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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

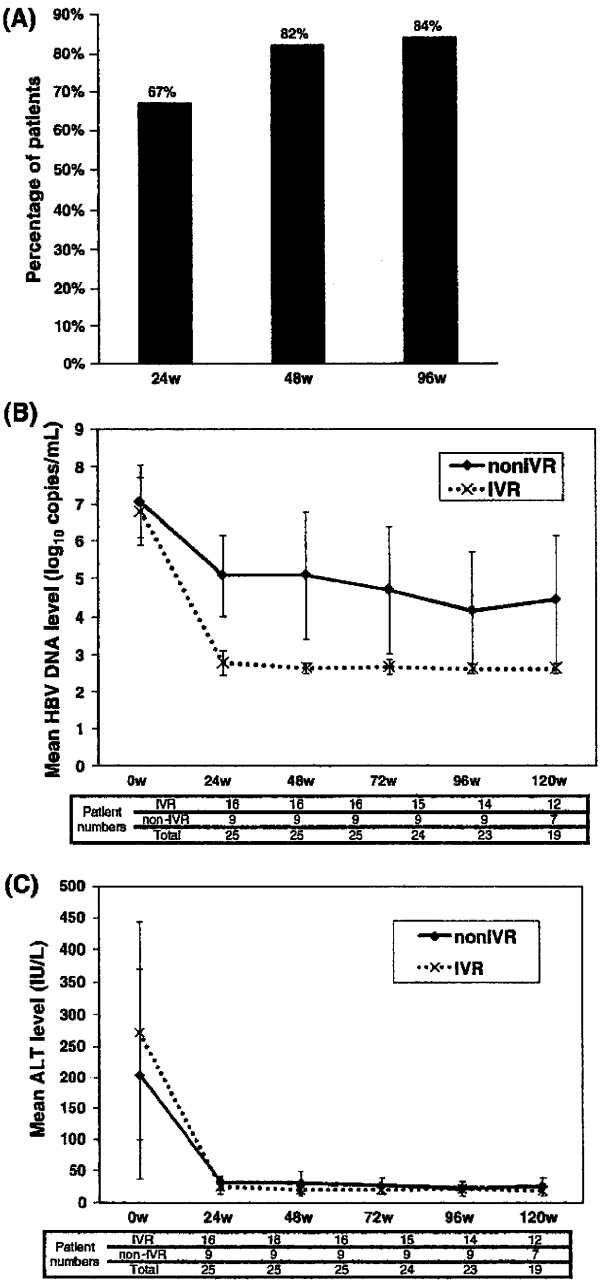

Fig. 1 **a** Percentages of patients with reduction in HBV DNA by <2.6 $\log_{10}$ copies/mL 24, 48, and 96 weeks after ADV add-on LAM. **b, c** Sequential HBV DNA and ALT levels according to initial virological response (IVR). Patients who achieved IVR are represented by the *dashed line*; those who 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

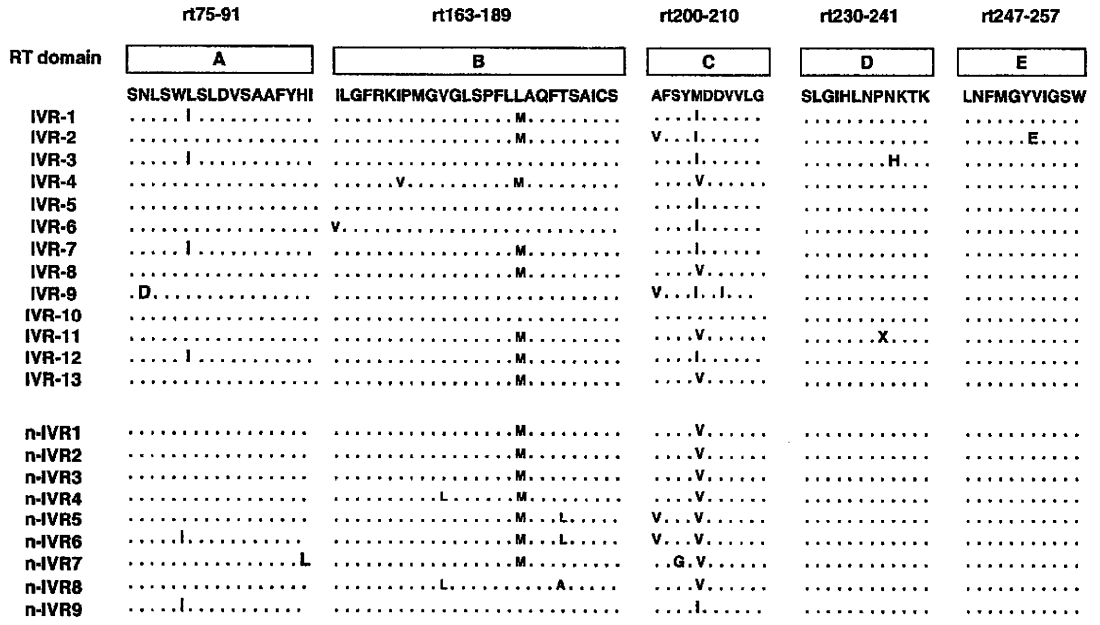

Fig. 2 Amino acid sequences of A, B, C, D, E domains of the RT motif are shown for the initial virological response (IVR) group and the non-IVR group. Double mutation of rtL180M + rtM204V is predominant in the non-IVR group compared with the IVR group (78% vs. 28%, $P = 0.014$)

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

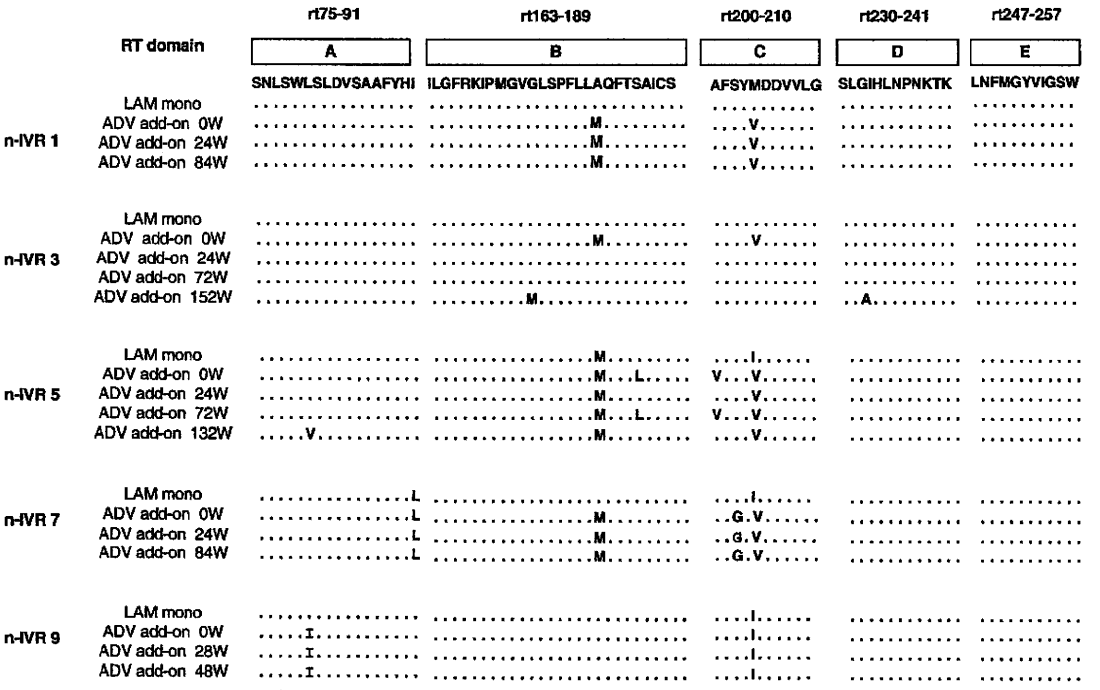

Fig. 3 Amino acid sequences of five representative patients in the non-IVR group are shown. Emergence of the rtL180M + M204V double mutation was observed in four of five patients from the commencement of ADV add-on LAM combination therapy

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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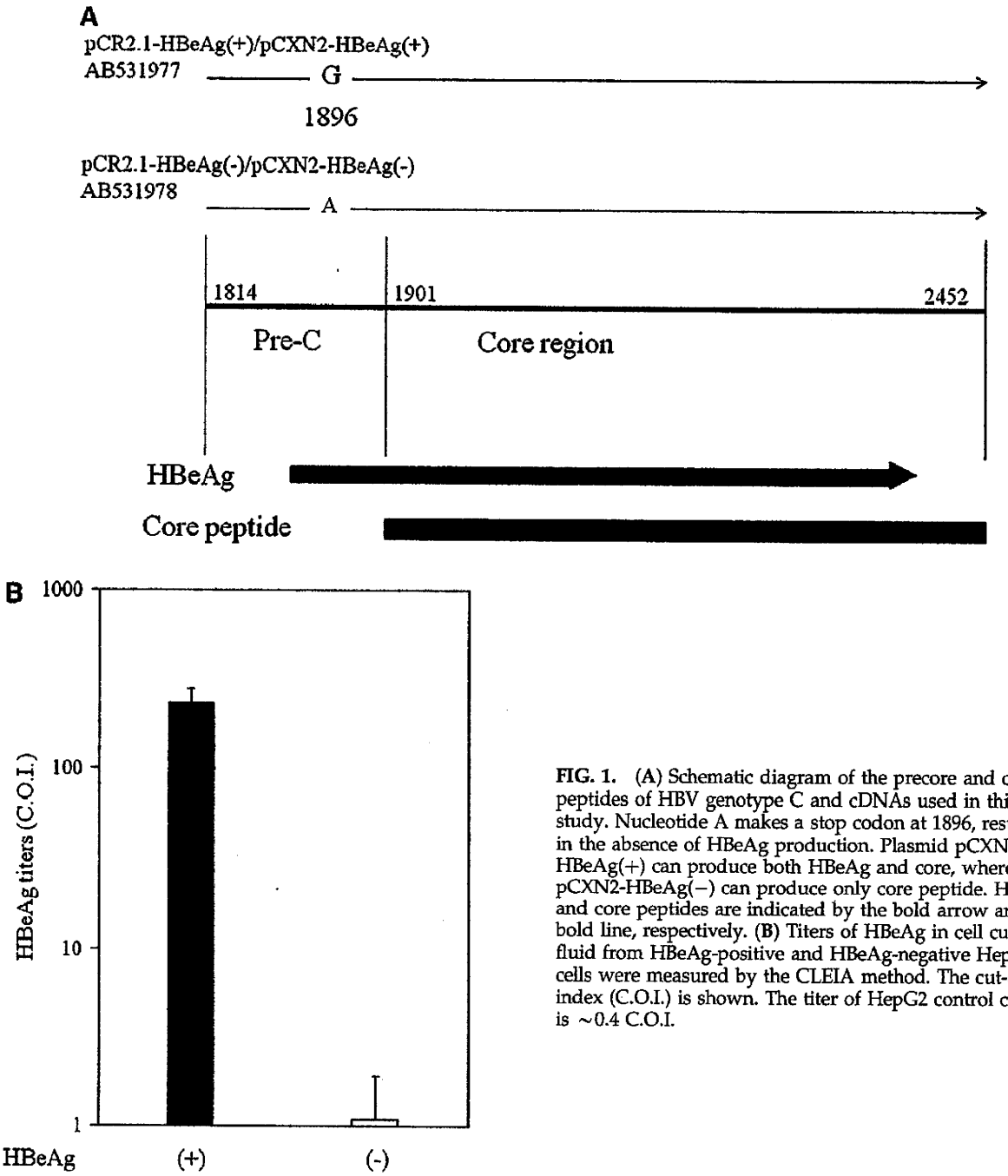

FIG. 1. (A) Schematic diagram of the precore and core peptides of HBV genotype C and cDNAs used in this study. Nucleotide A makes a stop codon at 1896, resulting in the absence of HBeAg production. Plasmid pCXN2-HBeAg(+) can produce both HBeAg and core, whereas pCXN2-HBeAg(−) can produce only core peptide. 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.

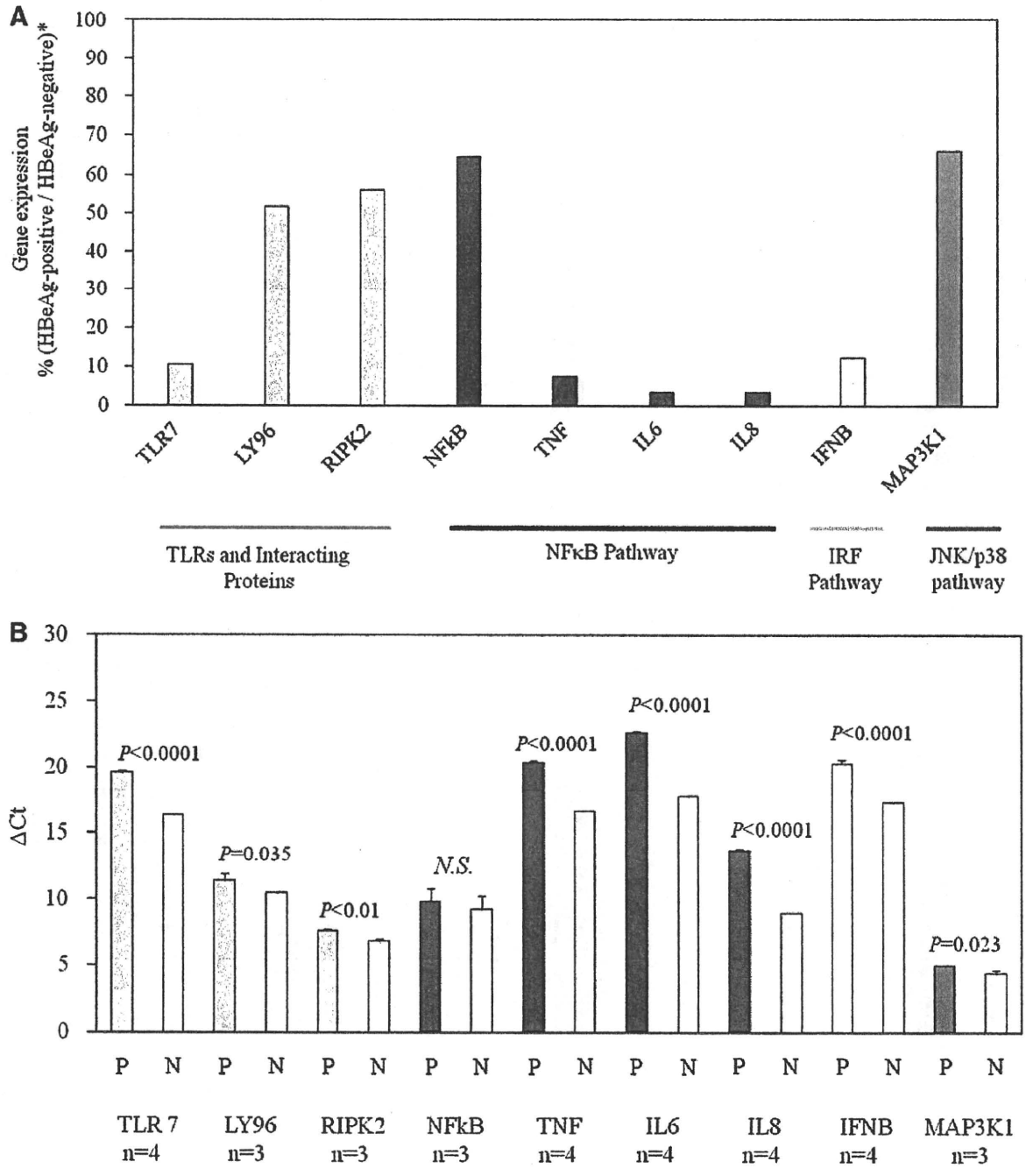

FIG. 2. Effects of HBeAg on toll-like receptor (TLR) signaling-related gene expression (comparison of genes expressed in HBeAg-positive HepG2 with those in HBeAg-negative HepG2). (A) TLR target gene expression examined by real-time RT-PCR in at least triplicate. GAPDH was used for normalization. These 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).

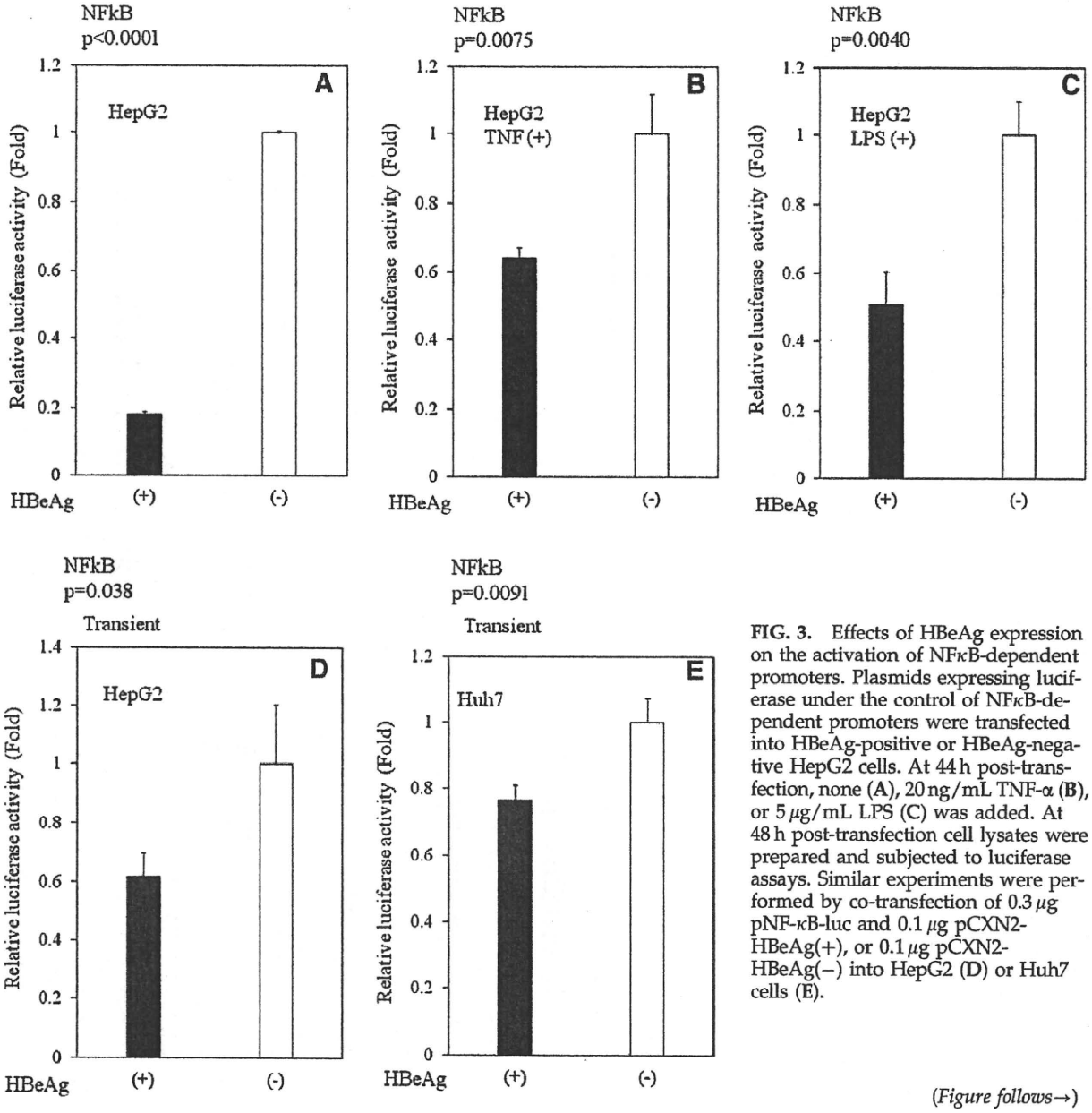

FIG. 3. Effects of HBeAg expression on the activation of NFκB-dependent promoters. Plasmids expressing luciferase under the control of NFκB-dependent promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 44 h post-transfection, none (**A**), 20 ng/mL TNF-α (**B**), or 5 μg/mL LPS (**C**) was added. At 48 h post-transfection cell lysates were prepared and subjected to luciferase assays. Similar experiments were performed by co-transfection of 0.3 μg pNF-κB-luc and 0.1 μg pCXN2-HBeAg(+), or 0.1 μg pCXN2-HBeAg(−) into HepG2 (**D**) or Huh7 cells (**E**).

(*Figure follows→*)

TLR4 plays an important role in the activation of NF-κB following exposure to extracellular LPS. When LPS was added to the cell culture medium of HepG2 and Huh7, approximately 23∼56-fold and 1.8∼3.0-fold activation, respectively, of NF-κB activity were observed (Table 2). Similarly, when TNF-α, another NF-κB activator, was added to the cell culture medium of HepG2 and Huh7, respectively, approximately 9∼14-fold and 1.4∼8.6-fold activation, respectively, of NF-κB activity were observed (Table 2). However, to examine for a functional TLR3 pathway by luciferase reporter assay, when poly(I-C) was added to the cell culture medium of HepG2 and Huh7, respectively, approximately 1.69-fold and 0.93-fold activation, respectively, of IFN-β-promoter activity were observed (Table 2), supporting the view that Huh7 cells are defective in the TLR3 and RIG-I pathway (16,39). Our results suggested that HepG2 possesses functional TLR3 and TLR4 pathways to some extent, but Huh7 does not possess a functional TLR3 pathway.

Downregulation of IFN and cytokine gene expression by HBeAg

Since HBeAg is associated with immune tolerance (3,42), we wanted to determine whether this might be related to HBeAg suppressing the host innate response, including the production of cytokines. To confirm the downregulation of IFN and cytokine genes, we performed real-time RT-PCR

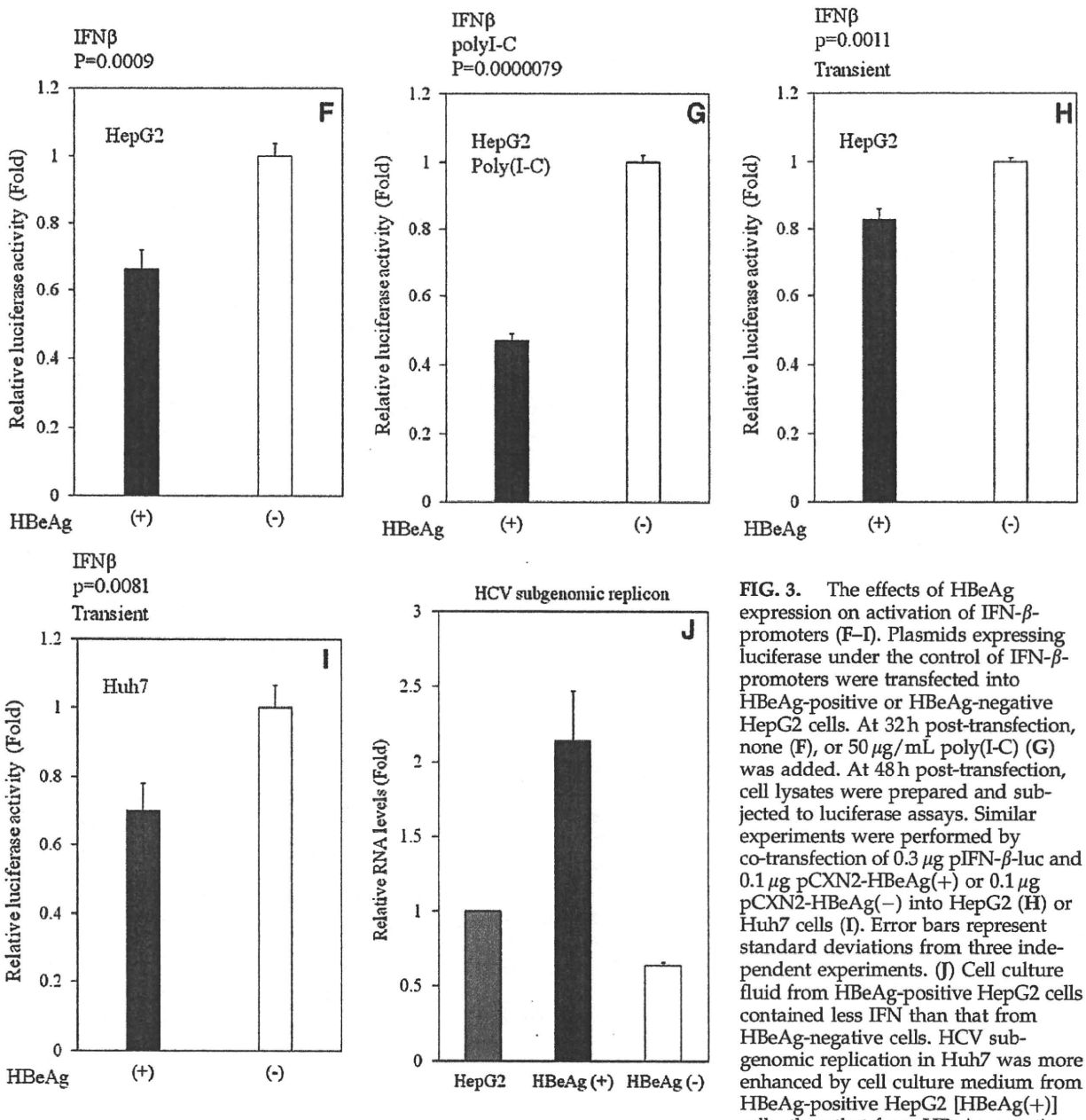

FIG. 3. The effects of HBeAg expression on activation of IFN-β-promoters (F–I). Plasmids expressing luciferase under the control of IFN-β-promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 32 h post-transfection, none (F), or 50 μg/mL poly(I-C) (G) was added. At 48 h post-transfection, cell lysates were prepared and subjected to luciferase assays. Similar experiments were performed by co-transfection of 0.3 μg pIFN-β-luc and 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) into HepG2 (H) or Huh7 cells (I). Error bars represent standard deviations from three independent experiments. (J) Cell culture fluid from HBeAg-positive HepG2 cells contained less IFN than that from HBeAg-negative cells. HCV subgenomic replication in Huh7 was more enhanced by cell culture medium from HBeAg-positive HepG2 [HBeAg(+)] cells, than that from HBeAg-negative HepG2 [HBeAg(−)] cells, and that from control HepG2 cells. Total cellular RNA was extracted at 24 h after adding cell culture medium. Intracellular gene expression levels of HCV and GAPDH were measured by real-time RT-PCR. The ratios of HCV/GAPDH are presented as n-fold relative to that in control HepG2 cells. The results are presented as means of data from three independent experiments.

assays. We compared six IFN and cytokine (IFN-α1, IFN-β, IL-6, IL-8, IL-12A, and TNF) gene expressions in HBeAg-positive HepG2 cells with those in HBeAg-negative HepG2 cells. The mRNAs of IFN-α1 and IL-12A were inhibited (6.7% and 11.6%, respectively, of those in HBeAg-negative HepG2), and ΔCt of HBeAg-positive HepG2/ΔCt of HBeAg-negative HepG2 in IFN-α1 mRNA and those in IL-12A mRNA were $14.36 \pm 0.11/10.47 \pm 0.02$ ($p < 0.001$, $n = 3$), and $17.74 \pm 0.11/14.65 \pm 0.17$ ($p < 0.001$, $n = 3$), respectively. As shown in Fig. 2, more inhibition of IFN-β, IL-6, IL-8, and TNF mRNA in HBeAg-positive HepG2 were also observed, compared with HBeAg-negative HepG2.

Effects of TLR-dependent target gene expression by HBeAg

To explore the upstream mechanism of IFN and cytokine production, we performed RT^2 profiler array assays to analyze important TLR-activated genes (84 target genes were included in the RT^2 profiler array), that could be modulated by

TLR-signaling, from HBeAg-positive HepG2. Expression profiling showed more than twofold inhibition of nine genes compared to that of HBeAg-negative HepG2 cells (TLR7, LY96, RIPK2, NFκB1, TNF, IL-6, IL-8, IFN-β, and MAP3K1) (Fig. 2A). To confirm these results, real-time PCR was performed. All of these genes except NF-κB1 were significantly downregulated in HBeAg-positive cells compared to HBeAg-negative cells. All of these genes have important roles in the immune response and activation of transcription (Fig. 2B).

Effects of HBeAg on NF-κB activation

Next, we assessed the mechanisms by which HBeAg affects cytokine and IFN production. HBV activates NF-κB, a major player in innate immune responses to viral infections (19). Therefore, we postulated that HBeAg inhibition of the activation of NF-κB might result in the inhibition of cytokine and IFN production, and the subsequent escape of an antiviral response. To test this assumption, we expressed luciferase reporter protein under the control of an NF-κB-dependent promoter in HBeAg-positive or HBeAg-negative HepG2 with or without TNF-α or LPS stimulation (Fig. 3A–E). As expected, HBeAg inhibited NF-κB promoter activity in HBeAg-positive HepG2 cells (Fig. 3A–C; $p < 0.001$ with no drug [n = 3]; $p = 0.004$ with LPS [n = 3]; $p = 0.0075$ with TNF [n = 3]). These results were also confirmed by the transient HBeAg-expression assay in HepG2 ($p = 0.038$, n = 3) and Huh7 cells ($p = 0.0091$, n = 3) (Fig. 3D and E). These findings suggest that HBeAg may affect cytokine production, at least in part, through NF-κB.

Effects of HBeAg on IFN-β activation

NF-κB stimulation leads to the expression of multiple cellular factors, including IFN-β, a central player in the innate immune response that is activated upon virus infection. In order to ascertain whether HBeAg inhibits IFN-β-promoters, we performed experiments using IFN-β-promoter luciferase reporter, essentially as described in the previous section. That is, we used the luciferase gene under the control of an IFN-β-stimulated promoter, and examined its expression in HBeAg-positive and HBeAg-negative HepG2 cells. HBeAg inhibited IFN-β-stimulated promoter activity in HBeAg-positive HepG2 cells with ($p < 0.001$, n = 3), or without poly(I-C) ($p < 0.001$, n = 3) (Fig. 3F and G). We also confirmed these results by transient transfection experiments with HepG2 ($p = 0.0011$, n = 3), and with Huh7 ($p = 0.0081$, n = 3) (Fig. 3H and I). These results demonstrated that HBeAg inhibits both NF-κB- and IFN-β-signaling pathways in hepatocytes.

Cell culture fluid from HBeAg-positive HepG2 enhanced HCV subgenomic RNA replication

To confirm the function of IFN production of these cell lines, we examined whether conditioned media from HBeAg-positive or HBeAg-negative HepG2 cells would cause any differences in HCV subgenomic RNA replication, which is IFN-sensitive replication (14), as it has been reported that there are no direct interactions between HBV and HCV replication in cell culture models and in a mouse study (1,9,11). Cell culture fluid from HBeAg-positive HepG2 cells enhanced HCV subgenomic RNA replication, more than that from HBeAg-negative HepG2 cells (Fig. 3J; $p = 0.0014$, n = 3), suggesting that HBeAg-expressing HepG2 cells contain less IFN than do HBeAg-negative cells, and that conditioned medium from HBeAg-positive HepG2 cells contains less IFN than that from HBeAg-negative cells. In this system, when we treated cells with 0, 1, 10, 100, and 1000 U/mL IFN-α, HCV subgenomic RNA levels were 100%, 57%, 39%, 28%, and 25%, respectively. We estimated that conditioned medium from HBeAg-negative HepG2 cells was equal to ~10 IU/mL IFN-α. Our results showed that HBeAg inhibits IFN production in cell culture medium.

Since the NF-κB target gene IL-6 has also been implicated in hepatitis B pathogenesis (30), the modulation of IL-6 involved in innate signaling by HBeAg was also verified at the protein level by ELISA. Our results demonstrated that IL-6 expression was downregulated in HBeAg-positive HepG2 cells (36.6 ± 30.1 pg/mL; 0 ± 0 pg/mL in conditioned medium from HepG2 control cells; 324.2 ± 15 pg/mL in conditioned medium from HBeAg-negative HepG2 cells). The concentration of IL-6 from HBeAg-positive HepG2 cells was significantly lower than that from HBeAg-negative HepG2 cells ($p = 0.00012$, n = 3).

Discussion

In this study, we investigated the regulation of HBeAg-induced suppression of IFN and cytokines in HepG2 stably expressing HBeAg protein as a model cell line. Our results demonstrated that HBeAg expression inhibits IFN and cytokine production. Transient expression of HBeAg also downregulated both NF-κB- and IFN-β-promoter activity in HepG2 or Huh7, although the mechanisms for this downregulation are unknown. In contrast to our findings, Yang *et al.* (46) observed that HBeAg activates NF-κB through IκBα degradation, and produces TNF-α and GM-CSF in the human hepatoma cell HA22T/VGH. These differences between their findings and ours may have been caused by the differences in the cell lines, and/or promoters (33). Extensive immunological studies by the Milich group (3,4,27) demonstrated that HBeAg appears more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. Our observations support the immune-modulating role of HBeAg.

Locarnini *et al.* (23) used the Tet-off tetracycline gene expression system in Huh7, and revealed that core/precore expression affected gene expression, including cytokines. The system used in our present study, with HepG2 stably expressing HBeAg, supports these findings. Our results provide further direct evidence that hepatocytes exposed to HBeAg have enhanced HCV subgenomic RNA replication, and are significantly influenced in their ability to replicate. Several recent reports have also suggested that there was no evidence of direct interaction between HBV and HCV (1,9,11), although clinical studies showed interaction between HBV and HCV replication (24). It is possible that HBV might interfere with another virus by IFN or another cytokine. A cytokine response is critical for clearance of HCV, as failure to mount a potent and broad T-cell-repertoire response results in persistent HCV replication. This would explain how patients dual-infected with HBV and HCV exhibit a selective deficit of anti-HCV immunity, while demonstrating preservation of a normal immune response to unrelated antigens.

We used RT-PCR to observe the expression of TLRs 1, 3, 4, 5, 6, and 7 in HepG2 cells. We also confirmed in the present study that HepG2 has functional TLRs 3 and 4. Preiss *et al.*

(32) could not detect an NF-κB response to 1 ng/mL–1 μg/mL LPS in HepG2, whereas we could detect such a response to 10–50 μg/mL LPS (Table 2). Downregulation of TLR2 mRNA by genotype C HBV-derived HBeAg was not observed in our study, in contrast to the results of a previous study (43), in which genotype D HBV-derived clone (23) was used. Xu *et al.* (45) reported that TLR7 was suppressed in HBV infection, supporting our results. We do not know why LY96, an important molecule for TLR4, is downregulated (Fig. 2). Viruses encode proteins that target various intracellular signaling pathways, causing their constitutive or prolonged activation, resulting in increased cell proliferation and survival (41). It is well known that HBV activates the MAPK pathway (5). It is also known that RIPK2 activates the NF-κB- and IFN-β-dependent antiviral responses (8). These findings were in accordance with HBeAg's inhibition of the production of IFNs and cytokines (Fig. 2).

What is the mechanism of the downregulation of cytokine production by HBeAg? From our results (Fig. 2), HBeAg appears to interact with the TLR signaling pathway upstream of NF-κB. In LPS stimulation, we observed downregulated TLR4 in HBeAg-positive HepG2 cells (data not shown). Although we are currently investigating this issue, TLR4 might be one of the more important molecules. Precore protein also may affect intracellular signal transduction pathways. Further studies will be needed to clear up these issues.

Many viruses have evolved strategies that block the effector mechanisms induced through IFN- and/or cytokine-signaling pathways (17). Although multiple mechanisms contribute to viral persistence, the ability of the virus to evade innate immune responses is likely to be particularly important. In this report, we have demonstrated that HBeAg suppresses IFN and cytokine mRNA expression. Exploration of the novel HBeAg-inhibiting signaling pathways could lead to the development of new therapeutic strategies for persistent HBV infection.

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Author Disclosure Statement

The authors have no conflicts with regard to financial interests. This material has not been previously reported and is not under consideration for publication elsewhere.

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Impact of *IL-28B* SNPs on control of hepatitis C virus infection: a genome-wide association study

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"...four independent studies have clearly shown a close association of *IL-28B* single nucleotide polymorphisms with treatment response to PEG-IFN plus ribavirin, with consistent results among patients of different ethnic origin."

Hepatitis C virus (HCV) infection is a problem worldwide affecting approximately 170 million individuals. HCV causes chronic hepatitis leading to cirrhosis and hepatocellular carcinoma (HCC). The incidence of HCC caused by HCV is increasing in European countries and the USA, with HCV infection a leading cause of liver transplantation in industrialized countries [1]. More than 20 years have passed since the discovery of HCV by Houghton and colleagues [2]. In the last decade, the efficacy of therapy for chronic hepatitis C has improved. Interferon (IFN) treatment enables the eradication of HCV in chronically infected patients. In genotype 1-naive patients with a high viral load, the ratio of sustained virological response (SVR), defined as undetectable HCV RNA 6 months after IFN therapy, has gone from approximately 10% with standard IFN for 6 months to approximately 50% with a combination of pegylated IFN (PEG-IFN) and ribavirin (RBV) for 1 year, which is the current standard therapy [3].

Efficacy of IFN treatment is determined by a number of factors associated with the virus, host and IFN [4]. Genetic variance could influence the difference in treatment response; however, to date, few single nucleotide polymorphisms (SNPs) have been identified. Recently it has become possible to examine the association of genetic variation with observable traits in the analysis of around 500,000 SNPs across a whole genome, although not all known SNPs (currently in excess of 25 million SNPs) can be analyzed.

Between August 2009 and January 2010, four research groups independently identified SNPs in the *IL-28B* region as associated with response to PEG-IFN plus RBV treatment among HCV-infected individuals of European, African and Asian ancestry [5–8]. Ge *et al.* identified rs12979860 (located ~3 kb upstream of *IL-28B*) as the variant most strongly associated with SVR [5]. In their study, patients of European ancestry showed an association of the CC genotype with a twofold (95% CI: 1.8–2.3) greater rate of SVR than the TT genotype. The rate of SVR was found to be similar in people of African–American ancestry with a threefold (95% CI: 1.9–4.7) greater rate of SVR and in Hispanics a twofold (95% CI: 1.4–3.2) greater rate of SVR. The frequency of the CC genotype was 39, 16 and 35% in European–Americans, African–Americans and Hispanics, respectively, indicating that the genome frequency is quite different among these populations, which might explain the distinct response rates to PEG-IFN and RBV among them [5].

Suppiah *et al.*, Tanaka *et al.* and Rauch *et al.* found the strongest association with rs8099917 (located ~8 kb upstream of *IL-28B*), which is in linkage disequilibrium with rs12979860 [6–8]. Suppiah *et al.* and Rauch *et al.* demonstrated *IL-28B* polymorphisms in European cohorts,

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