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Evolution of hepatitis B genotype C viral quasi-species during hepatitis B e antigen seroconversion

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Background & Aims: Although the evolution of viral quasi-species may be related to the pathological status of disease, little is known about this phenomenon in hepatitis B, particularly with respect to hepatitis B e antigen (HBeAg) seroconversion.

Methods: Nucleotide sequences of the hepatitis B virus (HBV) *X/precore/core* region was analyzed at five time-points in four groups of chronic hepatitis B patients, interferon-induced seroconverters (IS, $N = 9$), interferon non-responders (IN, $N = 9$), spontaneous seroconverters (SS, $N = 9$), and non-seroconverters (SN, $N = 9$) followed during 60 months on an average. Only patients with genotype C were studied.

Results: Analysis of 1800 nucleotide sequences showed that there was no statistical difference between the nucleotide genetic distances of seroconverters (IS and SS; 6.9×10^{-3} substitutions (st)/site and 6.7×10^{-3} st/site, respectively) and those of non-seroconverters (IN and SN; 5.3×10^{-3} st/site and 3.8×10^{-3} st/site, respectively) before seroconversion. Compared to non-seroconverters (IN and SN; 5.1×10^{-3} st/site and 5.9×10^{-3} st/site, respectively), the sequence diversity of seroconverters (IS and SS; 10.9×10^{-3} st/site and 9.9×10^{-3} st/site, respectively) was significantly higher after seroconversion ($p < 0.05$), and was higher in seroconverters after seroconversion than before seroconversion ($p < 0.05$), while this changed very little in non-seroconverters during the observation period. Phylogenetic trees showed greater complexity in secoconverters than non-seroconverters. Parsimony-based estimation of the direction of sequence change between descendants and ancestors before HBeAg seroconversion, revealed higher frequencies of transversional A to T substitution in seroconverters (0.06 *vs.* 0.02, $p = 0.0036$) that coincided with the dynamics of quasi-species possessing A1762T mutation.

Conclusions: The distinctly greater viral diversity in HBeAg seroconverters after seroconversion could be related to escape mutants resulting from stronger selection pressure.

Keywords: Chronic hepatitis B; Quasi-species; Hepatitis B e antigen seroconversion.

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Abbreviations: SC, seroconversion; ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IS, interferon-induced HBeAg seroconverters; IN, IFN non-responders; SS, spontaneous seroconverters; SN, non-seroconverters.

Introduction

Hepatitis B virus (HBV) is a major human pathogen which can cause severe hepatic disease, including chronic hepatitis, cirrhosis (LC), and hepatocellular carcinoma (HCC). Quasi-species comprises a complex and dynamic distribution of non-identical but related genomes [1]. The evolution of viral quasi-species has been reported as important in the pathogenesis of RNA viruses such as hepatitis C virus [2–6] and human immunodeficiency virus [7–10], but little is known about HBV. HBV is a hepatotropic, non-cytopathic DNA virus replicated by an error-prone polymerase through an RNA intermediate. Because of this feature, the replication of HBV lacks fidelity. This results in a complex distributions of genomes with naturally-acquired mutations or mutations selected by either antiviral therapy or the immune response of the host. HBV quasi-species have not been subjected to detailed investigation, especially in the context of hepatitis B e antigen (HBeAg) seroconversion (SC), an immunologically mediated event. Whether there is a causal relationship between HBV seroconversion and HBV quasi-species remains unclear. HBV-related disease is known to be mediated both virologically and immunologically. Several studies have depicted the dynamic evolution of HBV quasi-species during lamivudine resistance or multiple drug resistance. This highlights the importance of HBV molecular evolution in revealing the mechanism of drug resistance [11,12]. HBV-specific cytotoxic T-cells play a significant role in the control of replication of HBV, which has been well documented in the literature [13–16].

Precore/core protein is the target of immunologically mediated HBeAg seroconversion. When the *precore/core* gene in HBV DNA is transcribed and translated, HBeAg is produced and secreted into the circulation [17,18]. But the synthesis and secretion of HBeAg are aborted by the emergence of a point mutation from G to A at nucleotide (nt)1896 (G1896A). Convincing lines of evidence have indicated a close association between HBeAg/anti-HBe seroconversion and the emergence of *precore* and *core* promoter mutations [19,20].

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The purpose of this study was to elucidate the evolution of HBV quasi-species during HBeAg seroconversion. The results might help us to better understand the pathogenic mechanisms of HBV. We selected patients with well-characterized clinical phenotypes and compared their viral diversity based on the nucleotide sequences of the *X*/precore/core region. *Precore* and *core* promoter mutations were also investigated in detail before and after HBeAg seroconversion.

Materials and methods

Patients

Sera from 36 chronic hepatitis B patients with well-characterized clinical follow-up for >5 years were selected from a chronic hepatitis B database (77 seroconverters and 67 non-seroconverters) at Chiba University Hospital. Only patients with genotype C (subtype C2) were studied to ensure that differences found in viral evolution were not due to genotypic variation. Nine patients in each group were selected randomly if they fulfilled the following criteria and had sufficiently long follow-up. The index group comprised patients with documented HBeAg seroconversion (spontaneous seroconverters, SS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−25.2 ± 6.2/months), time-point II (−11.6 ± 2.7/months), time-point III (1 ± 2.3/months), time-point IV (12.5 ± 3.3/months), and time-point V (25 ± 3.6 months). Untreated control patients included those who were followed for a similar period of time and were persistently HBeAg positive (non-seroconverters, SN), and they were matched for average age of seroconversion and time-point intervals of the SS group. A second index group of patients with interferon (IFN)-induced HBeAg seroconversion (IFN seroconverters, IS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−24.3 ± 3.1/months), time-point II (−11.2 ± 1.9/months), time-point III (1 ± 1.2/months), time-point IV (12.7 ± 1.7/months), and time-point V (25.4 ± 2.2/months). Control patients were persistently HBeAg-positive despite IFN therapy (IFN non-responders, IN). Controls were matched for the average age of seroconversion, sex and time-point intervals of the IS group.

HBeAg seroconversion was defined as the loss of HBeAg and the development of anti-HBe. The serial serum samples in this study were taken at five time-points for each patient, as described above. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological examination

HBsAg, HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL). HBV genotype was determined from the patients' sera by ELISA (HBV genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan), based on the method described by Usuda et al. [21]. Serum HBV DNA levels were monitored using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 $\log_{10}$ copies/ml, at each time-point.

Cloning and sequencing

Total DNA was extracted from 200 μl of each serum sample using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 200 μl distilled water. Because HBeAg seroconversion is associated with a decrease in HBV DNA levels, nested PCR was performed for all the samples. The primers for the first round of PCR were 5′-TCG CAT GGA GAC CAC CGT GA-3′ (sense, nt1604–1623) and 5′-ATA GCT TGC CTG AGT GC-3′ (antisense, nt 2076–2060). The primers for the second round of PCR were 5′-CAT AAG AGG ACT CTT GGA CT-3′ (sense, nt 1653–1672) and 5′-GGA AAG AAG TCA GAA GGC-3′ (antisense, nt 1974–1957).

Amplification was performed with 2 μl of DNA template (extracted DNA from serum samples for the first round PCR and the first round PCR products for the second round PCR) in 50 μl reaction under the following conditions: an initial 2 min of denaturation at 94 °C and 36 cycles of 94 °C denaturation for 1 min, annealing at either 54 °C or 52 °C for 1 min, in the first and second round respectively, and 72 °C extension for 1 min. The last cycle was followed by a final extension at 72 °C for 10 min. A 473-base pairs fragment (nt 1604–2076) containing the *X/precore/core* region was amplified.

PCR reactions were followed by cloning using TOPO® TA cloning kits (Invitrogen, Carlsbad, CA). All PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), then cloned into the TOPO vector, and transformed into *Escherichia coli*. At least 15 clones per one cloning for samples from PCR reactions proceeded subsequent to the electrophoretic size separation on 1.2% agarose gel. Ten positive clones per cloning for samples from each PCR reaction were sequenced using BigDye® Terminator and a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The cloning PCR and sequencing primers were M13-forward, 5′-GTA AAA CGA CGG CCA GT-3′, and M13-reverse, 5′-GGA AAC AGC TAT GAC CAT G-3′.

Sequence analysis

The DNAPARS program from PHYLIP v3.65 package, implemented in Simmonic Sequence Editor version 1.5 [22], was used for sequence analysis. To evaluate quasi-species-based evolution of HBV strains in chronic patients, sequences of clones ($N = 10$) isolated at each time-point ($N = 5$) from individual patients ($N = 36$) were subjected to alignment and used to generate one parsimonious ancestral sequence. Maximum nucleotide composition distances were evaluated pair-wise between the ancestral sequence and the sequences of each of the 10 clones with a mean value estimated for each patient at a given time-point (*MEGA* version 4 [23]). All patients were categorized into four groups with respect to seroconversion status and the mean distance value for each group was calculated for each time-point.

The differences in genetic distance among clinical groups and time-points, and diversity at each time-point, were analyzed using ANOVA (analysis of variance). Student's *t*-test was also performed to determine the average of genetic diversities in non-seroconverters. All graphical data are presented as means ± standard deviation (SD). Results were considered statistically significant at $p < 0.05$. The statistical analysis was performed with SPSS (2004; SPSS Inc., Tokyo, Japan).

Construction of phylogenetic trees

To examine the evolution of the viral sequence and whether this evolution was elicited by quasi-species or mutagenesis, phylogenetic trees were constructed using the Neighbor-Joining (NJ) model with the Simmonic Sequence Editor version 1.5, based on the genomic sequences of HBV. Moreover, to investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 were further analyzed phylogenetically. Neighbor-Joining trees were constructed at time-points 1 and 2 (Fig. S1 and S2, respectively) using all groups of sequences.

Results

Baseline clinical characteristics of the patients and sequential levels of serum ALT and HBV DNA

The clinical and laboratory characteristics of all patients are listed in Table 1. The levels of alanine aminotransferase (ALT) and HBV DNA over time are illustrated in Fig. 1A and B, respectively. Serum ALT levels, a marker of hepatocyte damage, normalized after seroconversion and, for all groups except the interferon non-responders, were <40 IU/L at the end-point of observation. HBV DNA loads decreased markedly in seroconverters (<3 $\log_{10}$ copies/ml, $p < 0.0001$) but changed very little in non-seroconverters. It is noteworthy that, at the second year after seroconversion, serum HBV DNA loads increased in interferon-induced seroconverters compared to spontaneous seroconverters, without statistical significance ($p^H = 0.1087$) (Fig. 1B).

Viral nucleotide sequence diversity

Viral sequence diversity, phylogenetic trees, and mutation pattern based on 1800 HBV nucleotide sequences from clones of the *X/precore/core* region, were analyzed among selected patients.

Table 1. Baseline clinical features of patients.

	IFN Seroconverters (IS)	IFN Non-seroconverters (IN)	Spontaneous Seroconverters (SS)	Spontaneous Non-seroconverters (SN)
Age (y)	40 ± 8	40 ± 11	29 ± 10	34 ± 6
Male : Female	6:3	8:1	5:4	7:2
HBV DNA (log_{10} copies/ml)	6.8 ± 0.9	6.8 ± 1.0	6.8 ± 1.2	7.1 ± 0.8
ALT (IU/L)	88.3 ± 48.6	94.3 ± 144.4	89.8 ± 71.4	67.6 ± 48.7

Note: The IFN-induced group (seroconverters and non-responders) was older than the spontaneous group (seroconverters and non-responders). Males were the majority in all groups. Baseline serum HBV DNA and ALT levels are similar among the four groups. Data are shown as mean ± SD.

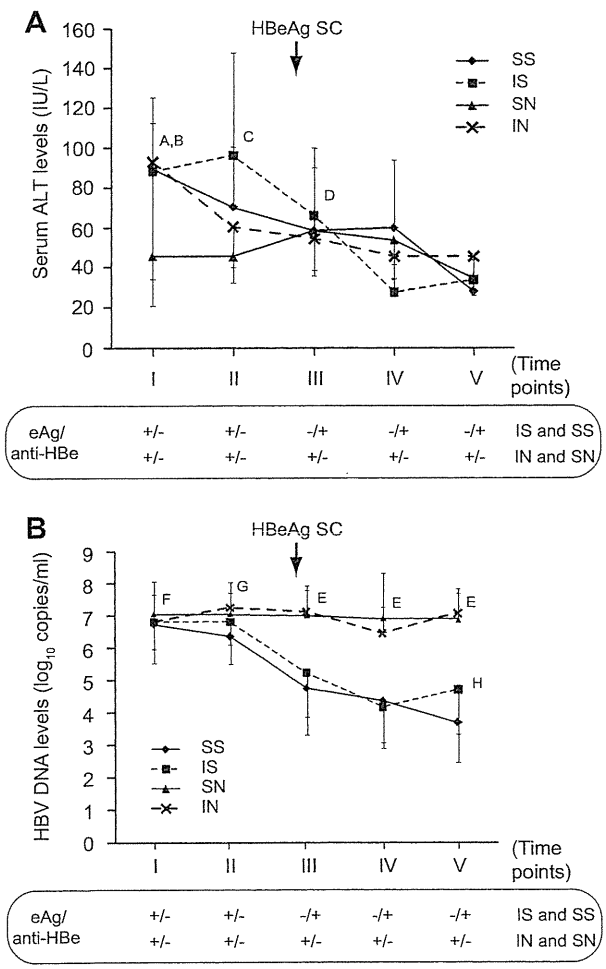

Fig. 1. Serum ALT and DNA levels in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk, and non-seroconverters controls (SN) is a solid line triangle. (A) $p^A = 0.0234$ comparing time-point I with time-point IV, $p^B = 0.0028$ comparing time-point I with time-point V, $p^C = 0.007$ comparing time-point II with time-point V, $p^D = 0.0068$ comparing time-point III with time-point V. (B) $p^E < 0.0001$ comparing seroconverters with non-seroconverters, $p^F < 0.0001$ comparing time-point I with III, IV, V, $p^G < 0.0001$ comparing time-point II with the other time-points, $p^H = 0.1087$ at time-point V in seroconverters.

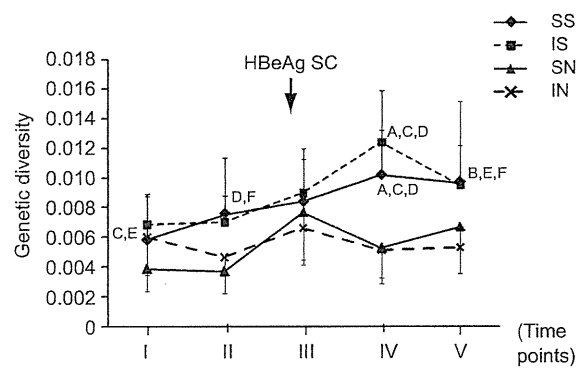

Fig. 2. Viral genetic diversity in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk and non-seroconverters controls (SN) is a solid line triangle. $p^A < 0.0001$ comparing seroconverters with non-seroconverters at time-point IV, $p^B = 0.0301$ comparing seroconverters with non-seroconverters at time-point V, $p^C = 0.0013$ and $p^D = 0.0025$ comparing I and II with time-point IV in seroconverters. $p^E = 0.0121$ and $p^F = 0.021$ comparing time-points I and II with V in seroconverters.

Striking differences in nucleotide sequence diversity were revealed between seroconverters and non-seroconverters before and after seroconversion (Fig. 2). The nucleotide sequence diversity of seroconverters was similar to that of non-seroconverters before seroconversion. Analysis of genetic distance showed that the viral sequence diversity of seroconverters was significantly greater than that of non-seroconverters after seroconversion (Fig. 2, $p^A < 0.0001$ at time-point IV, $p^B = 0.0301$ at time-point V) and was greater in seroconverters after seroconversion than before (Fig. 2, $p^C = 0.0013$ and $p^D = 0.0025$), while almost no changes were observed in non-seroconverters during the observation period.

It is noteworthy that, in interferon-induced seroconverters at the last time-point of observation, the nucleotide sequence diversity was less, although this increased clearly at the first year after seroconversion. This tendency of reversed change at the last two time-points was also seen in HBV DNA loads (Fig. 1B), namely, increase or decrease of the genetic diversity accompanied by decrease or increase of the viral load in interferon-induced seroconverters. On the other hand, the nucleotide sequence diversity increased continuously in spontaneous seroconverters, accompanied by a concurrent decrease of viral loads (Fig. 1B) during the follow-up period. Amino acid sequence diversity had an almost

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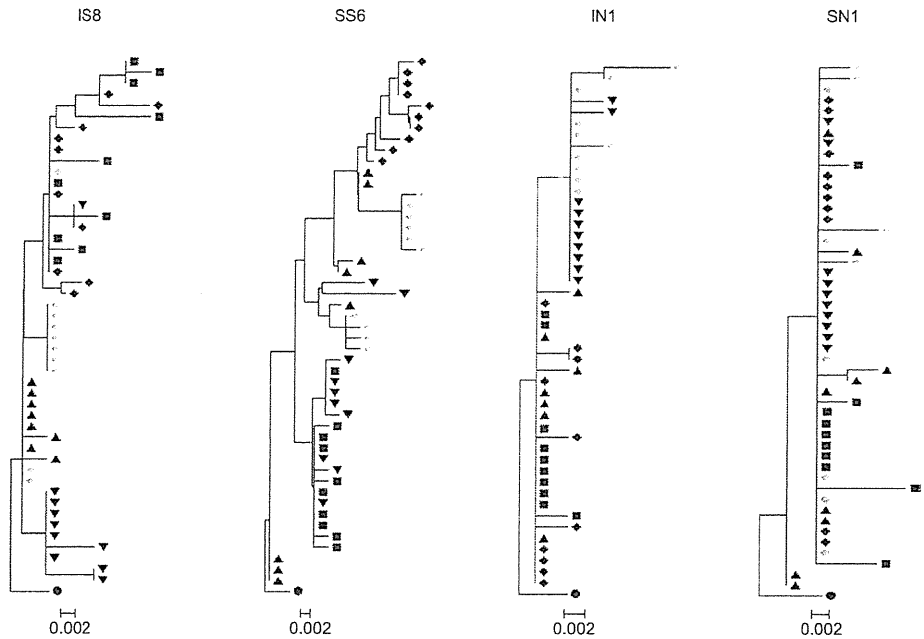

Fig. 3. Representative Neighbor-Joining phylogenetic trees of HBV sequences for each clinical group showing complex trees in seroconverters. HBV *X/precore/core* sequences from time-points I (purple filled triangle), II (blue filled inverted triangle), III (green filled square), IV (red filled diamond) and V (sky blue filled diamond) serum samples are analyzed phylogenetically and their positions are displayed on the trees. A sequence retrieved from the time-point I (red dot) of each group as outgroup in the trees, respectively. Scale bar represents 0.002% genetic variation. Seroconversion patients (IS, SS) show relatively complex branching patterns, forming clusters over time. With the pressure of seroconversion, the genetic diversity increased. In contrast, patients without seroconversion (IN, SN) were simply branching patterns and the genetic diversity in these patients changed very little over time.

identical pattern to that of DNA nucleotide sequence diversity (data not shown).

Construction of phylogenetic trees

Phylogenetic trees were complex for secoconverters and comparatively simple for non-seroconverters. In seroconverters (IS and SS), the arrangement and branch lengths of the trees were consistently more complex and longer than those for non-seroconverters. The genetic diversity was great after seroconversion in seroconverters (IS and SS) and less in non-seroconverters (IN and SN) (Fig. 3).

To investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 (before seroconversion) were further analyzed phylogenetically. Trees were reconstructed using Neighbor-Joining, ML (data not shown), and PAML methods (data not shown). In general, no clusters were seen to be supported by robust bootstrap values for any group or particular patient quasi-species. This indicates that the region of the HBV genome studied does not contain patterns of variability sufficient for robust phylogenetic relation reconstruction. However, variability of branch lengths in the tree indicated that seroconversion patient groups exhibit greater diversity of the quasi-species compared to patients without seroconversion. This is in agreement with the genetic distance plot (Fig. 2), showing greater deviation from the mean values observed in patients with seroconversion. The IN group exhibited least deviation on the distance plot (Fig. 2) and shortest branch lengths on the trees (Fig. 3).

Interclonal differences of the quasi-species

To investigate whether a particular mutation pattern of evolution of the quasi-species is associated with seroconversion, we further analyzed the sequence changes in all patients at time-points 1 and 2, corresponding to the time before seroconversion. Parsimony-based ancestral sequences were generated using the Simmonic Sequence Editor. Aligned sequences of time-points 1 and 2 from a single patient were used as the input. Frequencies of changes in 12 types of mutations, including 4 transitions (CT, TC, AG, and GA) and 8 transversions (AT, TA, AC, CA, CG, GC, GT, and TG) were evaluated between generated descendants and ancestral sequences for each clone of the patient. Statistical *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups is summarized in Table 2 and Supplementary Table 1.

Analysis of sequence changes indicated a higher frequency of transversional A to T in spontaneous seroconverters (SS *vs.* SN = 0.06 *vs.* 0.02, $p = 0.04$) and IFN-induced seroconverters (IS *vs.* IN = 0.05 *vs.* 0.01, $p = 0.05$) and A to C changes in IFN-induced seroconverters (IS *vs.* IN = 0.025 *vs.* 0.006, $p = 0.04$) before seroconversion. Comparison of seroconversion groups (SS and IS) indicated a higher frequency of transversional A to T mutation pattern ($p = 0.003$, Table 2) and the trend of G to A mutation is higher in seroconversion groups (SS and IS) (Table 2). Subsequently, alignments of the clones were generated. Visual inspection of the alignments indicated variation in the ratio of A1762T mutation in clones isolated from each patient at time-points 1 and 2 (Fig. 4). In contrast to non-seroconverters, seroconverters

Table 2. *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups.

	Seroconversion (n = 18)	Non-seroconversion (n = 18)	*p*
CT	0.117033	0.103750	0.637023
TC	0.156706	0.201328	0.155252
AG	0.125483	0.148372	0.498916
GA	0.196722	0.124511	0.073433
AT	0.061194	0.022128	0.003665
TA	0.049372	0.045417	0.778612
AC	0.027944	0.012550	0.145158
CA	0.017128	0.011094	0.523868
CG	0.009439	0.007744	0.835337
GC	0.018167	0.014894	0.748267
GT	0.009839	0.019217	0.272185
TG	0.041783	0.035528	0.731324

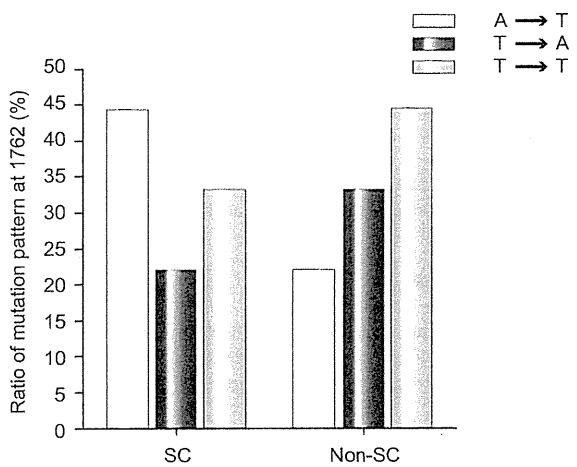

Fig. 4. The evolution of the *core* promoter mutation (A1762T) between seroconversion and control groups from time-point I to II. SC indicates seroconversion and non-SC, non-seroconversion. Alignment of the clones was carried out and the frequency of A1762T mutation in clones isolated from each patient at time-points 1 and 2 was determined. Subsequently, the evolutionary ratio of mutation from time-point I to II was calculated.

showed a higher frequency of A to T mutation pattern in the core promoter region from time-point I to II.

Core promoter (A1762T/G1764A) and precore (G1896A) mutations

Given that the *core* promoter/*precore* mutations influenced virus replication and HBeAg seroconversion, we analyzed the sequential change of *core* promoter (A1762T/G1764A)/*precore* (G1896A) mutations over time (Table 3). After seroconversion, patients with more than 50% *precore* mutant clone had higher HBV DNA loads than those with less than 50% of *precore* mutant clone (*precore* wild type) virus at time-point V [5.4 ± 1.3 (n = 5) *vs.* 3.8 ± 1.1 (n = 13), p = 0.0185] and 8 patients with a HBV DNA load less than 4.0 $\log_{10}$ copies/ml had all *precore* wild-type virus at time-point V (Table 3). Clinical progress of these patients was investigated over 10 years as median (range 1–20 years) after HBeAg seroconversion. HCC developed in 3 of 5 patients with *precore* mutant virus, compared to 1 of 13 patients with *precore* wild-type virus at time-point V (p = 0.017). On the other hand, 3 patients with ASC had all *precore* wild-type virus at time-point V (Table 3).

Discussion

In this study, analysis of 1800 nucleotide sequences from 36 HBV carriers showed that the viral diversity of seroconverters (IS and SS) after seroconversion was significantly greater than that of non-seroconverters (IN and SN) (Fig. 2, p <0.05) and was higher after seroconversion than before, in the seroconverters (Fig. 2, p <0.05). Phylogenetic analysis also generated complex trees for seroconverters and relatively simple trees for non-seroconverters. Analysis on interclonal differences in the quasi-specie showed a higher frequency of transversional A to T mutation pattern in seroconverters that coincided with the A1762T core promoter mutation. These findings suggested that HBeAg seroconversion involves dynamic shifts of the serum HBV quasi-species.

Osiowy et al. [24] examined viral quasi-species in eight HBeAg-negative patients at two time-points 25 years apart and obtained the evolutionary rate. Their results suggested that HBV diversity may be generated more rapidly than those estimated previously [25–29]. The higher evolutionary rate may be related to the seroconversion event driving quasi-specie complexity and diversification [24]. Our phylogenetic study showed that viral quasi-species populations appear to be replaced by new populations arising from a different clade after seroconversion.

Increased immune responses are accompanied by the reduction of viral loads and stronger immune pressure induces the selection of escape mutations, which leads to greater viral diversity [30]. According to this scenario, in our study, non-seroconverters have a high viral load and low quasi-species diversity and they obviously have a weak immune response.

Lim et al. [31] reported that viral genetic diversity in genotype B CHB patients was 2.4-fold greater in HBeAg seroconverters (spontaneous or IFN-induced) than in non-seroconverters before seroconversion. In this study of genotype C CHB patients, the nucleotide genetic distance was 1.49-fold greater in seroconverters (IS and SS) than in non-seroconverters before seroconversion but there was no statistical difference. This discrepancy might be due to the smaller region for analysis of genetic distance in our study than that of Lim et al. Another interpretation is that the host's immune response to the selection of mutant virus might differ between genotype B and genotype C. The natural course of CHB and the response to treatment could be affected by HBV genotype and there are some lines of evidence that indicate that the prevalence rates of *precore* and *core* promoter mutations vary among patients infected with HBV strains of different genotypes [32–34].

T-test comparison of mean values of nucleotide changes (Table 2) and linear logistic regression univariate analysis of mutations associated with seroconversion between seroconverters and non-seroconverters (data not shown) indicated a variation in the AT mutation pattern in the former (p = 0.003 and p = 0.006, respectively). This coincided with differences in the

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Table 3. *Core* promoter and *precore* mutations in seroconverters (IS and SS).

Patients	CP (ntA1762T/G1764A) (percent)			PC (ntG1896A) (percent)			DNA Loads (log_{10} copies/ml)			Histological diagnosis
	I	III	V	I	III	V	I	III	V	
IS1	100	100	100	0	0	70	5.7	3.8	4.8	CHB
IS2	100	100	100	90	100	90	7.6	7.2	7.6	HCC
IS3	70	100	100	10	0	10	6.5	5.2	5.5	CHB
IS4	90	100	10	0	10	0	7.6	6.2	3.3	CHB
IS5	100	40	20	0	0	0	7.6	3.6	4.1	CHB
IS6	70	90	90	20	10	90	5.7	4.1	4.5	HCC
IS7	100	100	90	0	10	10	7.2	3.1	3.4	LC
IS8	80	100	60	0	0	60	7.6	4.0	4.5	CHB
IS9	100	100	10	0	0	0	6.0	4.5	4.8	HCC
SS1	0	60	0	80	0	80	7.6	4.2	5.4	HCC
SS2	80	100	90	10	90	10	6.6	7.6	5.9	ASC
SS3	100	90	60	10	0	0	6.5	4.3	2.8	ASC
SS6	30	100	10	0	0	10	3.9	4.4	4.1	CHB
SS7	80	100	100	0	0	0	7.6	2.8	2.6	ASC
SS8	0	100	90	0	20	0	7.6	5.4	3.6	CHB
SS9	0	80	20	0	10	0	7.6	4.0	2.6	CHB
SS10	50	20	40	0	0	40	7.3	3.9	2.6	CHB
SS11	100	100	100	0	0	0	6.1	6.3	3.8	CHB

IS: interferon induced seroconverter; SS: spontaneous seroconverter; ASC: asymptomatic carriers; CHB: chronic hepatitis B; LC: cirrhosis; HCC: hepatocellular carcinoma.

ratio of T1762A quasi-species between seroconverters and non-seroconverters, indicating that it might be a marker preceding seroconversion in HBV/genotype C-infected patients as reported previously [35–37].

HBeAg seroconversion is an incomplete marker of immune control, although most patients experience some clinical benefit from it [38,39]. Previous studies have shown that the average rate of spontaneous HBeAg seroconversion in patients with chronic hepatitis B is about 10% per year [40,41]. HBeAg seroconversion associated with incomplete viral suppression may result in the emergence of the *precore* mutant and attendant chronic sequelae. Mutations in the *precore* and *core* promoter regions of the HBV genome have been reported in many HBeAg-negative CHB patients. Longitudinal studies found that the A1896 mutation emerges or is selected around the time of HBeAg seroconversion, and high *precore* mutant ratios have been associated with persistent hepatitis after anti-HBe seroconversion [42]. Patients who continued to have high HBV DNA titres after HBe seroconversion had a lower genetic heterogeneity but more often had the *precore* mutant.

The limitations of this study were, the small size of study group, only 10 clones per sample, and a small region for analysis of genetic distance. In addition, the *X/precore/core* region is a highly conserved region, investigation of another region of the HBV genome, such as the polymerase, might help us to better understand the evolution of quasi-species of HBV.

In conclusion, the distinctly greater viral diversity after seroconversion in HBeAg seroconverters could be related to increased HBV-specific T-cell responses and escape mutants which arise from selective pressure caused by host immune activity. Long-term follow-up is required to determine whether hepatitis B viral diversity decreases or remains at a high level. Further study will be needed to elucidate the relationship between seroconversion and viral quasi-species in relation to antiviral therapy.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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ORIGINAL ARTICLE

Initial Virological Response and Viral Mutation with Adefovir Dipivoxil Added to Ongoing Lamivudine Therapy in Lamivudine-Resistant Chronic Hepatitis B

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Abstract
Background Although adefovir dipivoxil (ADV) has been used for antiviral treatment of lamivudine (LAM)-resistant chronic hepatitis B (CHB) patients, the long-term efficacy of this treatment is not well understood. Initial virological response (IVR) has been reported to be an important factor in relation to the development of ADV-resistance.
Aims We therefore examined the factors associated with IVR and ADV mutation in these patients.
Methods Forty-nine LAM-resistant CHB patients with ADV add-on LAM therapy, 47% of whom were hepatitis B e-antigen (HBeAg)-positive with median treatment duration of 23 months, were enrolled in this study. Patients were classified into IVR and non-IVR groups on the basis of viral suppression status. Mutational analysis of the HBV polymerase/reverse transcriptase (rt) domain was performed by PCR-direct sequencing.
Results Serum HBV DNA was undetectable (<2.6 $\log_{10}$ copies/mL) in 67, 82, and 84% of patients at 24, 48, and 96 weeks, respectively, after ADV add-on LAM therapy. IVR was achieved in 82% of patients, and ALT normalized at week 24 in 90% of IVR and 78% of non-IVR patients. The lower pretreatment HBV DNA level and virus-containing mutations other than double mutation of rtL180M + rtM204V were significantly associated with IVR ($P = 0.002$ and $P = 0.014$, respectively). ADV-resistant mutations in the RT motif, reported previously, were not detected.
Conclusion IVR is useful for predicting the antiviral efficacy of ADV and LAM combination therapy in LAM-resistant CHB.

Keywords Chronic hepatitis B · Adefovir dipivoxil · Lamivudine · Initial virological response · Mutation

Abbreviations

ADV	Adefovir dipivoxil
ALT	Alanine aminotransferase
CHB	Chronic hepatitis B
HBV	Hepatitis B virus
IVR	Initial virological response
LAM	Lamivudine
rt	Reverse transcriptase

Introduction

Because of the frequent development of life-threatening sequelae, for example liver cirrhosis and hepatocellular carcinoma (HCC), chronic hepatitis B (CHB) infection is a major public health problem worldwide, affecting over 350 million people [1], especially in Asia and Africa [2–4]. The levels of circulating hepatitis B virus (HBV) DNA reflect the status of HBV replication in the liver and are thought to be related to future incidence of cirrhosis, HCC [2, 5–8], and HCC-related mortality [9]. Therefore, complete and sustained suppression of viral replication is the most important objective of treatment of chronic HBV infection. Long-term administration of nucleos(t)ide analogues may

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prevent these complications. Lamivudine (LAM) has been used as first-choice therapy for CHB patients, regardless of HBeAg status, because of its potency, safety profile, and relatively low cost [10]. However, the efficacy of long-term therapy with LAM is compromised by viral resistance; the annualized incidence rate of LAM-resistant mutations was 22% [11] and reached 71% in year 4 [12].

Adefovir dipivoxil, an oral pro-drug of adefovir (ADV), is a synthetic adenine nucleotide analogue that has been shown to be effective in suppression of HBV DNA, HBeAg seroconversion, alanine aminotransferase (ALT) normalization, and histological improvement, regardless of HBeAg status [13–15]. The drug has been shown to have antiviral activity against not only wild-type HBV [13, 14] but also LAM-resistant HBV mutants both in vitro and in vivo [16, 17]. In contrast with LAM therapy, the benefit of ADV therapy is the delayed and infrequent selection of drug-resistant viruses [14, 18–20]. The cumulative incidence of an ADV-resistant mutation emerging in nucleos(t)ide treatment-naïve CHB patients at 48, 96, 144, 192, and 240 weeks was 0, 0.8–3, 11, 18%, and up to 29%, respectively [13, 21–25].

The antiviral activity of ADV has been reported to be lower in LAM-resistant CHB patients than in treatment-naïve patients [26–28]. However, the factors associated with antiviral efficacy of ADV are still not well understood.

We have previously studied the association between lamivudine sensitivity and amino acid substitutions in the reverse transcriptase (RT) region of HBV polymerase and found that sequence analysis of the RT domain is useful for predicting sensitivity to LAM therapy [29].

In this study we assessed the long-term efficacy of ADV add-on therapy for CHB patients with LAM-resistance, analyzed the relationship between amino acid substitution in the RT domain and sensitivity to ADV add-on LAM therapy for LAM-resistant CHB patients, and determined the risk factors associated with the initial virological response (IVR).

Materials and Methods

Patients

CHB patients ($n = 49$) who received 10 mg daily of ADV as add-on therapy to ongoing LAM (100 mg daily) after the emergence of LAM resistance were enrolled at Chiba University Hospital between 2004 and 2009. All patients were negative for hepatitis C, hepatitis D, and human immunodeficiency virus antibodies. Sera obtained from patients at the commencement of ADV add-on LAM therapy were stored at −20°C until analysis. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological Examination

HBsAg, HBeAg, and anti-HBe antibody were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL, USA). HBV genotype was determined from patients' sera by ELISA (HBV Genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan) based on the method described by Usuda et al. [30]. Serum HBV DNA levels were monitored every four weeks using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 log copies/mL.

Viral Genome Sequencing

Pretreatment sera were obtained from 31 patients and nucleotide sequences could be analyzed in 22 patients. Sequence analysis for detection of HBV-DNA mutations in serum samples in the non-IVR group was performed after 24, 48, and 96 weeks of treatment. To amplify the region encompassing the polymerase reverse transcriptase (RT) domain, DNA extracted from 200 μL serum was used as a template and long-range PCR and nested PCR were performed in a 50-μL reaction using LA Taq polymerase (TaKaRa Bio, Kyoto, Japan) under the following conditions: 5-min activation at 94°C, 35 cycles or 30 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 90 s and 1 min in the first and second round, respectively. The last cycle was followed by a final extension at 72°C for 7 min. An 862 base-pair fragment (nt 242-1103) containing the polymerase RT domain was amplified. The primers for the first round of PCR were 5′-CCT CAG GCT CAG GGC ATA-3′ (sense, nt 3082-3099) and 5′-GAC GGG ACG TAG ACA AAG G-3′ (antisense, nt 1436-1418). The primers for the second round of PCR were 5′-CAG AGT CTA GAC TCG TGG-3′ (sense, nt 242-258) and 5′-GGC GAG AAA GTG AAA GCC-3′ (antisense, nt 1103-1086). The PCR product was sequenced using the primers: 5′-TGG CTC AGT TTA CTA GTG CC -3′ (nt 668-687), 5′-GGC ACT AGT AAA CTG AGC CA-3′ (nt 687-668), and the primers for the second round of PCR. The amino acid sequence of each protein was deduced from the nucleotide sequence. The HBV genotype was also confirmed on the basis of the viral sequence data obtained.

Definition of Initial Virological Response and Undetectable HBV DNA

An initial virological response (IVR) was defined as HBV DNA < 4 $\log_{10}$ copies/mL after treatment for 24 weeks [26]. HBV DNA < 2.6 $\log_{10}$ copies/mL was regarded as "serum HBV DNA undetectable".

Statistical Analysis

Categorical variables between groups were compared by use of Fisher's exact test. The Mann–Whitney U test was used for assessing the association between baseline factors and the occurrence of IVR. Results were considered statistically significant at $P < 0.05$.

Results

Clinical and Biochemical Data of the Patients

A total of 49 patients were included in this analysis. Thirty-six (71%) were men, the median age when ADV was added to LAM treatment was 55 years (range: 35–71 years), and 24 patients (47%) were HBeAg-positive. Pretreatment ALT levels ranged from 14 to 1495 IU/L (median: 129 IU/L), and the median pretreatment HBV DNA level was 6.9 $\log_{10}$ copies/mL (range: 2.8–8.8 $\log_{10}$ copies/mL). The median duration of treatment with LAM was 25.5 months (range: 3–78 months). The median duration of combination treatment with ADV and LAM was 29 months (range: 8–63 months) (Table 1).The median duration of treatment with LAM was 26 months (range: 3–78 months) and 23 months (range: 12–50 months) in the IVR and non-IVR groups, respectively (P = N.S.).

Frequency of Undetectable HBV DNA Levels

In all patients, sequential monitoring revealed that 24, 48, and 96 weeks after addition of ADV to ongoing LAM therapy serum HBV DNA levels were undetectable (<2.6 $\log_{10}$ copies/mL) in 67, 82, and 84%, respectively, (Fig. 1a). Among the 24 HBeAg-positive patients, HBeAg seroconversion was observed to be 5 and 16% after 48 and 96 weeks, respectively.

Table 1 Clinical and biochemical data of patients infected with hepatitis B virus

Number of patients	49
Median age, years (range)	55 (35–71)
Male sex, number (%) of patients	36 (71%)
HBeAg positive, number (%) of patients	24 (47%)
Median pretreatment ALT level, IU/L (range)	129 (14–1495)
Median pretreatment HBV DNA level, $\log_{10}$ copies/mL (range)	6.9 (2.8–8.8)
Median duration of LAM therapy, months (range)	26 (3–78)
Median duration of ADV therapy, months (range)	29 (8–63)

ALT, alanine aminotransferase; HBV, hepatitis B virus; ADV, adefovir dipivoxil

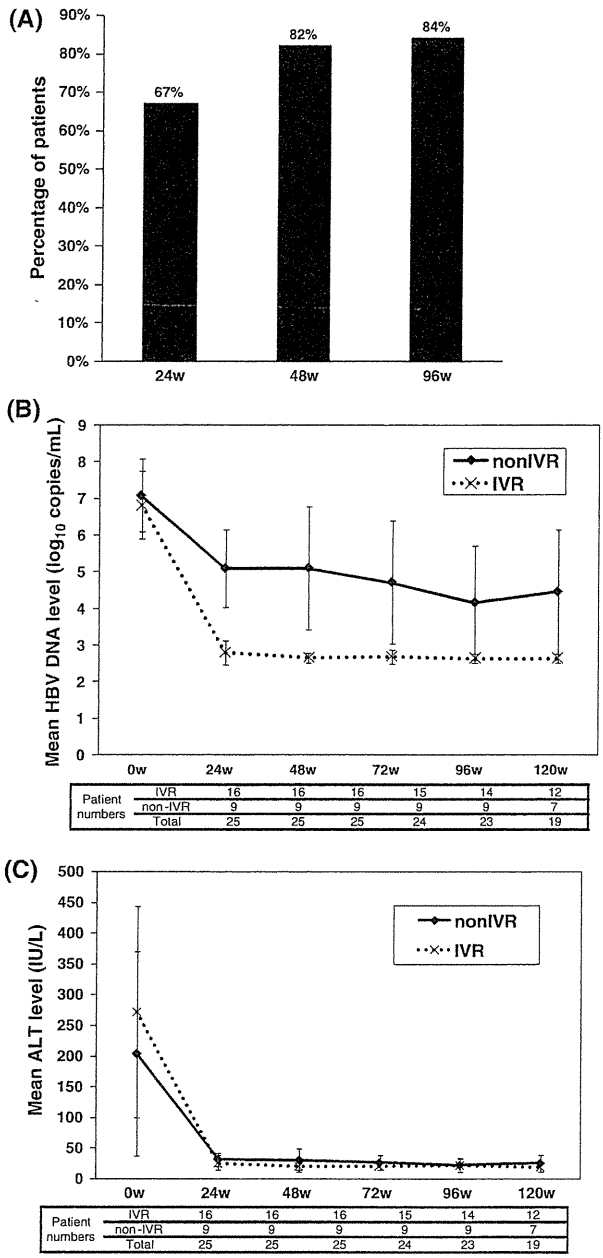

Fig. 1 **a** Percentages of patients with reduction in HBV DNA by <2.6 $\log_{10}$ copies/mL 24, 48, and 96 weeks after ADV add-on LAM. **b**, **c** Sequential HBV DNA and ALT levels according to initial virological response (IVR). Patients who achieved IVR are represented by the *dashed line*; those who did not are represented by the *solid line*. Data represent mean ± SD. An HBV DNA level below 2.6 $\log_{10}$ copies/mL was regarded as being approximately equal to 2.6 $\log_{10}$ copies/mL. The numbers of IVR and non-IVR patients, and total patient numbers, at each time point are also shown in the table

Comparison of Characteristics Between the IVR and Non-IVR Groups

According to the IVR definition, patients were classified into two groups, an IVR group and a non-IVR group, for further analyses (Table 2). IVR was achieved in 82% of the 49 patients. As expected, patients who achieved IVR had a more marked drop in HBV DNA levels during the first 24 weeks and this reduction lasted throughout the follow-up period (Fig. 1b). The rates of ALT normalization at week 24 were 90 and 78% in the IVR and non-IVR groups, respectively (Fig. 1c).

Comparison of patient characteristics in the IVR and non-IVR groups showed that the HBV DNA level at baseline was lower in the IVR group than in the non-IVR group ($P = 0.002$). The non-IVR group had a high percentage of HBeAg-positive patients at baseline compared with the IVR group (78% vs. 42%, $P = 0.054$). More women than men achieved IVR (Table 2, $P = 0.036$). There were no significant differences between the baseline characteristics age, body mass index, or baseline serum ALT levels of the two groups. Genotype was determined in 27 patients. There were 25 with genotype C and 2 with genotype A; genotype C was detected in 16/18 of the IVR group and in 9/9 of the non-IVR group (P = N.S.).

Amino Acid Sequences of RT Motif Domains Between the IVR and Non-IVR Groups

The polymerase RT domains were sequenced to investigate the relationship with sensitivity to ADV add-on LAM therapy. We compared the deduced amino acid sequences of A, B, C, D, and E domains of the RT motif between the two groups (13 and 9 patients in the IVR and non-IVR groups, respectively), but found neither ADV-resistant mutations nor any significant differences, except for the substitutions at rtL180 and rtM204. Double mutation (rtL180M + M204V/I) or single mutation (rtM204I, rtM204V) was observed among the patients studied (Fig. 2). However, the HBV-containing double mutant (rtL180M + M204V) was observed more frequently in the non-IVR group than in the IVR group (78% vs. 28%, $P = 0.014$).

Characteristics of Patients with Lasting High HBV DNA Levels

Most of the patients achieved undetectable levels of HBV DNA after 48 weeks of ADV add-on LAM therapy, but six patients in the non-IVR group were found to have sustained high HBV DNA levels (>5 $\log_{10}$ copies/mL) beyond 48 weeks of ADV add-on LAM treatment. Two of these patients (n-IVR 5 and n-IVR 9) developed virological breakthrough (VBT, elevation of >1 $\log_{10}$ copies/mL from nadir). VBT occurred at weeks 68 and 48 in patients n-IVR 5 and n-IVR 9, respectively. To investigate the additional amino acid substitution in the RT domain during treatment of these patients we analyzed the amino acid sequences at several time points during combination therapy. Four cases were found to be infected with HBV carrying the rtL180M + M204V double mutation at the commencement of ADV add-on LAM therapy (Table 3). Sequential analysis of RT mutations of the four patients is shown in Fig. 3. Because no additional amino acid substitutions, including ADV-resistant mutations, were detected in any samples tested, an alternate mechanism is likely to be responsible for the insufficient response of these patients to therapy.

Discussions

In LAM therapy for patients with chronic HBV infection, emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. For this reason, LAM has been replaced

Table 2 Comparison of patient characteristics between IVR and non-IVR groups at enrollment

	IVR group ($n = 40$)	Non-IVR group ($n = 9$)	P value
Median age, years (range)	54 (35–71)	50 (38–67)	0.588[b]
Male/female	26/14	9/0	0.036[a]
Median body mass index, kg/m^2 (range)	22.0 (17.5–27.9)	22.8 (19.4–25.2)	0.795[b]
HBeAg positive rates	42%	78%	0.054[a]
Median ALT level, IU/L (range)	117 (14–1495)	199 (35–710)	0.439[b]
Median HBV DNA level, $\log_{10}$ copies/mL (range)	6.8 (2.8–8.7)	8.0 (7.1–8.8)	0.002[b]
rtL180M + M204V	28%	78%	0.014[a]

ALT, alanine aminotransferase

[a] Fisher's exact test

[b] Mann–Whitney U test

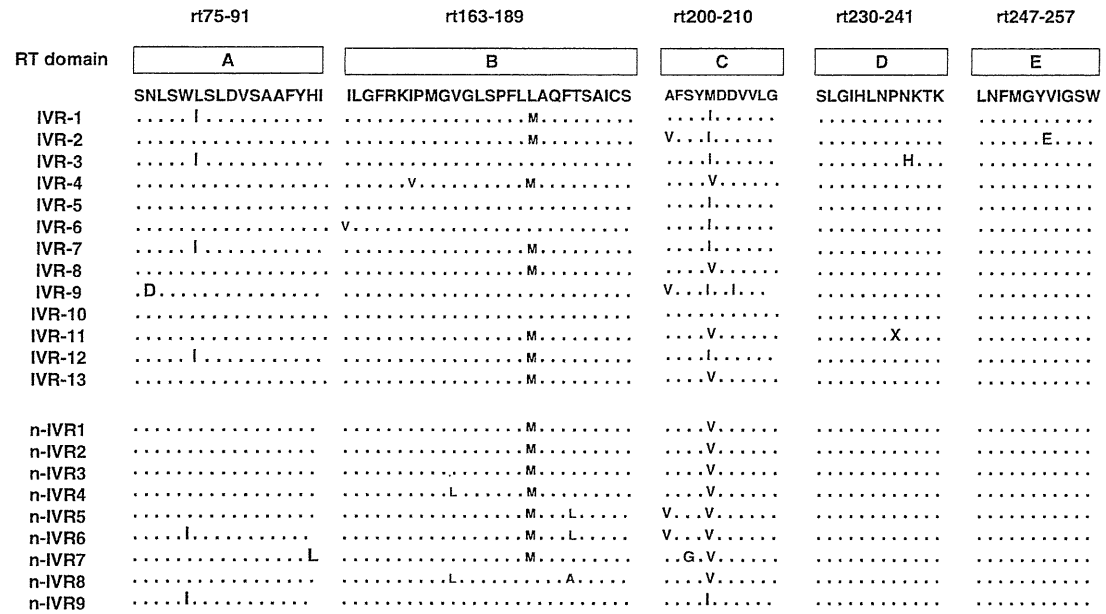

Fig. 2 Amino acid sequences of A, B, C, D, E domains of the RT motif are shown for the initial virological response (IVR) group and the non-IVR group. Double mutation of rtL180M + rtM204V is predominant in the non-IVR group compared with the IVR group (78% vs. 28%, $P = 0.014$)

Table 3 Pretreatment characteristics of patients with sustained elevation of HBV DNA levels after 48 weeks of ADV treatment

	n-IVR 1	n-IVR 3	n-IVR 5	n-IVR 7	n-IVR 8	n-IVR 9
Age (years)	61	41	44	42	51	64
Gender	Male	Male	Male	Male	Male	Male
HBeAg	Positive	Positive	Positive	Negative	Positive	Positive
HBV-DNA level (log_{10} copies/mL)	8	7.5	7.3	7.1	8	8
ALT level (IU/L)	358	389	35	416	85	56
Mutation in the RT region	rt180M + rtM204V	rt180M + rtM204V	rt180M + rtM204V	rt180M + rtM204V	rtM204V	rtM204I
Virological breakthrough	Negative	Negative	Positive	Negative	Negative	Positive

RT, reverse transcriptase

by newly developed nucleos(t)ide analogues, for example ADV and entecavir (ETV), for treatment of chronic hepatitis B. ETV has been reported to be more effective at reducing HBV DNA, and induces the drug-resistant mutant virus less frequently than LAM in nucleos(t)ide-naïve patients [24, 31].

The IVR, which was recently defined as HBV DNA < 4 log_{10} copies/mL after 24 weeks on treatment [26], was reported to be associated with the antiviral efficacy of ADV and the emergence of an ADV-resistant mutation in LAM-resistant CHB [32–34]. Several previous studies have suggested that lower pretreatment HBV DNA levels, higher pretreatment ALT, HBeAg negativity, and the presence of liver cirrhosis were associated with the virological response. In agreement with a previous report [33], this study showed that patients without IVR exhibited higher baseline HBV DNA levels than patients with IVR (8.0% vs. 6.8%, $P = 0.002$). Other studies have identified HBV virological rebounds during LAM or ADV treatment in the absence of mutation associated with drug resistance [22, 35]. The possibility of patient dosing adherence may be one of the factors leading to non-IVR.

The analyses of the amino acid sequence of the RT motif at the commencement of ADV add-on therapy revealed that it was difficult to achieve optimum viral suppression in patients who were infected with the virus carrying the rtL180M + M204V double mutation compared with other mutational patterns, for example the rtL180M + rtM204I double mutation, or rtM204V and rtM204I single mutations. Because the number of samples

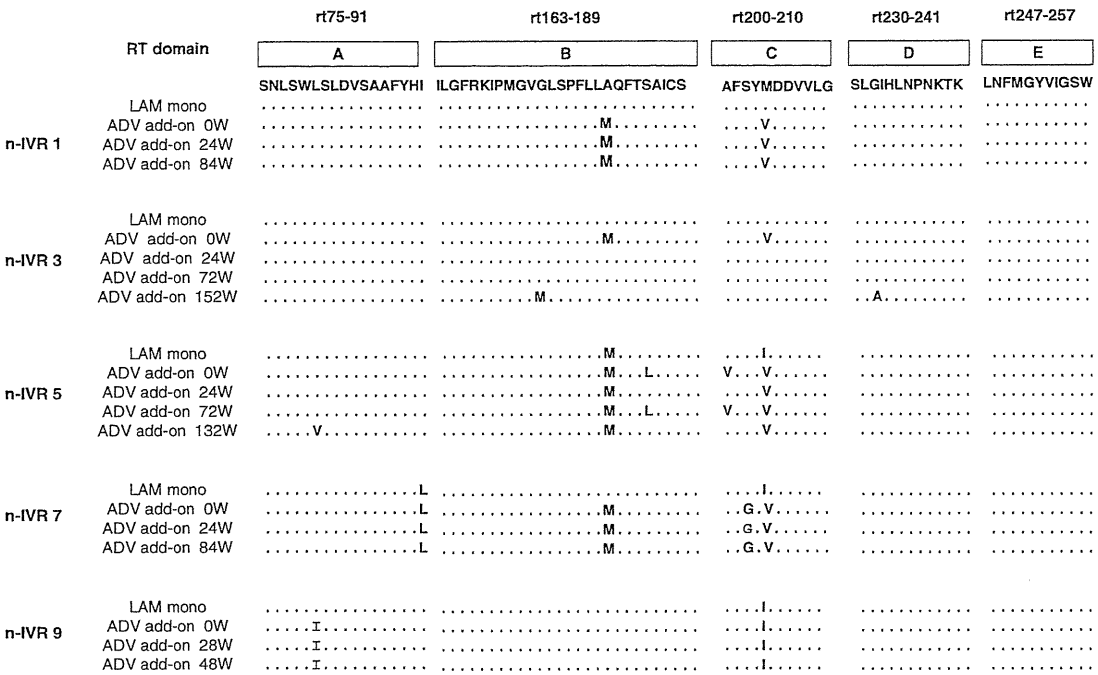

Fig. 3 Amino acid sequences of five representative patients in the non-IVR group are shown. Emergence of the rtL180M + M204V double mutation was observed in four of five patients from the commencement of ADV add-on LAM combination therapy

detected containing these mutations was small, and the alleged association was negative, further study will be needed to confirm this result.

Suzuki et al. [36] reported that the rtM204I mutant was associated with an earlier virological response as compared with the rtM204V mutant, and virological suppression of the mutation rtL180M was linked to that of rtM204I or rtM204V [36]. Furthermore, Suzuki et al. [36] showed that when viral loads of both mutants (rtM204V and rtM204I) were similar at the commencement of ADV therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. In our study, six patients in the non-IVR group had sustained elevation of HBV DNA levels (>5 $\log_{10}$ copies/mL), yet endured ADV add-on LAM co-administration for more than 48 weeks, and four of the six patients had mutant virus carrying the rtL180M + M204V double mutation (Table 3).

Cha et al. [37] assessed the patterns of LAM-resistant mutations and the effect of such mutations on virological response to ADV monotherapy in LAM-resistant CHB. They established the mutational patterns, for example rtM204V ± rtL180M ± rtV173L, rtM204I ± rtL180M, rtM204I ± rtL80I, compared the IVR status with these mutations, and found that the antiviral effect of ADV did not differ significantly among these patterns. Lada et al. [38] studied the susceptibility of LAM-resistant HBV to ADV in vitro. They reported that in samples with triple LAM resistance-associated amino acid changes rtV173L + L180M + M204V, HBV DNA reduction at week 48 was lower than for samples which had only the rtL180M + M204V mutations. In our study, rtV173L was observed only in the non-IVR group but the incidence did not differ significantly between groups. Our results are partially discordant with these previous studies, and differences between the studies, for example the additional mutations and use of ADV monotherapy, may be a possible explanation for the different outcomes.

In a randomized controlled study of ADV therapy in 42 patients who had genotypic LAM resistance with virological and clinical breakthrough, Rapti et al. [39] found that ADV resistance was not detected in the 28 patients undergoing ADV add-on LAM combination therapy but was detected in three patients (21%) upon viral/biochemical breakthrough after switching to ADV monotherapy. In our study, most of the patients treated with ADV add-on LAM therapy exhibited sustained viral suppression, except for two patients who had emergent virological breakthroughs. The sequencing analyses, however, demonstrated no ADV-resistant mutations (rtN236T, rtA181V/T, and rtI233V), suggesting the other mechanisms, for example viral mutation in the remaining part of the sequences or host factors, may be responsible for the reduced efficacy of the combination therapy in these two patients.

In conclusion, ADV add-on LAM therapy for LAM-resistant CHB patients was effective in suppressing viral replication and normalizing ALT levels. However, in cases

with high pre-treatment HBV DNA levels and the rtL180M + rtM204V double mutation, the antiviral effect of ADV is likely to be weak. Careful monitoring for the emergence of ADV-resistant mutation during prolonged treatment is critical.

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Hepatitis B Virus e Antigen Downregulates Cytokine Production in Human Hepatoma Cell Lines

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Abstract

Disease activities of hepatitis B are affected by the status of hepatitis B e antigen (HBeAg). The function of the hepatitis B virus (HBV) precore or HBeAg is unknown. We assumed that HBeAg blocks aberrant immune responses, although HBeAg is not required for viral assembly, infection, or replication. We examined the interaction of HBeAg and the immune system, including cytokine production. The inflammatory cytokine TNF, IL-6, IL-8, IL-12A, IFN-α1, and IFN-β mRNA were downregulated in HBeAg-positive HepG2, which stably expresses HBeAg, compared to HBeAg-negative HepG2 cells. The results of real-time RT-PCR-based cytokine-related gene arrays showed the downregulation of cytokine and IFN production. We also observed inhibition of the activation of NF-κB- and IFN-β-promoter in HBeAg-positive HepG2, as well as inhibition of IFN and IL-6 production in HBeAg-positive HepG2 cell culture fluids. HBeAg might modify disease progression by inhibiting inflammatory cytokine and IFN gene expression, while simultaneously suppressing NF-κB-signaling- and IFN-β-promoter activation.

Introduction

More than 2 billion people have been exposed to hepatitis B virus (HBV), and 350 million remain chronically infected worldwide. HBV is a noncytopathic DNA virus with a partially double-stranded 3.2-kb genome. HBV causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (2,6,21,25,37). Viral clearance and its pathogenesis during acute HBV infection require the induction of a vigorous $CD8^+$ T-cell response, and the induction of hepatic immunopathology, including cytokine responses.

The HBV genome consists of four open reading frames coding for the surface, core, polymerase, and X proteins. Viral DNA, upon entry into cells during productive infection, undergoes a repair process and forms covalently closed circular DNA. Transcription of this DNA produces longer (precore) and shorter (pregenomic) 3.5-kb RNAs. The pregenomic RNA is packaged into nucleocapsides along with the viral polymerase, and serves as the template for viral genome replication. Precore and pregenomic RNAs encode core, polymerase (by pregenomic RNA), and hepatitis B e antigen (HBeAg) (by precore RNA) (47).

Disease severity of hepatitis B is affected by the status of HBeAg. The presence of HBeAg in serum is also known to be a marker of a high degree of viral infectivity. Although there are diverse opinions, fulminant hepatitis may occur in persons who are negative for HBeAg in highly endemic areas (29). Infants born to HBeAg-positive mothers tend to be HBsAg-positive more than those born to HBeAg-negative mothers (44). HBeAg-positive asymptomatic carriers (ASCs) have higher viral load, but most do not display any liver dysfunction (10). These clinical cases can be assumed to have immune tolerance for HBeAg.

The core gene of 183 codons (at least for genotypes B and C) is preceded by an in-frame pre-ATG codon that extends the protein by 29 hydrophobic amino acids (Fig. 1A). Proteins like this are translated from a 3.5-kb precore RNA and converted to HBeAg by two proteolytic cleavage events in the secretory pathway (12,26,38). First, the N-terminal 19 residues encoded by the precore region serve as the signal peptide for translocation of the precore/core protein into the endoplasmic reticulum lumen, where the peptide is clipped away by a signal peptidase. Next, 30 residues are removed from the C terminus in a post-endoplasmic reticulum compartment to generate mature HBeAg of ~17 kDa (12). A single point mutation has been reported to produce a stop codon in the precore region of HBV DNA and prevent the formation of the precore protein required to make HBeAg (7). HBeAg is thought to involve immune tolerance via an unknown mechanism, although it is not required for viral

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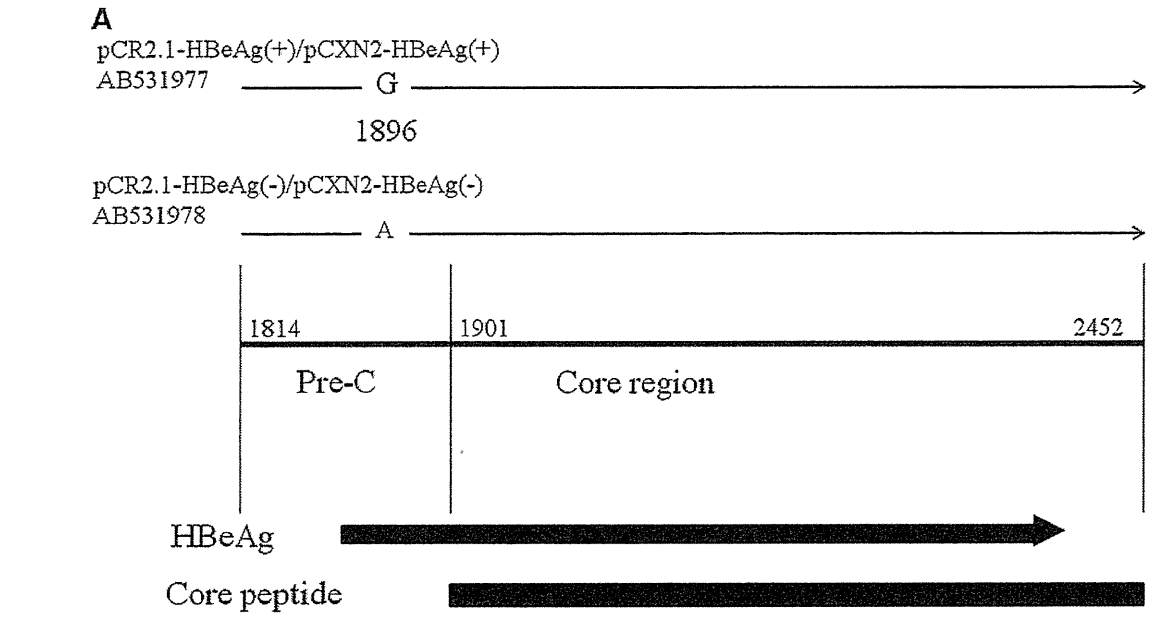

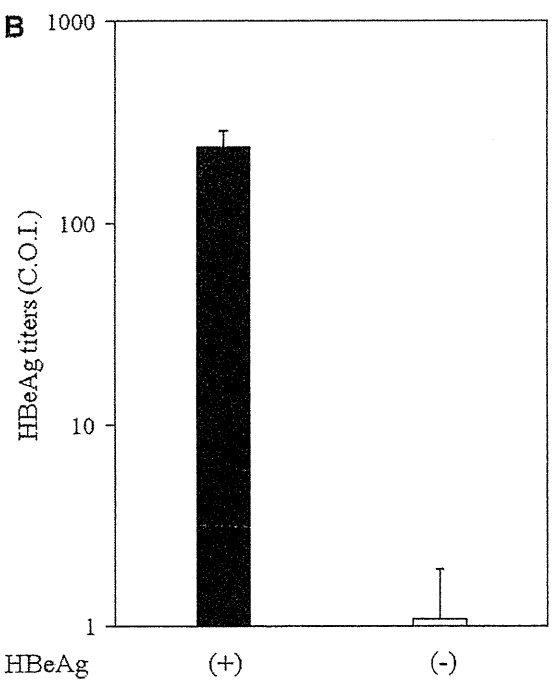

FIG. 1. (**A**) Schematic diagram of the precore and core peptides of HBV genotype C and cDNAs used in this study. Nucleotide A makes a stop codon at 1896, resulting in the absence of HBeAg production. Plasmid pCXN2-HBeAg(+) can produce both HBeAg and core, whereas pCXN2-HBeAg(−) can produce only core peptide. HBeAg and core peptides are indicated by the bold arrow and bold line, respectively. (**B**) Titers of HBeAg in cell culture fluid from HBeAg-positive and HBeAg-negative HepG2 cells were measured by the CLEIA method. The cut-off index (C.O.I.) is shown. The titer of HepG2 control cells is ~0.4 C.O.I.

assembly, infection, or replication (3,42). Visvanathan *et al.* (43) reported that the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes, was significantly reduced in HBeAg-positive chronic hepatitis B patients. Although the precise function of HBV precore or HBeAg is unknown, it is possible that HBeAg suppresses the TLR pathways, thereby allowing HBV to establish persistent infection in the host (43).

Toll-like receptors (TLRs) play important roles in the innate immune response and are thought to have therapeutic potential for infectious diseases and cancers (18). Some of them are expressed on many different cells, including hepatocytes (32,36). Preiss *et al.* (32) demonstrated mRNA transcription for most TLRs, with the exception of TLR8. TLR5 mRNA was not detectable in HepG2 cells. Hepatocytes may themselves play an active role in innate immune responses to viruses such as HBV (32). Once these pattern recognition receptors (PRRs) have identified the pathogen-associated molecular patterns (PAMPs), the effector cells function and respond immediately. Ligand recognition by TLRs leads to the recruitment of various TIR domain-containing adaptors, such as myeloid differentiation primary

response gene (88) (MyD88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), TIR domain-containing adapter inducing interferon-β (TRIF), and TRIF-related adapter molecule (TRAM), which in turn triggers the cascade of the signaling pathway, and ultimately the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs), leading to the expression of various cytokines (e.g., tumor necrosis factor [TNF], interleukin-6 [IL-6], IL-8, interferon-α1 [IFN-α1], and IFN-β). Hepatic cytokines also play an important role in the progression of hepatitis B-associated liver diseases. A number of viruses have been shown to encode proteins that have the potential to inhibit antiviral activity of the innate and adaptive immune responses. Inflammatory cytokines contributing to viral clearance in HBV infection may have therapeutic value (20). In the present study, we assumed that HBeAg blocks aberrant immune responses, and we examined the role of HBeAg protein in cytokine production to test the interaction between HBeAg and the immune system in human hepatocytes. Our results demonstrated that cytokine production is inhibited by HBeAg, and that it also enhances IFN-sensitive hepatitis C virus (HCV) replication.

Materials and Methods

Plasmids

pNF-κB-luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene (La Jolla, CA). This vector has five repeats of the binding site for NF-κB (TGGGGACTTTCCGC). pIFN-β-luc, which expresses luciferase under the control of an IFN-β-dependent promoter, was kindly provided by Dr. N. Kato (Institute of Medical Science, University of Tokyo, Japan). To construct plasmids including HBV precore and core regions, HBV DNA was used from the serum of a genotype C HBeAg-positive asymptomatic carrier (ASC) patient as previously described (10). The DNA sequence information from this study will appear at GenBank (accession numbers AB531977 and AB531978). To make pCR2.1-HBeAg(+), the PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Using the Quickchange II site-directed mutagenesis kit (Stratagene), precore stop codon mutant G1896A was induced into pCR2.1-HBeAg(+) to pCR2.1-HBeAg(−) according to the manufacturer's instructions. To obtain the mammalian cell expression vectors, we performed subcloning using the EcoRI site of pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Osaka, Japan), a mammalian expression vector with a β-actin-based CAG promoter and SV40 origin (28). The constructs pCXN2-HBeAg(+) and pCXN2-HBeAg(−) were generated by this method (Fig. 1A). All sequences of these plasmids were confirmed using Big Dye Terminator on a 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Cell culture

Human hepatoma cells, HepG2 and Huh7 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Approximately 1×10^5 HepG2 cells were placed on 35-mm tissue culture dishes (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection (13). The cells were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 was added at 1000 μg/mL for the selection of stable cell lines, and HBeAg-positive and HBeAg-negative HepG2 cells were designated. After 3 wk, to avoid monoclonal selection, all cells were collected for further analysis.

RNA extraction, cDNA synthesis, and real-time PCR

The cells were seeded into 6-well plates, and total cellular RNA was extracted 48 h later using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were then stored at −80°C until use. RNA quality was examined using the A_{280}/A_{260} ratio (Pharmacia Biotech, Bedford, MA). cDNA synthesis was performed using a random hexamer. For RNA quantitation, real-time PCR was conducted using SyBr Green I (ABI PRISM 7300; Applied Biosystems). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization, and data were analyzed by the comparative threshold cycle (C_T) method (16). The primers used are shown in Table 1.

Real-time PCR arrays

Gene expression profiling for TLR target genes was performed using RT^2 profiler PCR arrays (SuperArray, Frederick, MD) according to the manufacturer's instructions. In brief, 1 μg RNA was reverse-transcribed with the RT^2 profiler PCR array first-strand synthesis assay (SuperArray), followed by

TABLE 1. PRIMERS USED FOR QUANTITATIVE REAL-TIME PCR

Gene name	*Sequences (forward/reverse)*
GAPDH	5′-ACCCACTCCTCCACCTTTG-3′ / 5′-CTCTTGTGCTCTTGCTGGG-3′
TLR7	5′-GGAGGTATTCCCACGAACACC-3′ / 5′-GACCCCAGTGGAATAGGTACAC-3′
TNF	5′-CCAGACCAAGGTCAACCTC-3′ / 5′-CCAGATAGATGGGCTCATACC-3′
IL-6	5′-AAAAGTCCTGATCCAGTTC-3′ / 5′-GAGATGAGTTGTCATGTCC-3′
IL-8	5′-ACATACTCCAAACCTTTCCAC-3′ / 5′-CCAGACAGAGCTCTCTTCC-3′
IL-12A	5′-CCCTTGCACTTCTGAAGAG-3′ / 5′-AGGCAACTCTCATTCTTGG-3′
IFN-α1	5′-GGGATGAGGACCTCCTAGAC-3′ / 5′-GGAGTCCGCATTCATCAGG-3′
IFN-β	5′-GATTCATCTAGCACTGGCTGG-3′ / 5′-CTTCAGGTAATGCAGAATCC-3′
LY96 (MD-2)	5′-ATTTGCCGAGGATCTGATG-3′ / 5′-GGTGTAGGATGACAAACTCC-3′
RIPK2	5′-AGACACTACTGACATCCAAG-3′ / 5′-CACAAGTATTTCCGGGTAAG-3′
NF-κB1	5′-GAAGAAAATGGTGGAGTCTG-3′ / 5′-GGTTCACTAGTTTCCAAGTC-3′
MAP3K1	5′-CCACTGCATGTCAATTTGGG-3′ / 5′-CGTGGCTGTAGAAATCATGAG-3′
HCV	5′-TCTGCGGAACCGGTGAGTA-3′ / 5′-TCAGGCAGTACCACAAGGC-3′

real-time PCR with RT^2 real-time PCR master mix SyBr green (SuperArray). Gene expression was normalized to two internal controls (GAPDH and β-actin), to determine the fold change in gene expression between the test sample (HBeAg-positive HepG2) and the control sample (HBeAg-negative HepG2) by the 2^{-ddCT} (comparative cycle threshold) method (17). Data were analyzed with RT^2 Profiler™ PCR Array Data Analysis software (http://www.superarray.com/pcrarraydataanalysis.php). Genes with more than twofold change were also confirmed by real-time RT-PCR in at least triplicate. For this we used GAPDH for normalization.

Transfection and reporter assay

Approximately 1×10^5 cells were placed on 6-well plates (Iwaki Glass) 24 h prior to transfection. Cells were transfected with 0.4 μg of plasmid pIFN-β-luc or pNF-κB-luc in Effectene (Qiagen). For luciferase assay of NF-κB activation, cells were treated for 4 h with 0.5 or 5 ng/mL TNF-α, 10 or 50 μg/mL TLR4 ligand:lipopolysaccharide (LPS), or none at 44 h post-transfection (22,31,34,35,40). For IFN-β promoter assay, 50 μg/mL TLR3 ligand:poly(I-C), or none was added to cell culture fluid at 32 h post-transfection (16). At 48 h post-transfection, the cells were lysed with reporter lysis buffer (Promega, Madison, WI), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO Bio Instruments, Tokyo, Japan) as previously described (16). Relative luciferase activity was measured at 48 h post-transfection and compared with that of an untreated control. Relative luciferase activity of HBeAg-negative cells was set as 1.

Chemiluminescent enzyme immunoassay

The supernatants of these cell lines were used for measuring the levels of HBeAg by the chemiluminescent enzyme immunoassay (CLEIA) system (Fujirebio Inc., Tokyo, Japan).

ELISA

Cell culture fluid was analyzed for IL-6 by enzyme-linked immunosorbent assay (ELISA; KOMA Biotech Inc., Seoul, Korea) following the manufacturer's protocol. Briefly, cell culture fluid samples were incubated in plates at 4°C overnight, followed by incubation with biotinylated monoclonal antibodies. Avidin-conjugated peroxidase was added to the plates, and enzyme activity was detected with an ELISA plate reader.

MTS assay

MTS assays were performed with the CellTiter 96 AQ One Solution Cell Proliferation Assay (Promega) (15). Twenty microliters/well of the MTS reagent was added to 100 μL of media containing cells in each well of 96-well plates, and left for 4 h at 37°C in a humidified 5% CO_2 atmosphere. For analysis, absorbance at 490 nm was measured using a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA).

Antiviral assay using HCV subgenomic replicon

Huh7 cells harboring HCV genotype 1b subgenomic replicon, termed C13-3 cells, were used for antiviral bioassay (14). Intracellular HCV subgenomic RNA was measured by real-time RT-PCR. C13-3 cells were incubated in cell culture supernatant from HBeAg-positive, HBeAg-negative HepG2, or control HepG2 cells for 24–48 h. Post-incubation, RNA was extracted and stored at −80°C until analysis.

Statistical analysis

Results were expressed as mean ± SD. Student's *t*-test was used to determine statistical significance.

Results

Detection of stable expression of HBeAg by CLEIA

First, we examined the HBeAg production in cell culture fluid in HepG2 stably expressing HBV precore and core regions. HBeAg was detected in cell culture supernatants of HBV precore and core region-expressing cells (HBeAg-positive HepG2, 241 ± 47.9 C.O.I.) by CLEIA (cut-off index [C.O.I.]). On the other hand, expression of the core region without precore did not produce HBeAg in cell culture fluid (HBeAg-negative HepG2, 1.1 ± 0.84 C.O.I.) (Fig. 1B). Next, we performed an MTS assay to examine whether HBeAg affected cell proliferation or cell viability in our system. Cell proliferation/viability of HBeAg-positive cells (100 ± 0.87% at 24 h [n = 4]; 98.5 ± 0.7% at 48 h [n = 4]) was not statistically different from that of HBeAg-negative HepG2 (100 ± 0.4% at 24 h [n = 4]; 100 ± 1.21% at 48 h [n = 4]).

HepG2 cells respond to TLR3 ligand, TLR4 ligand, and tumor necrosis factor

Next we examined whether human hepatoma cell lines HepG2 and Huh7 respond to TLR3 ligand, TLR4 ligand, and tumor necrosis factor (TNF). Here we examined the NF-κB- and IFN-signaling pathways in HepG2 and Huh7 cells. To examine whether HepG2 possesses a functional TLR4 pathway, we initially characterized LPS-induced activation of NF-κB in HepG2 and Huh7 by luciferase reporter assay.

TABLE 2. NUCLEAR FACTOR (NF)-κB ACTIVATION FOLLOWING EXPOSURE TO LIPOPOLYSACCHARIDE (LPS), AND FOLLOWING EXPOSURE TO TUMOR NECROSIS FACTOR (TNF)-α, AND INTERFERON (IFN)-β-PROMOTER ACTIVATION FOLLOWING EXPOSURE TO POLY(I-C) BY LUCIFERASE ASSAYS

Ligand	*HepG2 (fold)*	*Huh7 (fold)*
NF-κB activation		
LPS (10 μg/mL)	23.3 ± 3.11**	1.82 ± 0.17*
LPS (50 μg/mL)	56.0 ± 13.6*	3.01 ± 0.69*
TNF-α (0.5 ng/mL)	9.47 ± 1.37**	1.45 ± 0.27
TNF-α (5 ng/mL)	14.4 ± 0.82***	8.59 ± 1.18**
IFN-β-promoter activation		
Poly (I-C) (50 μg/mL)	1.69 ± 0.14*	0.93 ± 0.10

Cells were transfected with 0.4 μg of plasmid pIFN-β-luc or pNF-κB-luc in Effectene (Qiagen). For the luciferase assay of NF-κB activation, cells were treated for 4 h with 0.5 or 5 ng/mL TNF-α, 10 or 50 μg/mL LPS, or none, at 44 h post-transfection (22,31,34,35,40). For the IFN-β promoter assay, 50 μg/mL poly(I-C) or none was added to cell culture fluid at 32 h post-transfection (16). Relative luciferase activity was measured at 48 h post-transfection and compared with that of an untreated control. Results are expressed as mean ± SD.

*$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ in HepG2 or Huh7 induced by each ligand compared with untreated controls.

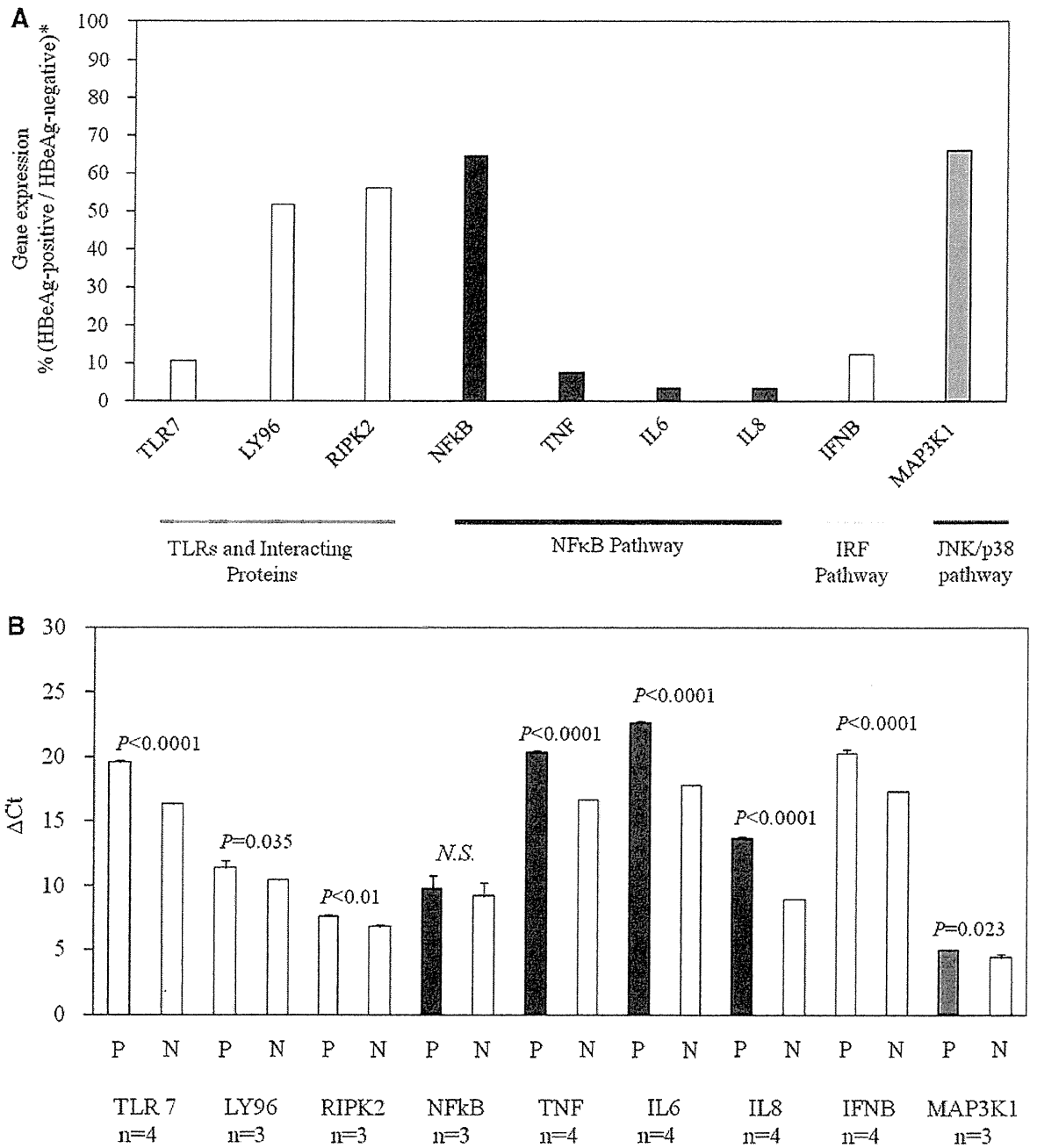

FIG. 2. Effects of HBeAg on toll-like receptor (TLR) signaling-related gene expression (comparison of genes expressed in HBeAg-positive HepG2 with those in HBeAg-negative HepG2). (**A**) TLR target gene expression examined by real-time RT-PCR in at least triplicate. GAPDH was used for normalization. These genes were screened by real-time PCR arrays, as described in the materials and methods section *(value of HBeAg-positive cells/value of HBeAg-negative cells)×100. (**B**) Statistical analysis of TLR signaling-related gene expression in HBeAg-positive and HBeAg-negative HepG2 cellular RNA by real-time RT-PCR by ΔCt. Results are expressed as mean ± SD (N.S., not statistically significant by Student's *t*-test; P, HBeAg-positive HepG2; N, HBeAg-negative HepG2; TLR7, toll-like receptor 7; LY96 [MD-2], lymphocyte antigen 96; RIPK2, receptor-interacting serine-threonine kinase 2; NFκB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 [p105]; IL-6, interleukin-6 [interferon-β2]; IL-8, interleukin-8; IFN-β, interferon-β1; MAP3K1, mitogen-activated protein kinase kinase kinase 1).