3.6×10^9 copies per milliliter) at 6 weeks after inoculation (Fig. 1A), similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was 1.0×10^7 copies per milliliter (range: 1.2×10^6–0.8×10^7 copies per milliliter) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g per day of intramuscular IFN-α for 2 weeks. Treatment resulted in a decrease of only 0.3 log in mice serum HBV DNA level compared to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a rapid decrease in HCV RNA to undetectable levels, as confirmed by quantitative polymerase chain reaction (PCR; Fig 1B).

To investigate the direct interference of the two viruses, we performed double-infection experiments. Ten chimeric mice were first inoculated intravenously with 50 μL of HCV-positive human serum samples. Six weeks after HCV infection when the mice developed HCV viremia, 50 μL of HBV-positive human serum samples were inoculated intravenously in 5 of 10 HCV-infected mice. All five mice became positive for both HBV and HCV at 2 weeks after HBV infection. No significant decrease in HCV RNA levels was observed in these superinfected mice before or after the development of HBV viremia (Fig. 2A). After HBV infection, there was no apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in HBV–HCV-coinfected mice was comparable with that of only HBV-infected mice (Fig. 2B). These results sug-

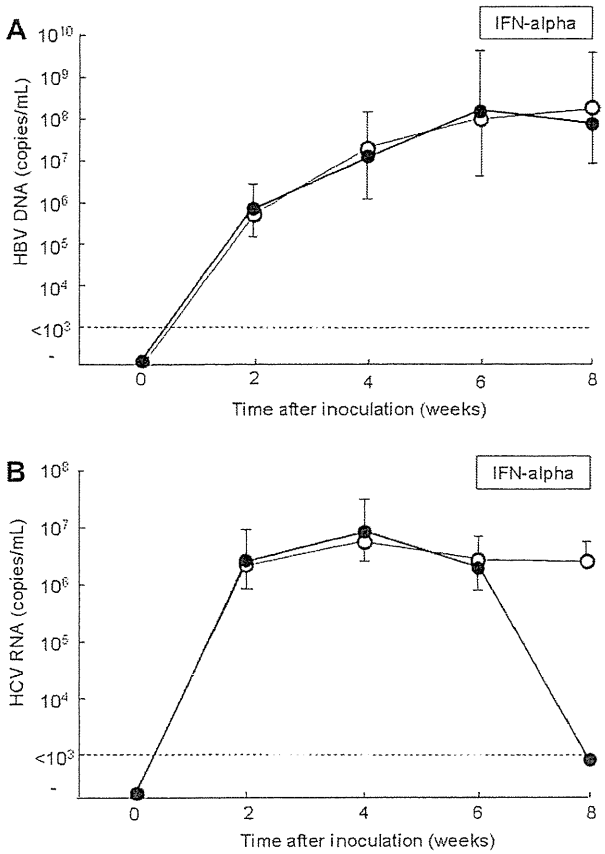

Fig. 1. Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g per day of interferon-alpha intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean plus or minus standard deviation ($n = 3$). The horizontal dashed line represents the detection limit (10^3 copies per milliliter).

gest no interference between the two viruses in mice, which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the effect of IFN against the other virus, three HBV–HCV-coinfected mice were treated with IFN-α (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast, no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B). These results are similar to the reduction of HCV RNA and HBV DNA in mice that were infected with either of these hepatitis viruses. These results indicate that HCV is more susceptible to IFN-α than HBV and that each virus does not alter the effect of IFN on the other virus. Because the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. It is possible, however, that HBV and HCV replicated in different cells in these mice. Because it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histologic examination, we performed *in vitro* experiments.

3.2. Production of both HBV- and HCV-producing cells and the effect of interferon

To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stable HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42, and -53) that produced HBsAg, HBeAg, and HBV DNA into the supernatant were selected (Table 1). These cell lines continuously produced HBV for more than 3 months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after JFH1 transfection (Fig. 4A). In contrast, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than in Huh7 cells, and the level was not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

3.3. Effects of interferon on HBV and HCV in vitro

The effects of IFN on virus production in both HBV- and HCV-producing cell lines was examined by adding different amounts of IFN-α (0, 50, and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as MxA, OAS, and PKR increased in a dose-dependent manner in all three cell lines as well as in parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV–HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN, and the decrease was dose-dependent (Fig. 5C).

4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.

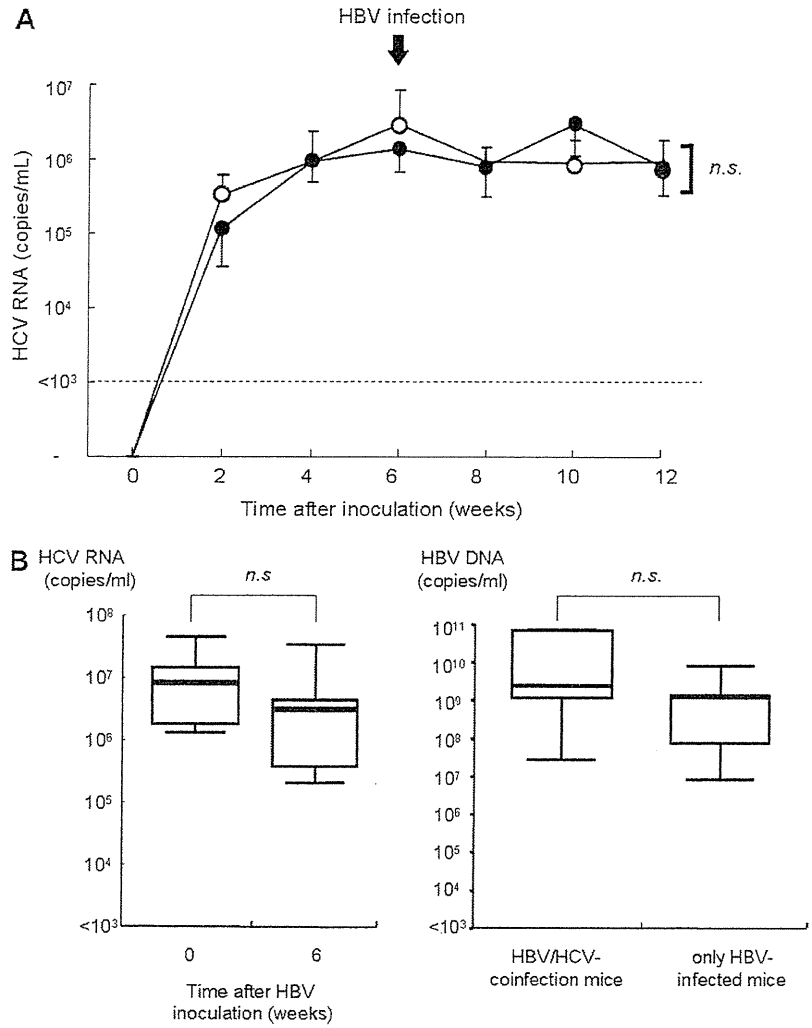

Fig. 2. Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining five mice (open circles) did not receive HBV inoculation. Data are mean plus or minus standard deviation ($n = 3$). (B) Serum HCV RNA titers in five mice infected with HCV before and at 6 weeks after HBV superinfection (left panel). Serum HBV DNA titers in five mice coinfected with HBV and HCV were compared with those of five mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry or expansion in HBV-infected chimpanzee livers and suggested that HBV was a stealthy virus early in the infection [19]. Because no reduction in HCV was noted during and after the development of high-level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without

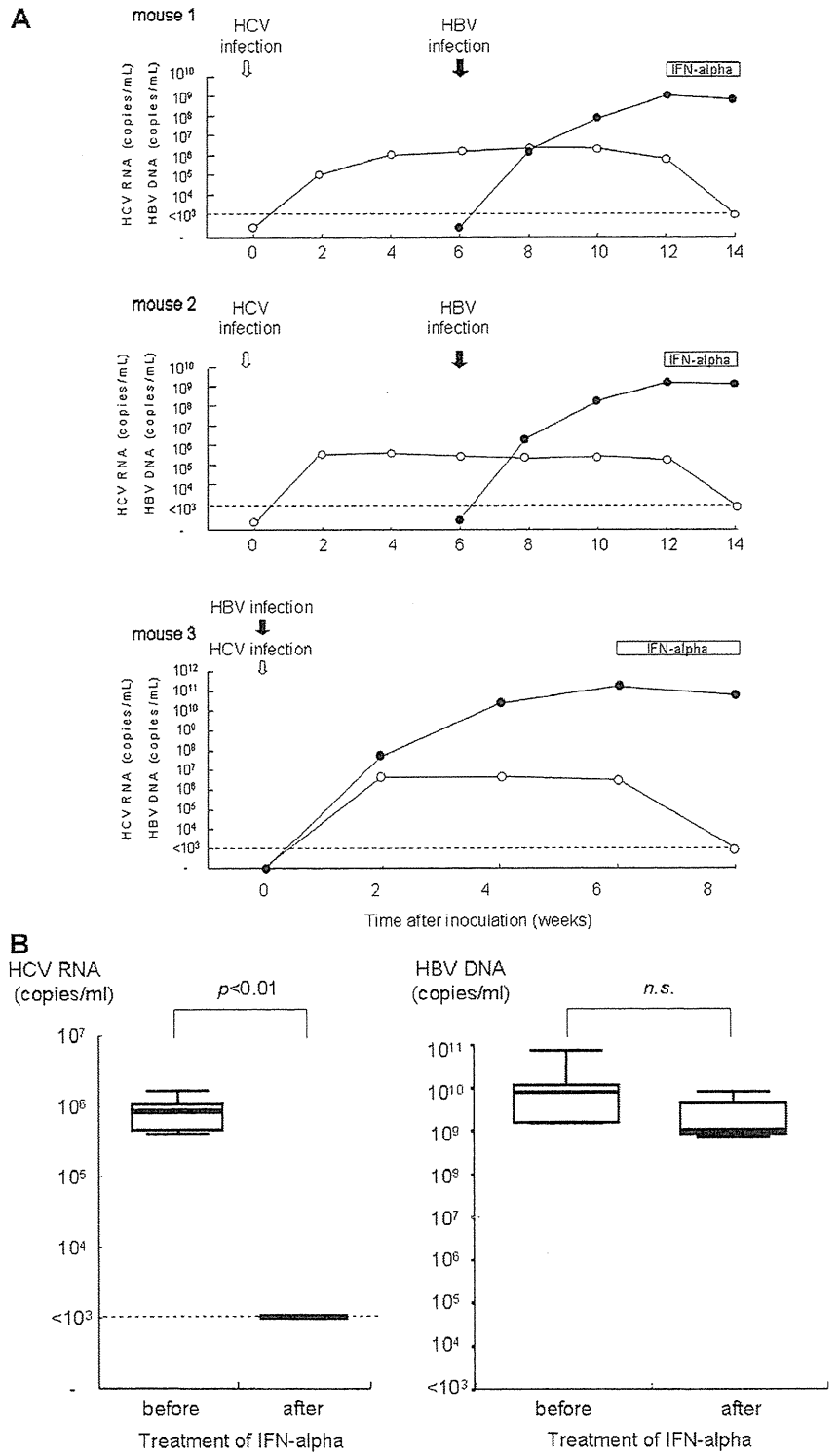

Fig. 3. Changes in serum hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA levels and effects of IFN on HBV–HCV-coinfected mice. Three mice (mouse 1, 2, and 3) were inoculated with both HBV- and HCV-positive human serum samples and treated daily with 7000 IU/g per day of interferon-alpha (IFN-α) intramuscularly for 2 weeks. Mice sera samples were obtained every 2 weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles) were analyzed by quantitative polymerase chain reaction. (A) The horizontal dashed line represents the detectable limit (10^3 copies per milliliter). (B) Serum HCV RNA and HBV DNA titers in mice before and after 2-week IFN-α treatment. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Table 1
Hepatitis B virus (HBV) markers in supernatants of stable HBV-transfected cell lines.

Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies per milliliter)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

evoking the IFN production system in liver cells. Further study using double-infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in mice infected with HCV only and in those coinfected with HBV and HCV (Figs. 1 and 3). This finding indicated that HBV does not disturb the effect of IFN through signal transduction from the IFN receptor through the Jak-STAT pathway. It was, however, considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same liver cell could be infected with both HBV and HCV [20,26], but it was difficult in the present study to confirm that these two viruses replicate in the same liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathologic sections of the mouse liver. To address this issue, we transfected HCV to stable HBV-producing cell lines (Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stable HBV-producing cells. Presence of the both hepatitis viruses in the same hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments that only HBV-transfected cell lines produced HBV and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are consistent with a recent report [20] that showed that HCV could infect cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV-transfected HBV-producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain machinery to disrupt the innate immune system; however, once exposed a large concentration of IFN, the virus shows high sensitivity, as shown in the replicon system [16,27]. Thus, HCV seems to have a relatively weak ability to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [28]. The fact that HBV does not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts the actions of IFN at IFN-induced antiviral product levels.

Although the culture environment is different from the replicon system, the JFH1 strain seems relatively resistant to IFN [29]. This suggests that the core and envelope proteins, which are absent in the replicon system, might play a role in IFN resistance; however, we could not show any effect for HCV infection on the actions of IFN on HBV replication. This finding sug-

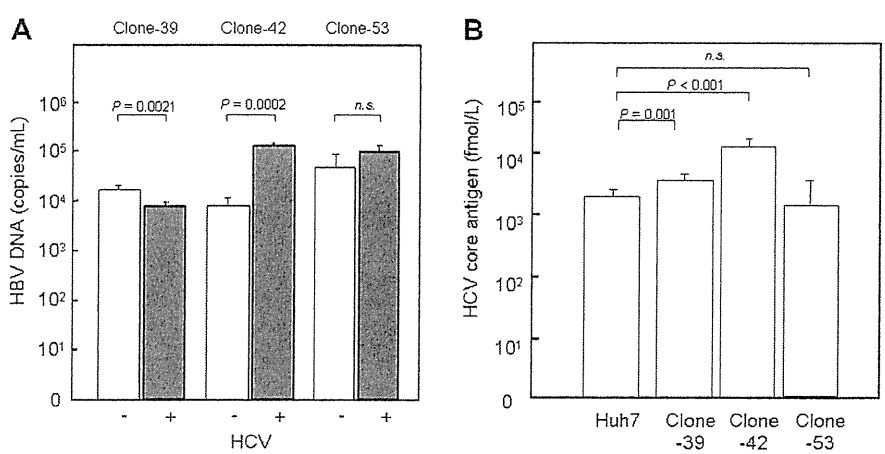

Fig. 4. Virus titers in supernatants of hepatitis B virus (HBV)–transfected or hepatitis C virus (HCV)–transfected cell lines. Huh7 cells were initially stably transfected with 1.4 genome-length HBV DNA. Three cell lines (Clone-39, -42, and -53) producing HBV DNA into the supernatant were selected. (A) HBV DNA levels in supernatants of HBV-producing cell lines 72 hours after transfection with JFH1 RNA (HCV positive) or control plasmid (HCV negative). (B) HCV core antigen levels in the supernatant of parental Huh7 cells and HBV-producing cell lines 72 h after transfection with JFH1 RNA. Data are mean plus or minus standard deviation ($n = 3$).

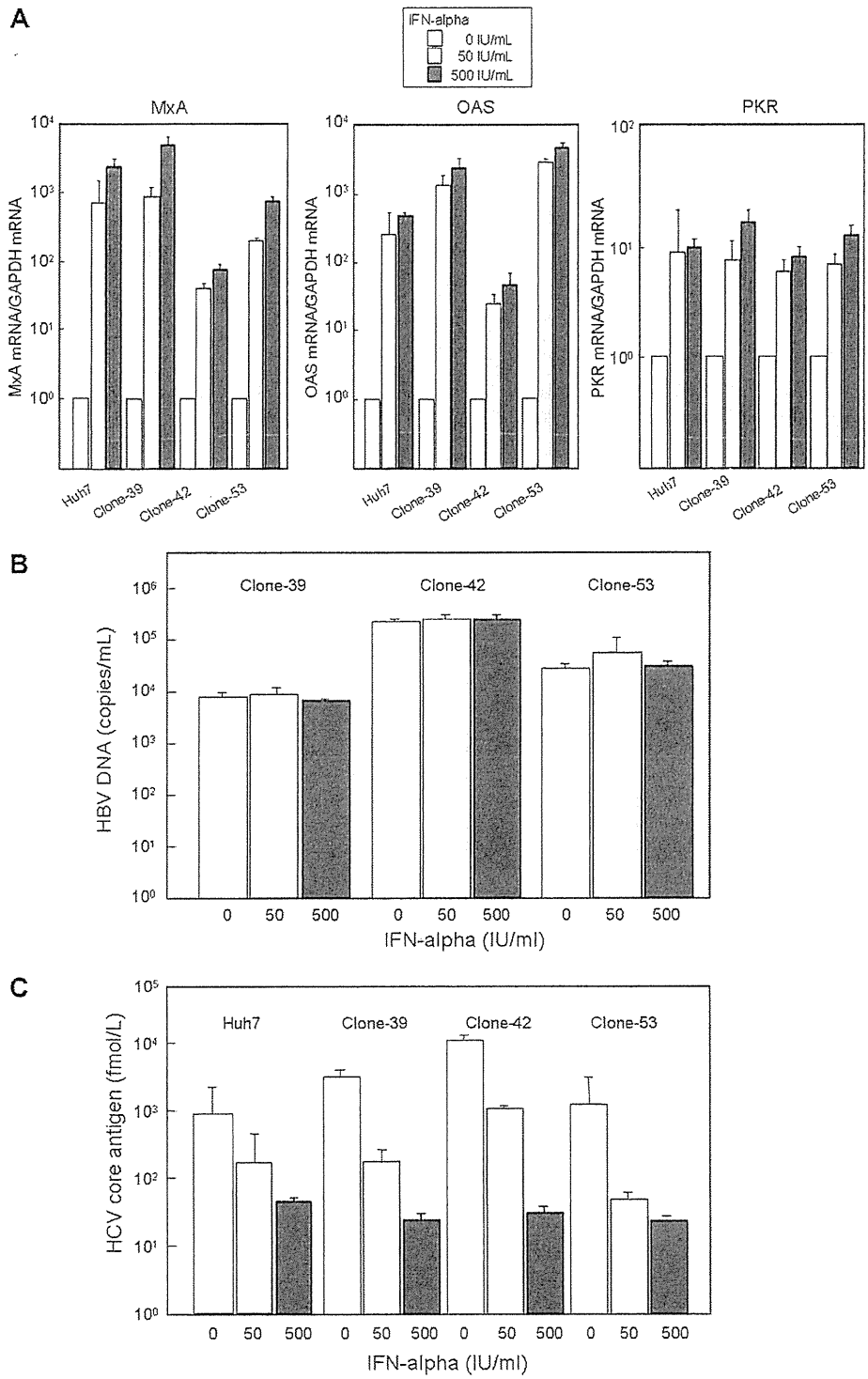

Fig. 5. Effects of interferon (IFN) treatment on hepatitis B virus (HBV) and hepatitis C virus (HCV) *in vitro*. Parental Huh7 cells and three HBV-transfected Huh7 cell lines (Clone-39, -42, and -53) were transfected with JFH1 RNA. Immediately after JFH1 transfection, the cell lines were treated with IFN-α (0, 50, and 500 IU/mL) for 72 h. (A) Intracellular gene expression levels of mixovirus resistance protein A (MxA), 2′,5′-oligoadenylate synthetase (OAS), and RNA-dependent protein kinase (PKR) were measured. RNA levels were expressed relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) messenger RNA. (B) HBV DNA and (C) HCV core antigen in supernatants were measured. Data are mean plus or minus standard deviation ($n = 3$).

gests that the core and envelope proteins have only a weak effect on IFN resistance.

In clinical practice, HBV shows high resistance against IFN therapy. This is also the case in the cell culture system, as we showed in this study and has been reported in previous studies [20,28]. The mechanism by which hepatitis viruses resist IFN needs to be clarified in order to develop new and effective therapies for eradication of these viruses.

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Research Paper

Efficacy of Lamivudine or Entecavir on Acute Exacerbation of Chronic Hepatitis B

Tatsuo Kanda[1*]✉, Masami Shinozaki[2*], Hidehiro Kamezaki[1], Shuang Wu[1], Shingo Nakamoto[1], Makoto Arai[1], Keiichi Fujiwara[1], Nobuaki Goto[2], Fumio Imazeki[1] and Osamu Yokosuka[1]

1. Department of Medicine and Clinical Oncology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
2. Department of Medicine, Numazu City Hospital, Numazu, Shizuoka 410-0302, Japan.

* These authors contributed equally to this article.

✉ Corresponding author: Tatsuo Kanda, MD, PhD, Associate Professor, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan. Tel.: +81-43-226-2086; Fax: +81-43-226-2088; E-mail: kandat-cib@umin.ac.jp

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Abstract

Background/Aims: Spontaneous acute exacerbation of chronic hepatitis B virus (HBV) infection occasionally occurs in its natural history, sometimes leading rapidly to fatal hepatic failure. We compared the effects of lamivudine (LAM) with those of entecavir (ETV) treatments in acute exacerbation of chronic hepatitis B with 500 IU/L or higher alanine aminotransferase (ALT) levels.

Methods: Thirty-four patients with acute exacerbation were consecutively treated with LAM /ETV. Their clinical improvements were compared.

Results: Among LAM-treated and ETV-treated patients, none showed a reduction of <1 log IU/mL in HBV DNA after 1 or 3 months of treatment. Initial virological response, defined as a reduction of 4 log IU/mL in HBV DNA at 6 months, with LAM and ETV, respectively, was 83.3% and 100%. One LAM patient developed hepatic encephalopathy, but all patients in both groups survived. Twelve months after treatment, 41.6% of 24 LAM group patients switched to another drug or added adefovir to their treatment due to the emergence of LAM-resistant mutants. On the other hand, patients receiving ETV did not need to change drugs.

Conclusions: ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should carefully start to treat these patients as soon as possible.

Key words: acute exacerbation, ALT, entecavir, HBV, lamivudine

INTRODUCTION

Chronic hepatitis B infection is associated with the development of hepatocellular carcinoma [1]. Infection with hepatitis B virus (HBV) also leads to wide a spectrum of liver injury, including acute, self-limited infection, fulminant hepatitis, and chronic hepatitis with progression to cirrhosis and liver failure, as well as to an asymptomatic chronic carrier state [2, 3].

Reactivation of hepatitis B is a well-characterized syndrome marked by the abrupt reappearance or rise of HBV DNA in the serum of a patient with previously inactivated or resolved HBV infection [4]. Reac-

tivation is often spontaneous, but can also be triggered by cancer chemotherapy and immune suppression. Spontaneous acute exacerbation of chronic hepatitis B infection is seen with a cumulative probability of 15-37% after 4 years of follow-up [5]. Prognosis is generally poor in HBV carriers with spontaneous acute exacerbation together with high alanine aminotransferase (ALT) levels, jaundice, and liver failure [4, 6, 7]. This condition has been defined as acute-on-chronic liver failure according to a recent Asia-Pacific consensus recommendation [8]. Acute exacerbation occasionally leads to a critical scenario, meaning that clinicians need to treat this condition immediately.

Lamivudine (LAM) is a reverse-transcriptase inhibitor of viral DNA polymerase with an excellent profile of safety and tolerability, causing inhibition of viral replication, and it is approved for antiviral treatment of hepatitis B patients [9, 10]. LAM suppresses serum HBV DNA values in up to 98% of patients within a median period of 4 weeks, leading to aminotransferase normalization, increased hepatitis B e antigen (HBeAg) seroconversion rate, and improvement of histological parameters [11, 12]. A study from Taiwan showed that LAM had a survival benefit and was effective for patients with baseline bilirubin levels below 20 mg/dL [7].

Entecavir (ETV), a deoxyguanosine analogue, is a potent and selective inhibitor of HBV replication; its *in vitro* potency is 100- to 1,000-fold greater than that of LAM, and it has a selectivity index (concentration of drug reducing the viable cell number by 50% [CC_{50}]/concentration of drug reducing viral replication by 50% [EC_{50}]) of ~8,000 [13, 14]. At present, the Japanese national health insurance system approves ETV as the first-line therapy for chronic hepatitis B, although some patients are treated with standard interferon-alfa. ETV is a nucleoside analogue (NUC) belonging to a new subgroup, cyclopentane [15], and it has been shown to be highly effective in suppressing HBV replication to an undetectable level and normalizing ALT, although NUCs do not eradicate the virus. ETV develops less resistance than LAM.

We undertook a retrospective study to compare the efficacy of LAM with that of ETV in the reduction of HBV DNA levels and associated improvement in disease severity and biochemical recovery in patients with acute exacerbation together with higher ALT levels due to HBV reactivation.

MATERIALS AND METHODS

Patients

A retrospective analysis of LAM/ETV-treated chronic hepatitis B patients at Chiba University Hospital and Numazu City Hospital, Japan, between May 2003 and December 2009 was performed. The inclusion criteria were: acute exacerbation of chronic hepatitis B characterized by an elevation of ALT level ≥ 500 IU/L along with HBV DNA ≥ 4.5 log IU/mL presenting in a patient with diagnosed chronic liver disease. The exclusion criteria were: acute hepatitis B, superinfection with other viruses (hepatitis E, A, D, or C), other causes of chronic liver failure [16, 17], coexistent hepatocellular carcinoma, portal thrombosis, coexistent renal impairment, pregnancy, coinfection with human immunodeficiency virus (HIV), or patients who had received a previous course of NUC treatment. This retrospective study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Ethics Committee of Chiba University, Graduate School of Medicine [18].

Baseline assessment of patients

Retrospectively collected data included patient demographics, clinical findings, all laboratory variables including virological tests and abdominal ultrasound. HBsAg, HBeAg, anti-HBe antibody and immunoglobulin M (IgM) anti-HBc antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan) [19]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al [20]. HBV DNA was measured by Roche Amplicor™ PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan).

Definitions

Primary antiviral treatment failure was defined as a reduction of < 1 log IU/mL in HBV DNA after 3 months of therapy. Initial virological response (IVR) was defined as a reduction of ≥ 4 log IU/mL in HBV DNA after 6 months of therapy [21].

Follow-up

Clinical assessment and routine investigations were done every 15 days or every month for at least 6 months. HBV DNA measurements were repeated monthly.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010 for Windows™ 7 and StatView 5 (SAS Institute Inc, Cary, NC). Continuous variables were expressed as mean ± standard deviation and were compared by two-factor analysis of variance (ANOVA) and two-way repeated measures ANOVA. Categorical variables were compared by Chi-square

test. Baseline was taken as the date when the first dose of LAM/ETV was administered. Statistical significance was considered at a *P*-value < 0.05.

RESULTS

Patients

Between May 2003 and December 2009, 34 patients with spontaneous acute exacerbation of chronic hepatitis B, with ALT levels ≥ 500 IU/mL and treated with LAM or ETV, were consecutively enrolled and retrospectively analyzed. 24 (70.5%) were treated with LAM at 100 mg daily and 10 (29.4%) were treated with ETV at 0.5 mg daily. All patients were followed for at least 6 months. Mean follow-up in the LAM and ETV groups was 55.5 ± 25.4 and 16.5 ± 9.9 months, respectively.

Baseline characteristics

Baseline characteristics in the two patient groups were similar **(Table 1)**. Median age was 37 (21-73) years and 79.4% were men. One patient of the LAM group developed hepatic encephalopathy, but recovered. All patients in both groups survived. At admission, the serological profile showed HBsAg positivity in all 34 (100%); 22 (64.7%) were HBeAg positive. The median HBV DNA level was 7.4 log IU/mL in the LAM group and 7.9 log IU/mL in the ETV group **(Table 1)**.

Table 1 Demographic, Clinical, and Laboratory Variables of Patients at Entry.

Parameters	Total Patients (N=34)	LAM (N=24)	ETV (N=10)	*P*-value
Age (years)	37 (21-73)	37 (21-73)	39 (24-67)	NS
Male (%)	27 (79.4)	18 (75)	9 (90)	NS
Cirrhosis (+/-)	2/32	2/22	0/10	NS
ALT (IU/L)	986 (523-2,450)	995 (523-2,450)	1,046 (523-2,140)	NS
T. Bil (mg/dL)	2.0 (0.8-22.0)	2.4 (0.8-20.6)	1.6 (1.9-22.0)	NS
PT (%)	83 (24-121)	81.5 (24-119)	83.6 (35-121)	NS
HBeAg (+/-)	22/12	18/6	4/6	NS
HBV DNA (log IU/mL)	7.6 (4.8-8.7)	7.4 (5.2-8.7)	7.9 (4.8-8.7)	NS

LAM, lamivudine; ETV, entecavir; ALT, alanine aminotransferase; T. BIL, total bilirubin; PT, prothrombin time; NS, statistically not significant.

Reduction in HBV DNA of total patients

LAM significantly reduced HBV DNA levels from baseline 7.24 log IU/mL to 3.27 log IU/mL at 1 month ($P < 0.001$), to 2.21 log IU/mL at 3 months ($P < 0.001$), and to 1.53 log IU/mL at 6 months ($P < 0.001$). ETV also significantly reduced HBV DNA levels from baseline 7.56 log IU/mL to 3.12 log IU/mL at 1 month ($P < 0.001$), to 2.14 log IU/mL at 3 months ($P < 0.001$), and to 1.77 log IU/mL at 6 months ($P < 0.001$). There were no differences in HBV DNA levels from baseline to 6 months between the two groups. None with primary antiviral treatment failure was identified in either group. There were no significant differences in IVR between the two groups **(Figure 1)**.

Reduction in ALT levels of total patients

LAM significantly reduced ALT levels from baseline 1,130 IU/mL to 102 ($P < 0.001$) at 1 month, to 28.6 ($P < 0.001$) at 3 months, and to 23.1 ($P < 0.001$) at 6 months. ETV also significantly reduced ALT levels from baseline 1,210 IU/mL to 117 ($P < 0.001$) at 1 month, to 25 ($P < 0.001$) at 3 months, and to 24.4 ($P < 0.001$) at 6 months. There were no differences in ALT levels from baseline to 6 months between the two groups **(Figure 2)**.

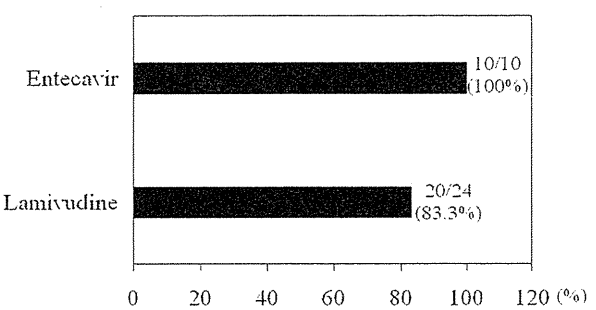

Figure 1 Initial virological response (IVR). IVR was defined as a reduction of ≥ 4 log IU/mL in HBV DNA after 6 months of therapy [21].

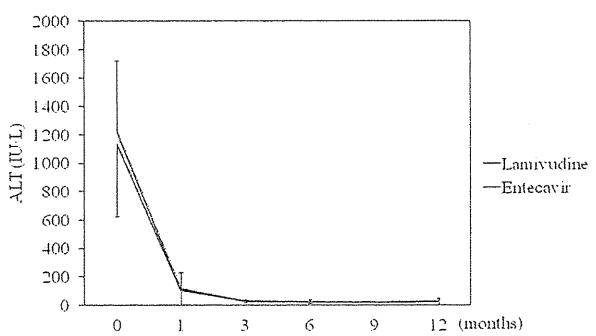

Figure 2 Efficacy of lamivudine and entecavir for ALT levels. Lamivudine (N=24) vs. entecavir (N=10); data are shown as mean ± SD.

Reduction in HBV DNA of HBeAg-positive patients

It has been demonstrated that the levels of HBV DNA in the HBeAg-positive phase were generally higher than those in the ant-HBe-positive phase [19, 22]. HBeAg positivity is also associated with HBV viremia and increased ALT levels in HIV/HBV co-infected patients [23]. Next, we compared the response to LAM or ETV in 18 or 4 HBeAg-positive patients, respectively **(Table 2)**. LAM significantly reduced HBV DNA levels from baseline 7.52 log IU/mL to 3.35 log IU/mL ($P < 0.001$) at 1 month, to 2.38 log IU/mL ($P < 0.001$) at 3 months, and to 1.55 log IU/mL ($P < 0.001$) at 6 months. ETV also significantly reduced HBV DNA levels from baseline 8.42 log IU/mL to 3.87 log IU/mL ($P < 0.001$) at 1 month, to 2.90 log IU/mL ($P < 0.001$) at 3 months, and to 2.22 log IU/mL ($P < 0.001$) at 6 months. There were no differences in HBV DNA levels from baseline to 6 months between the two groups. Primary antiviral treatment failure was not observed in either group. Four patients in the LAM group did not achieve IVR.

Table 2 Demographic, Clinical, and Laboratory Variables of HBeAg-positive Patients at Entry.

Parameters	Total Patients (N=22)	LAM (N=18)	ETV (N=4)	*P*-value
Age (years)	34.5 (21-51)	36.5 (21-51)	30 (24-33)	NS
Male (%)	18 (81.8)	14 (77.7)	4 (100)	NS
Cirrhosis (+/-)	1/21	1/17	0/4	NS
ALT (IU/L)	1,030 (523-2,450)	1,990 (523-2,450)	1,363 (980-1,620)	NS
T. Bil (mg/dL)	1.75 (0.8-20.6)	2.0 (0.8-20.6)	1.5 (1.0-18.7)	NS
PT (%)	77 (24-119)	73.6 (24-119)	95.0 (44.1-113)	NS
HBeAg (+)	22	18	4	
HBV DNA (log IU/mL)	7.6 (5.5- 8.8)	7.6 (5.5- 8.7)	8.6 (7.6- 8.7)	NS

LAM, lamivudine; ETV, entecavir; ALT, alanine aminotransferase; T. BIL, total bilirubin; PT, prothrombin time; NS, statistically not significant.

Reduction in ALT levels of HBeAg-positive patients

LAM significantly reduced ALT levels from baseline 1,150 IU/mL to 84 ($P < 0.001$) at 1 month, to 27.5 ($P < 0.001$) at 3 months, and to 22.0 ($P < 0.001$) at 6 months. ETV also significantly reduced ALT levels from baseline 1,460 IU/mL to 230 ($P = 0.0038$) at 1 month, to 22.2 ($P = 0.0016$) at 3 months, and to 24.0 ($P = 0.0016$) at 6 months. At 1 month after treatment, the ALT levels of the LAM groups were lower than those of the ETV group ($P < 0.0001$) **(Figure 3)**. During follow-up periods, 10 and 1 sero-converters of HBeAg to anti-HBe antibody phase were seen in 18 LAM-treated and in 4 ETV-treated patients, respectively.

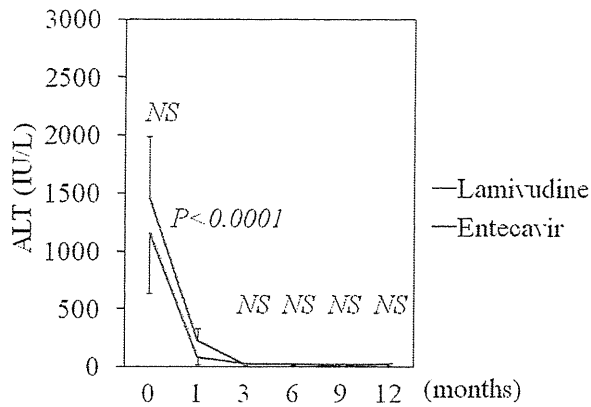

Figure 3 Efficacy of lamivudine and entecavir for ALT levels in HBeAg-positive patients. Lamivudine (N=18) vs. entecavir (N=4); data are shown as mean ± SD.

Safety

No patient stopped taking medications. Twelve months after treatment, 10 of 24 patients (41.6%) in the LAM group switched from LAM to ETV (n=4) or added adefovir (n=6) due to the emergence of LAM-resistant mutants. On the other hand, patients receiving ETV did not need to change their medication.

DISCUSSION

The present study compared the use of NUCs, LAM and ETV, for the treatment of acute exacerbation of chronic hepatitis B. The results clearly showed significant benefits of a rapid reduction of HBV DNA levels, compared with untreated patients in a previous report [4].

It was reported that ETV treatment is associated with increased short-term mortality in patients with severe acute exacerbation of chronic hepatitis B, but that it achieves better virological response in the long run [24]. We used LAM or ETV for patients with acute exacerbation of chronic hepatitis B presenting with ALT ≥ 500 IU/L in the present study. The effects of LAM on HBV DNA levels were the same as those of ETV (Figure 1). But the effects of LAM on ALT levels after 1 month were stronger than those of ETV in HBeAg-positive patients **(Figure 3)**. In spite of the limited number of these patients, the effects were possibly related to immunomodulating activities of LAM [25]. The patients' prognoses were more favorable than in the previous report [4]. This might have

depended on the fact that, in the present study, treatment was begun as soon as possible, and some patients may have had a milder grade of acute exacerbation of chronic hepatitis B than those in the previous report [4]. We believe that patients with acute exacerbation of chronic hepatitis B need to be subjected to treatment as promptly as possible.

The major routes of HBV infection in our country have been mother-to-child transmission and blood transfusion. However, cases with HBV transmitted through sexual contact are increasing, especially among HIV-1-seropositive patients [26]. One should bear in mind that knowledge about interactions between ETV and anti-HIV nucleoside analogues is limited [27]. Because long-term use of LAM induces LAM-resistant mutants [28], we can only use LAM for short-term treatment of patients with acute exacerbation of chronic hepatitis B. On the other hand, the present study also revealed that patients receiving ETV did not need to change drugs.

Recently, there have been several reports that reactivation of HBV is a fatal complication following systemic chemotherapy or other immunosuppressive therapy including rituximab and steroid therapies mainly in HBsAg-positive and -negative lymphoma patients. It is important to enable early diagnosis of HBV reactivation as well as initiation of antiviral therapy [29, 30].

In conclusion, ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should start to treat these patients with NUCs as soon as possible.

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ABBREVIATIONS

ETV: Entecavir; HIV: Human immunodeficiency virus; IVR: Initial virological response; LAM: Lamivudine; NUC: Nucleoside analogue.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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Clinical importance of serum hepatitis B surface antigen levels in chronic hepatitis B

S. Togo, M. Arai, A. Tawada, T. Chiba, T. Kanda, K. Fujiwara, F. Imazeki and O. Yokosuka

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

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SUMMARY. Quantitative serology for hepatitis B surface antigen (HBsAg) is a new candidate marker for prediction of clinical outcome. The aim of this study was to investigate the clinical significance of quantifying HBsAg in patients with hepatitis B virus (HBV) infection. A total of 424 patients who tested positive for HBsAg and were referred to Chiba University Hospital between January 1985 and April 2008 were included in the study, and the following characteristics were analyzed: age, gender, status of hepatitis B e antigen (HBeAg), alanine aminotransferase level (ALT), HBV DNA level, number of platelets and development of hepatocellular carcinoma. Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay method. The study group consisted of 239 men and 185 women, and their average age was 40.6 ± 14.0 years. HBsAg showed a positive correlation with HBV DNA level (Pearson's product moment correlation, $r = 0.586$, $P < 0.001$) and a weak inverse correlation with age ($r = 0.3325$, $P < 0.001$). A control study, matched with age and sex, was performed between two groups with and without HBeAg seroconversion during follow-up period. Compared with the age and sex-matched controls, the change in HBsAg levels per year showed a significant decrease 2 years before seroconversion (paired *t*-test, $P < 0.05$). The serial measurement of quantitative HBsAg level has the possibility of predicting the occurrence of HBeAg seroconversion.

Keywords: chronic hepatitis B, HBeAg seroconversion, HBs antigen quantification.

INTRODUCTION

An estimated 350 million persons worldwide are chronically infected with HBV [1]. Chronic infection with HBV can progress to cirrhosis, liver failure and hepatocellular carcinoma (HCC), and is a major cause of mortality worldwide [2,3]. Loss of hepatitis B e antigen (HBeAg), accompanied by seroconversion to anti-HBe antibody, usually results in normalized serum alanine aminotransferase (ALT) and decreased HBV DNA levels, and may lead to improved hepatic necroinflammation and confer a better clinical outcome [4–6]. In a recent study of the natural history of chronic hepatitis B (CHB) in 3233 Asian patients, the median age of HBeAg seroconversion was 35 years [7]. HBeAg seroconversion may occur spontaneously at a rate of 5–10% per year [8]. Thus, in clinical practice, HBeAg seroconversion is recognized as a successful serologic response to the treatment of HBeAg-positive CHB.

Determining an accurate prognosis for HBV carriers, based on clinical presentation, is important for clinical management of the disease. Various studies have been performed to distinguish the positive and negative prognostic factors for HBV carriers. The level of HBV DNA, evaluated by TaqMan® PCR method, is an important predictor of clinical outcome in patients with HBV infection [9], but its efficacy is limited [10]. Therefore, we need another marker for predicting the clinical outcome of HBV carriers. Recently, quantitative serology for hepatitis B surface antigen (HBsAg) has been developed as one of the promising candidates. Chan *et al.* [11] found that peginterferon (PegIFN) alfa-2a provided a significant reduction in HBsAg level in the sera of patients with HBeAg-positive CHB. Moreover, HBsAg decline was significantly associated with HBeAg seroconversion

Abbreviations: ALT, serum alanine aminotransferase; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CI, confidence interval; CLEIA, chemiluminescent enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HBcrAg, hepatitis B virus core-related antigens; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV DNA, hepatitis B virus deoxyribo nucleic acid; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IU, international units; LC, log copies; NA, nucleoside/nucleotide analogues; OR, odds ratio; PCR, polymerase chain reaction; PegIFN, peginterferon; PLTs, number of platelets; SD, standard deviation.

Correspondence: Makoto Arai, MD, Department of Medicine and Clinical Oncology (K1), Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chiba-City, 260-8670, Japan.
E-mail: araim-cib@umin.ac.jp

1 year post-treatment, and on-treatment HBsAg levels could be used as an early predictor of durable off-treatment response to PegIFN-based therapy in the individual patient. Recently, Chan *et al.* [12] reported about HBsAg reduction and the fluctuation in titre before and after HBeAg seroconversion of untreated patients.

In this study, based on a cohort of patients with CHB with long-term follow-up, we investigated the HBsAg levels at various stages of CHB. We also aimed to investigate the value of quantitative HBsAg for predicting clinical outcomes in HBeAg-positive CHB patients. Our results clarified the importance of evaluating serum HBsAg levels in patients with CHB.

MATERIAL AND METHODS

Patients

This was a retrospective analysis. Between January 1985 and April 2008, all patients visiting the Chiba University Hospital and who were HBsAg-positive carriers (n = 676) were approached for participation in the study. This study was reviewed and approved by the institutional review board of Chiba University School of Medicine. The patients' consent was obtained for the storage and use of serum. Patients who were positive for hepatitis C virus antibody and those who had another potential cause of chronic liver diseases (autoimmune hepatitis and primary biliary cirrhosis) were excluded from the study. Those patients with <1 year of observation or who had been given antiviral drugs (lamivudine or entecavir) at entry also were excluded from the analysis. As a result, 424 patients were selected for further analysis. To clarify the relationship between the level of HBsAg and other factors, HBV DNA, alanine aminotransferase (ALT) and the number of platelets (PLTs) were analyzed. In addition, we analyzed whether the level of HBsAg was related to the occurrence of HCC. The serum samples from the patients were stored at −20 °C, and the oldest sample obtained from each patient was used to define the level of HBV DNA and HBsAg at entry.

Laboratory assays

Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay (CLEIA) method and the HISCL-2000i (Sysmex Corporation, Kobe, Japan). HBeAg and anti-HBe levels were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL, USA). Anti-HCV was detected by ELISA (Ortho Diagnostics, Tokyo, Japan). The serum HBV DNA level was quantified by polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a linear range of quantification of 2.6–7.6 log copies (LC) per mL. The six major genotypes of HBV (A–F) were determined by ELISA (HBV Genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). HBV serum core–related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan).

Serial changes in HBsAg levels during long-term follow-up of HBeAg-positive patients

To observe the serial changes in the HBsAg levels in HBeAg-positive patients, we extracted the HBeAg-positive patients at the beginning of the observation period. Among 424 HBsAg-positive patients, 183 were HBeAg positive. To clarify the long natural history of HBV carriers, we excluded those who could not be followed for more than 5 years. Finally, 120 patients who could be followed for more than 5 years were enrolled and their HBsAg levels were evaluated every year with an error of <2 months.

Statistical analysis

The baseline data are presented as mean ± SD or median and range. The difference in the values of clinical parameters between the two groups was analyzed by paired t-test, unpaired t-test, Welch t-test and chi-square test. Pearson's product moment correlation coefficient analysis was used for statistical analyses, as appropriate, with the statistical program SPSS 16.1 (SPSS Inc., Chicago, IL, USA); a P value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics and the relationship between HBsAg quantification and other clinical markers

The baseline clinical and virological characteristics of the 424 HBsAg-positive carriers are shown in Table 1. First, we investigated the relationship between HBsAg and other virological and clinical markers. The relationships of HBsAg (log IU/mL) with age, gender, HBV genotype and HBeAg status are illustrated in Fig. 1. Gender was not associated with HBsAg titre (Fig. 1a). In contrast, the level of HBsAg in the patients with HBV genotype C differed significantly from those with genotype B ($P < 0.05$, unpaired t-test) (Fig. 1b). The average of HBsAg titre was significantly higher in HBeAg-positive patients compared with those who were HBeAg negative, with statistical difference (unpaired t-test, $P < 0.05$, Fig. 1c). HBsAg showed a significant positive correlation with the HBV DNA level (Pearson's product moment correlation, $r = 0.586$, $P < 0.001$, Fig. 2a), and a weak and inverse correlation between HBsAg and age is also shown (Fig. 2b). In contrast, HBsAg did not show a good correlation with ALT level or PLTs (Figs 2c,d). Next, we used the Cox proportional hazards model to investigate whether HBsAg could be a predictive marker for the occurrence of HCC. Screening for the detection of HCC was performed based on the typical findings of abdominal ultrasonography, dynamic computed tomog-