Table 1. Characteristics of Patients With Genotype A or a Non-A Genotype Acutely Infected With Hepatitis B Virus

Features	Genotype A (n = 107)	Non-A Genotypes (n = 105)*	*P* Value
Age (years)	36.3 ± 12.0	40.7 ± 14.3	0.032
Male sex	102 (95.3)	75 (71.4)	<0.001
HBeAg positive	104 (97.2)	79 (75.2)	<0.001
ALT (IU/L)	1210 ± 646	2225 ± 2851	0.045
Total bilirubin (mg/dL)	9.9 ± 9.4	7.5 ± 6.7	0.115
HBV DNA (log copies/mL)	7.0 ± 1.5	5.8 ± 1.5	<0.0001
Duration until disappearance of HBsAg (month)	6.7 ± 8.5	3.4 ± 6.5	<0.0001
Persistence of HBsAg positivity more than 6 months	25 (23.4)	9 (8.6)	0.003
Persistence of HBsAg positivity more than 12 months	8 (7.5)	1† (0.9)	0.018
Sexual transmission	81/84 (96.4)‡	71/79 (89.9)§	0.095
Treatment with NAs	61 (57.0)	42 (40.0)	0.013

Data are presented as n (%), mean ± standard deviation. HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; NAs, nucleotide analogs.

*Non-A genotypes include genotypes B, C, D, F and H (n = 25, 77, 1, 1, and 1, respectively).

†One patient had genotype C.

‡Transmission routes were unknown for 23 patients.

§Transmission routes were unknown for 26 patients.

Helsinki and was approved by the Ethics Committees of the institutions involved. Every patient gave informed consent for this study.

Serological Markers of HBV Infection. HBsAg, HBeAg, antibodies to HBsAg (anti-HBs), HBeAg (anti-HBe), and HBcAg, and anti-HBc of the IgM class were tested by a chemiluminescent enzyme immunoassay (CLIA) by ARCHITECT (Abbott Japan, Tokyo, Japan). HBV DNA measurements were performed using a real-time polymerase chain reaction (PCR) assay (Cobas TaqMan HBV Auto; Roche Diagnostics, Tokyo, Japan).

Genotyping of HBV. The six major HBV genotypes (A through F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan). This method is based on the pattern of detection by monoclonal antibodies of a combination of epitopes on preS2-region products, which is specific for each genotype.[17,18] Samples for which EIA could not determine the genotype were examined by direct sequencing of the pre-S2/S gene, followed by phylogenetic analysis.

Treatment With NAs. Treatments with NAs were performed using lamivudine or entecavir for more than 3 months. The individual clinicians determined if NAs were administered to patients, and when the treatment was to be started. The time to onset of treatment with NAs was measured in days from onset of AHB.

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and noncategorical variables by the Mann-Whitney *U* test. A *P* value less than 0.05 was considered significant. Multivariate analysis was performed using a backward stepwise logistic regression model to determine independent factors for viral persistence following AHB. Variables in the multivariate analysis were selected based on variables that were marginally significant with $P < 0.1$ in univariate analysis. Maintenance of HBsAg positivity was analyzed using the Kaplan-Meier method and significance was tested with the log-rank test. STATA Software (StataCorp, College Station, TX) v. 11.0 was used for analyses.

Results

Comparison of Characteristics Between Genotype A and Non-A Genotype AHB Patients. A total of 107 AHB patients (50.5%) were infected with genotype A while 105 AHB patients (49.5%) were infected with non-A genotypes, including genotypes B (25 [11.8%]), C (76 [35.8%]), D (1 [0.5%]), F (1 [0.5%]), and H (1 [0.5%]). Compared to those infected with non-A genotypes, genotype A patients were significantly younger (36.3 ± 12.0 versus 40.7 ± 14.3 years, $P = 0.032$), predominantly men (95.3% versus 71.4%, $P < 0.001$), and more frequently positive for HBeAg (97.2% versus 75.2%, $P < 0.001$). Moreover, genotype A patients had a lower peak ALT levels (1,210 ± 646 versus 2,225 ± 2,851 IU/L, $P = 0.045$) and a higher peak level of HBV DNA (6.7 ± 8.5 versus 3.4 ± 6.5 log copies/mL, $P < 0.0001$). A significantly higher percentage of genotype A patients were treated with NAs (57% versus 40%, $P = 0.013$). These data are summarized in Table 1.

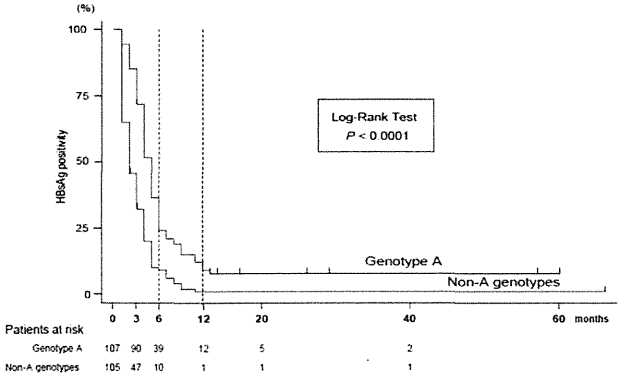

Fig. 1. Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between genotype A and non-A genotypes, analyzed using the Kaplan-Meier test. $P < 0.0001$, genotype A: red line, non-A genotypes: blue line.

Cumulative Maintenance of HBsAg Positivity During Follow-up in Patients With Genotype A and Non-A Genotypes. In the patients infected with genotype A and non-A genotypes, the mean durations of HBsAg positivity maintenance were 6.7 ± 8.5 and 3.4 ± 6.5 months, respectively ($P < 0.0001$; Table 1, Fig. 1). For 6 months after AHB onset, the number of patients with genotype A and non-A genotypes maintaining HBsAg positivity were 39/107 (36.4%) and 10/105 (9.5%), respectively ($P < 0.001$). However, in many patients HBsAg disappeared between 7 and 12 months after AHB onset; that is, HBsAg disappeared in 31/107 (29.0%) of patients with genotype A and in 9/105 (8.6%) of patients with non-A genotypes during this time period. However, in some patients HBsAg never disappeared after persisting for more than 12 months following AHB onset. When chronicity after AHB was defined as the persistence of HBsAg for more than 12 months, chronicity developed in 7.5% (8/107) of patients with genotype A and in 0.9% (1/105) of patients with non-A genotypes ($P = 0.018$).

Comparison of Characteristics Between Patients in Whom HBsAg Persisted More Than 6 or 12 Months and Those With Self-Limited AHB Infection. Table 2 compares the demographic and clinical characteristics between patients in whom HBsAg disappeared within 6 months and those in whom HBsAg persisted for more than 6 months from AHB. The peak ALT levels ($1{,}882 \pm 2{,}331$ versus $1{,}018 \pm 696$ IU/L, $P = 0.0024$) and peak HBV DNA levels (6.3 ± 1.6 versus 7.4 ± 1.6 mg/dL, $P = 0.0004$) were significantly higher and lower in the former group than in the latter group, respectively. Moreover, marked differences were present in the distribution of genotypes between the two groups. The percentage of the HBV genotype A (46.1% versus 73.5%, $P = 0.003$) was significantly higher among patients in whom HBsAg was persistent for more than 6 months. In addition, we compared the demographic and clinical characteristics between patients in whom HBsAg disappeared within 12 months and those in whom HBsAg persisted for more than 12 months from AHB. Peak ALT ($1{,}787 \pm 2{,}118$ versus 775 ± 513 IU/L, $P = 0.0089$) and peak total bilirubin (8.7 ± 8.2 versus 3.8 ± 6.6 mg/dL, $P = 0.0039$) levels were significantly higher in the former group than in the latter group. In contrast, the peak HBV DNA levels (6.4 ± 1.6 versus 7.9 ± 1.4 mg/dL, $P = 0.0046$) were significantly lower

Table 2. Comparison Between Patients With Chronicity Following Acute Hepatitis B and Those With Self-Limited Acute Infections Determined by the Persistence of HBsAg for More Than 6 or 12 Months

Features	Disappearance of HBsAg Within 6 Months (n = 178)	Persistence of HBsAg for More Than 6 Months From AHB (n = 34)	*P* Value	Disappearance of HBsAg Within 12 Months (n = 203)	persistence of HBsAg for More Than 12 Months From AHB (n = 9)	*P* Value
Age (years)	38.2 ± 13.1	40.0 ± 14.5	0.454	38.1 ± 13.2	46.7 ± 14.0	0.061
Male sex	147 (82.6)	30 (88.2)	0.416	169 (83.3)	8 (88.9)	0.677
HBeAg positive	150 (84.3)	32 (94.1)	0.131	175 (86.2)	8 (88.9)	0.815
ALT (IU/L)	1882 ± 2331	1018 ± 696	0.0024	1787 ± 2118	775 ± 513	0.0089
Total bilirubin (mg/dL)	8.6 ± 7.5	8.7 ± 11.3	0.137	8.7 ± 8.2	3.8 ± 6.6	0.0039
HBV DNA (log copies/mL)	6.3 ± 1.6	7.4 ± 1.6	0.0004	6.4 ± 1.6	7.9 ± 1.4	0.0046
HBV genotype						
Non-A	96 (53.9)	9 (26.5)		104 (51.2)	1 (11.1)	
A	82 (46.1)	25 (73.5)	0.003	99 (48.8)	8 (88.9)	0.018
Sexual transmission	128/137 (93.4)*	24/26 (92.3)†	0.711	146/157 (93.0)‡	6/6 (100.0)§	0.356
NAs treatment (+)	82 (46.1)	21 (61.8)	0.093	98 (48.3)	8 (88.9)	0.017

Data are presented as n (%) and mean ± SD. HBsAg, hepatitis B surface antigen; AHB, acute hepatitis B, HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; NAs, nucleotide analogs.

*Transmission routes of 41 patients were unknown.

†Transmission routes of 8 patients were unknown.

‡Transmission routes of 46 patients were unknown.

§Transmission routes of 3 patients were unknown.

Table 3. Multivariate Analysis of Factors Independently Associated With Persistence of HBsAg Positivity Following Acute Hepatitis B

	Persistence of HBsAg More Than 6 Months From AHB		
Factors	Odds Ratio	95% CI	*P* Value
ALT (per 1 IU/L increase)	1.000	0.999-1.000	0.035
HBV DNA (per 1 log copy/mL increase)	1.176	0.931-1.484	0.173
Genotypes			
Non-A	1.00		
A	4.224	1.853-9.631	0.001

95% CI, 95% confidence interval; ALT, alanine aminotransferase; HBV, hepatitis B virus.

in the former group than in the latter group. The percentages of HBV genotype A (48.8% versus 88.9%, $P = 0.018$) and NAs treatment (+) (48.3% versus 88.9%, $P = 0.017$) were significantly higher among patients in whom the HBsAg persisted for more than 12 months.

Factors Independently Associated With Viral Persistence Following AHB. A stepwise logistic regression model was used to perform multivariate analysis which explains relationships between some factors and persistence of HBsAg positivity more than 6 months following AHB. Peak ALT level, peak HBV DNA level, genotype A, and treatment with NAs were retained in the final multivariate logistic model in a backward stepwise manner ($P < 0.1$). For predicting the persistence of HBsAg for more than 6 months, only genotype A was independently associated with progression of AHB to the persistence of HBsAg (odds ratio [OR]: 4.224, $P = 0.001$, Table 3).

Characteristics of Patients Who Progressed to Chronicity That Was Defined as the Persistence of HBsAg for More Than 12 Months Following Acute Hepatitis B. Table 4 shows the clinical and virological characteristics of nine patients who progressed to chronicity defined as the persistence of HBsAg for more than 12 months following AHB. Among the nine patients who progressed to chronicity from AHB, eight (88.9%) were men and eight (88.9%) were HBeAg-positive. In general, among the patients who progressed to chronicity following AHB, the peak HBV DNA levels were high, and the peak total bilirubin and ALT levels were low. In eight (88.9%) patients, entecavir was administered; however, the duration until the onset of NA treatment from AHB onset was long (75-570 days).

Early Onset of Treatment With NAs Was Able to Prevent Viral Persistence After AHB Caused by Genotype A. The cumulative proportion maintaining HBsAg positivity during follow-up, expressed in terms of time after AHB onset, were significantly longer in patients with NAs treatment than in those without NAs treatment ($P = 0.046$, Fig. 2A). Table 5 shows the percentages of patients in whom HBsAg persisted for more than 6 or 12 months among patients categorized based on the period of time (i.e., duration) until the onset of NAs treatment. For patients in whom the onset of NAs treatment was less than 4 weeks from the onset of AHB, 12.7% of the patients showed persistent HBsAg for more than 6 months, while none showed HBsAg positivity for more than 12 months. For patients in whom the onset of NAs treatment was at 5-8 weeks, 37.5% of the patients showed persistent HBsAg for more than 6 months, whereas none showed persistent HBsAg for more than 12 months. For all groups, the period of HBsAg positivity in patients starting NAs treatment within 8 weeks from AHB onset was significantly shorter than that in patients beginning NAs treatment after more than 8 weeks from AHB onset ($P < 0.0001$, Fig. 2B). Patients starting NAs treatment within 8 weeks from AHB onset never progressed to chronicity after AHB caused by genotype A.

Table 4. Characteristics of Patients Who Progressed to Chronicity Following Acute Hepatitis B

Case	Age	Gender	HIV	HBeAg	HBV DNA (log copies/mL)	Total Bilirubin (mg/dL)	ALT (IU/L)	Observation Period (Months)	NAs Treatment	Duration Until NAs Treatment (Days)	Transmission Routes	Genotype
1	23	Male	(−)	(+)	7.6	1.7	1271	26	ETV	570	Heterosexual	A
2	40	Male	(−)	(−)	8.8	1.4	568	13	ETV	240	Heterosexual	A
3	45	Male	(−)	(+)	7.7	0.9	867	57	ETV	135	Heterosexual	A
4	37	Male	(−)	(+)	7.6	3.4	384	29	ETV	75	Unknown	A
5	54	Male	(−)	(+)	9	2	455	17	ETV	155	Homosexual	A
6	45	Male	(−)	(+)	4.8	21.2	512	60	(−)	(−)	Homosexual	A
7	61	Male	(−)	(+)	9.1	1.5	804	17	ETV	88	Unknown	A
8	56	Male	(−)	(+)	9.0	1.1	1820	14	ETV	118	Unknown	A
9	31	Female	(−)	(+)	7.4	0.8	296	66	ETV	150	Blood transfusion	C

HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; NAs, nucleotide analogs; ETV, entecavir.

Table 5. Proportion of Patients in Whom HBsAg Persisted for More Than 6 or 12 Months Among Patients Categorized Based on the Number of Weeks Until the Onset of NAs Treatment

Duration Until Onset of NAs Treatment (Weeks)	Persistence of HBsAg for More Than 6 Months	Persistence of HBsAg for More Than 12 Months	Total Patients
<4 weeks (n, %)	9 (12.7)	0 (0)	71
5-8 weeks (n, %)	6 (37.5)	0 (0)	16
9-12 weeks (n, %)	1 (33.3)	1 (33.3)	3
13-16 weeks (n, %)	4 (100)	1 (25.0)	4
>17 weeks (n, %)	9 (100)	6 (66.7)	9
Total	29	8	103

HBsAg, hepatitis B surface antigen; NAs, nucleotide analogs.

Discussion

A multicenter nationwide study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in Japanese patients who contracted AHB in adulthood. The study was feasible in Japan, where a universal vaccination program for HBV has not been implemented because of the extremely high efficacy of the immunoprophylaxis that is given to babies born to carrier mothers. The implementation of this program has resulted in a decrease in the persistent HBV carrier rate from 1.4% to 0.3%.[19] Selective vaccination means that Japanese are more likely to be infected with HBV by way of horizontal transmission since the percentage of the population possessing anti-HBs is much lower than that in countries in which universal vaccination programs have been established.[20] In addition, Japan is faced with the ever-increasing impacts of globalization: as many as 17 million Japanese travel abroad and over 7 million people visit Japan from overseas each year. This "population mixing" may help to explain the increased prevalence in Japan of AHB due to genotype A, which is transmitted through indiscriminate sexual contact. Consequently, Japan may be the only country in the world where the influences of HBV genotypes, including genotype A (as is predominant in Western countries) and genotypes B and C (as are predominant in Asian countries), on chronic outcomes after AHB can be compared.

Currently, the persistence of HBsAg in serum for more than 6 months is considered to represent a progression to chronic infection.[21] However, our data showed that HBsAg frequently disappeared between 7 to 12 months after the onset of AHB in patients with genotype A (31/107 [29.0%]) and non-A genotypes (9/105 [8.6%]) (Fig. 1). These patients were considered to exhibit prolonged cases of AHB, rather than persistent infection. This finding reflects the higher sensitivity of the most up-to-date assays for HBsAg as compared with previous methods. In the present study, HBsAg was measured by CLIA, which has been reported to be about 150 times more sensitive in the detection of HBsAg than reverse passive hemagglutination (RPHA)-HBsAg, which has been used for the last 30 years in Japan.[22] The use of a more sensitive assay for HBsAg results in a longer period during which HBsAg may be detected. In this study, HBsAg did not disappear in nine patients after remaining continuously detectable for more than 12 months. Therefore, the persistence of HBsAg for more than 12 months, as measured with a highly sensitive method for detecting HBsAg, may be suitable for defining the progression of AHB to chronicity; however, further study is necessary to determine whether this definition is appropriate worldwide.

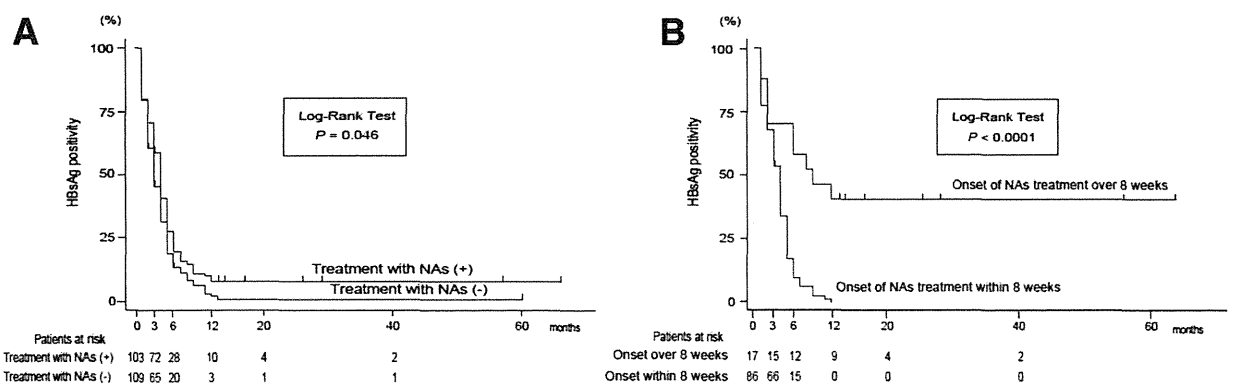

Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. $P = 0.046$, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the cumulative proportion of AHB patients in genotype A maintaining HBsAg positivity between treatment onset with NAs within 8 weeks and treatment onset with NAs over 8 weeks after onset of AHB, as analyzed using the Kaplan-Meier test. $P < 0.0001$, treatment onset with NAs over 8 weeks: red line, treatment onset with NAs within 8 weeks: blue line.

It has been reported that ~10% of patients who contract HBV as adults do not clear HBsAg from their serum and become carriers.[23] Meanwhile, a wide variation has been seen in the rate of persistence after AHB infection in adults. For example, viral persistence following AHB was seen in 0.2% (1/507) of adults in Greece,[24] 7.7% (5/65) of adult Alaskan Eskimos, and 12.1% (7/58) of adults in Germany.[25] The difference in the proportion of patients progressing from AHB to chronicity in different regions may be attributable to virological and host factors. In this study, 4.2% (9/212) of patients progressed to chronicity after AHB: 7.5% (8/107) of those infected with genotype A and 0.9% (1/105) of those infected with non-A genotypes. The non-A genotypes included genotypes B, C, D, F, and H (n = 25, 77, 1, 1, and 1, respectively). Genotypes B and C are predominant in eastern Asian countries, where the majority of those infected with HBV acquired the virus during the perinatal period by way of vertical transmission.[26] On the other hand, genotype A is predominant in Western countries, where the main route is horizontal transmission later in life.[26,27] Because HBeAg persists long after the infection in the genotype C as compared to other genotypes, this genotype has been shown to be a risk factor for perinatal and horizontal transmission in newborns and children.[28] The predominance of genotype A in Western countries may be attributable to a higher chronicity rate following AHB by way of horizontal transmission in adults.

In this study the characteristics of AHB associated with genotype A were a higher peak level of HBV DNA and a lower peak level of ALT. These findings were similar to those for patients with HBV-HIV coinfection.[29] Such characteristics of genotype A or coinfection with HIV are assumed to be attributable to milder hepatitis associated with weaker cellular immune responses. More slowly replicating viruses have been reported to evoke weaker cellular responses, enhancing the likelihood of persistence.[30] Indeed, our prior study showed that the replication of genotype A was significantly slower than that of genotype C in immunodeficient, human hepatocyte chimeric mice.[31] Moreover, variation among genotypes in the expression pattern of HBeAg may affect the progression of AHB to chronicity. Another previous study of ours revealed that a single form of HBeAg was detected by western blot analysis in serum samples from patients infected with genotypes B through D, but that two additional larger forms of HBeAg were detected in patients with genotype A.[32] Milich and Liang[33] reported that HBeAg may modulate the host immune response as a tolerogen to promote chronicity. Therefore, the different expression pattern of HBeAg by genotype A HBV may contribute to chronicity following AHB.

Early NAs initiation appeared to enhance the viral clearance across genotypes, although treatment with NAs did not show any overall benefit in duration of HBsAg. Previous studies examining the efficacies of NAs for preventing progression to chronic infection after AHB have reported conflicting results. Some small-scale studies have suggested the efficacy of lamivudine and entecavir in preventing the progression of AHB to chronic hepatitis.[34,35] Another study showed a lower seroconversion rate of HBsAg in lamivudine users.[36] Further, a randomized placebo-controlled trial showed no significant difference in clinical outcomes.[37] However, these previous studies did not mention the prevalence of HBV genotypes in the respective study populations. Although this was a retrospective study, our study included data on the prevalence of HBV genotypes. Additionally, our findings suggested that larger prospective randomized studies for every HBV genotype should be performed to determine whether early treatment with NAs prevented the progression of AHB to a chronic state.

In conclusion, in Japan genotype A was an independent risk factor for progression to chronic infection following AHB in adults. Confirmation of this association in patients with AHB in other countries is desirable and may provide insight into the pathogenetic mechanisms underlying this association. Early NA treatment appeared to reduce the likelihood of chronicity but this potentially important intervention needs to be prospectively studied before recommendations can be made.

Appendix

Members of the Japanese AHB Study Group include Yasuharu Imai (Ikeda Municipal Hospital), Norie Yamada, Hideaki Takahashi (St. Marianna University School of Medicine), Koji Ishii (Toho University School of Medicine), Hideyuki Nomura (Shin-Kokura Hospital), Jiro Nishida (Tokyo Dental Collage Ichikawa General Hospital), Shigeru Mikami (Kikkoman Hospital), Tsuneo Kitamura (Juntendo University Urayasu Hospital), Akihito Tsubota (Kashiwa Hospital Jikei University School of Medicine), Noritomo Shimada (Shinmatsudo Central General Hospital), Tetsuya Ishikawa (Nagoya University Graduate School of Medicine), Yoshiyuki Ueno (Tohoku University Graduate School of Medicine), Tomoyoshi Ohno (Social Insurance Chukyo Hospital), Etsuro Orito (Nagoya

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J Gastroenterol (2013) 48:397–404
DOI 10.1007/s00535-012-0645-5

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Entecavir and interferon-α sequential therapy in Japanese patients with hepatitis B e antigen-*positive* chronic hepatitis B

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Received: 8 April 2012 / Accepted: 5 July 2012 / Published online: 2 August 2012

Abstract
Background The outcomes of sequential therapy with lamivudine followed by interferon have been unsatisfactory in Japanese patients with hepatitis B envelope antigen (HBeAg)-*positive* chronic hepatitis B. However, the efficacy of sequential therapy with entecavir and interferon remains unclear.
Methods Twenty-four HBeAg-*positive* patients (23 men and 1 woman; mean age 39 ± 7 years) received entecavir 0.5 mg alone for 36–52 weeks, followed by entecavir plus interferon-α for 4 weeks, and lastly by interferon-α alone for 20 weeks. Twenty-three patients had genotype C infection, and one had genotype A infection.
Results No entecavir-resistant mutant variants emerged in any patient. Hepatitis flare occurred in three patients during interferon-α treatment after the withdrawal of entecavir, but none had hepatic decompensation. Serum hepatitis B surface antigen levels did not change during or after therapy. Serum hepatitis B core-related antigen levels were significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$), but returned to baseline levels after treatment. Twenty-four weeks after the completion of the sequential therapy, a sustained biochemical, virological, and serological response was achieved in 5 (21 %) patients. The proportion of patients in whom HBeAg was lost during entecavir treatment was significantly higher among those with a sustained response than among those with no response ($P = 0.015$).
Conclusions The rate of response to sequential therapy with entecavir and interferon-α in Japanese patients with HBeAg-*positive* chronic hepatitis B was not higher than the rate in previous studies of lamivudine followed by interferon.

Keywords Chronic hepatitis B · Genotypes · Interferon-α · Entecavir · Sequential therapy

For the B-SHOT Study Group.
Other members of the B-SHOT Study Group are listed in the Appendix.

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Introduction

Infection with hepatitis B virus (HBV) remains an important public health problem and a leading cause of liver-related morbidity worldwide [1, 2]. The natural course of chronic HBV infection acquired perinatally or during infancy consists of three distinct phases: 'immune tolerant', 'immune reactive', and 'inactive carrier'. During the immune-reactive phase, rises in alanine aminotransferase (ALT) are attributable to the host's immune response to HBV, and the occurrence of hepatitis will eventually be followed by spontaneous seroconversion from hepatitis B

envelope antigen (HBeAg) to anti-HBe. HBeAg seroconversion usually results in clinical remission and a life-long inactive state; however, patients with persistently detectable HBeAg and high HBV DNA levels who have recurrent hepatitis flares are at increased risk of developing cirrhosis and hepatocellular carcinoma [3, 4].

Currently available antiviral treatment for chronic hepatitis B includes nucleos(t)ide analogues such as lamivudine, adefovir, entecavir, and tenofovir, and the immunomodulator interferon [5–7]. The direct, potent antiviral effects of nucleos(t)ide analogues induce biochemical and virological responses in most patients, but viral relapse and exacerbations of hepatitis commonly occur after discontinuation of treatment. Long-term use of nucleos(t)ide analogues is associated with the emergence of drug-resistant variants possessing mutations in the HBV polymerase gene. In contrast, interferon-induced remission of chronic hepatitis B is durable, but is achieved in only a minority of patients. In randomized controlled trials, concomitant treatment with lamivudine and interferon-α has offered little clinical benefit, in terms of the rates of sustained therapeutic response, as compared with interferon-α alone [8, 9].

Serfaty et al. [10] reported that sequential therapy with lamivudine followed by interferon-α was effective in patients with chronic hepatitis B. In their pilot study in France, sustained virological and biochemical response was achieved in 8 (57 %) of the 14 patients who received lamivudine 100 mg alone for 20 weeks, followed by interferon-α 5 MU 3 times/week plus lamivudine for 4 weeks, and lastly by interferon-α alone for 24 weeks [10]. Some other groups have studied similar protocols for sequential therapy, but results have been conflicting [11–17]. The inconsistent results may have been caused, at least in part, by differences in the included HBV genotypes among studies, because HBV genotypes have specific geographic distributions and can affect the response to interferon [18, 19]. In our previous study [14], the rate of response to sequential therapy with lamivudine and interferon in 24 Japanese HBeAg-*positive* patients with chronic HBV genotype C infection was 29 %, considerably lower than the rate reported by Serfaty et al. [10].

Randomized controlled trials have shown that entecavir has higher antiviral activity against HBV than lamivudine [20, 21]. Among licensed nucleos(t)ide analogues, entecavir is used as a first-line treatment of choice for chronic hepatitis B, similar to tenofovir disoproxil fumarate [22]. Use of a potent nucleoside analogue before the initiation of interferon may improve the outcomes of sequential therapy.

In this study, we evaluated the efficacy of sequential therapy with entecavir and interferon-α in Japanese patients with HBeAg-*positive* chronic hepatitis B. In addition to the monitoring of serum HBeAg and HBV DNA levels, serum hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) [23, 24] levels were monitored during and after sequential therapy. The clinical characteristics of patients who had a sustained response to the sequential therapy were compared with those of patients who had no response.

Patients and methods

Patients

The subjects were 24 Japanese patients with HBeAg-*positive* chronic hepatitis B (23 men and 1 woman; mean age 39 ± 7 years) who had received sequential therapy with entecavir alone and then entecavir plus interferon-α followed by interferon-α alone between September 2006 and August 2011. The inclusion criteria were as follows: (1) persistent or fluctuating elevations of serum ALT levels for at least 6 months before the start of therapy; (2) presence of HBsAg in serum; (3) presence of HBeAg and absence of anti-HBe; (4) presence of HBV DNA $>10^5$ copies/mL (equivalent to 20,000 IU/mL); (5) no use of corticosteroids or immunomodulatory drugs, including interferon, within 1 year before the start of therapy; (6) no use of nucleos(t)ide analogues, such as lamivudine, within 1 year before the start of therapy; (7) absence of resistance to nucleos(t)ide analogues; (8) absence of antibodies to hepatitis C virus and other likely causes of chronic liver disease; and (9) no clinical signs of decompensated cirrhosis or hepatocellular carcinoma. The study procedures were in accordance with the Helsinki Declaration of 1975 (1983 revision) and were approved by the ethics committee of each participating center. Written informed consent was obtained from each patient. This study was registered in the UMIN Clinical Trials Registry (registration ID number, UMIN000000808).

Treatment

Patients were treated with entecavir alone for 36–52 weeks, followed immediately by both entecavir and interferon-α for 4 weeks, and lastly by interferon-α alone for 20 weeks. Entecavir (Baraclude; Bristol-Myers, Tokyo, Japan) was given orally at a dose of 0.5 mg once daily. Natural interferon-α (Otsuka Pharmaceutical, Tokyo, Japan) was given by intramuscular injection, at a dose of 5 MU, three times a week for 24 weeks (a protocol commonly used in Japan during the study period). All patients were followed up for at least 24 weeks after the completion of treatment, and responses to therapy were assessed as follows: *biochemical response* was defined as a decrease in

serum ALT levels to within the normal range; *virological response* was defined as a decrease in serum HBV DNA to $<10^4$ copies/mL; and a *serological response* was defined as loss of serum HBeAg. A sustained response was defined as fulfillment of the criteria for combined biochemical, virological, and serological responses 24 weeks after the end of therapy.

Assays

The following variables were determined for all enrolled patients: complete blood counts; serum ALT level; HBsAg, HBeAg, anti-HBe, HBcrAg, and HBV DNA levels; HBV genotypes; proportion of mutants in the precore and basal core promoter regions of HBV DNA; and drug-resistant mutations in the HBV polymerase gene.

Complete blood counts and serum ALT (upper limit of normal, 30 IU/L) were determined by standard procedures. HBsAg was measured with a chemiluminescent microparticle immunoassay (Architect HBsAg QT; Abbott Japan, Tokyo, Japan) as described elsewhere [25]. HBeAg and anti-HBe were detected with chemiluminescence enzyme immunoassays. HBcrAg was also detected with a chemiluminescence enzyme immunoassay (Fuji-Rebio, Tokyo, Japan) [23]. HBV DNA was measured with a real-time polymerase chain reaction (PCR) assay (COBAS TaqMan HBV Test v2.0; Roche Diagnostics, Tokyo, Japan) [26]. Genotypes of HBV were identified by enzyme-linked immunosorbent assay with monoclonal antibodies to type-specific epitopes in the preS2-region (Institute of Immunology, Tokyo, Japan) [27]. Mutations at nucleotide (*nt*) 1896 in the precore region and at *nt* 1762 and *nt* 1764 in the basal core promoter region of HBV DNA were found by means of an enzyme-linked minisequence assay (Genome Science Laboratory, Tokyo, Japan). Drug-resistant mutations (at codons 180, 181, 184, 202, 204, 236, and 250 of the HBV reverse transcriptase domain) were detected by PCR-Invader technology (BML, Tokyo, Japan) [28].

Histopathology

When informed consent had been obtained, a liver biopsy was performed before the patient started therapy. Histopathological findings were assessed by grading inflammatory activity and staging fibrosis according to the METAVIR scoring system [29]. An experienced pathologist blinded to the clinical data performed these evaluations.

Statistical analysis

Statistical analysis was performed with SAS, version 9.2 for Windows (SAS Institute, Cary, NC, USA). Distributions of continuous variables were analyzed with the non-parametric Mann–Whitney *U*-test. Differences in proportions were tested by Fisher's exact test. The significance of changes in values between two time points was evaluated by the Wilcoxon signed-rank test. A two-tailed *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Rate of response to therapy

Although common interferon-α-related side effects included pyrexia, fatigue, headache, and myalgia, the therapy was well tolerated, and all patients completed the treatment according to the protocol. The proportions of patients with biochemical, virological, and serological responses during and after sequential therapy with entecavir and interferon-α are shown in Fig. 1. Drug-resistant mutant variants did not emerge in any patient during entecavir treatment. At the start of interferon-α treatment (about 1 year after the start of the entecavir treatment), most patients had normal ALT levels and serum HBV DNA levels of $<10^4$ copies/mL

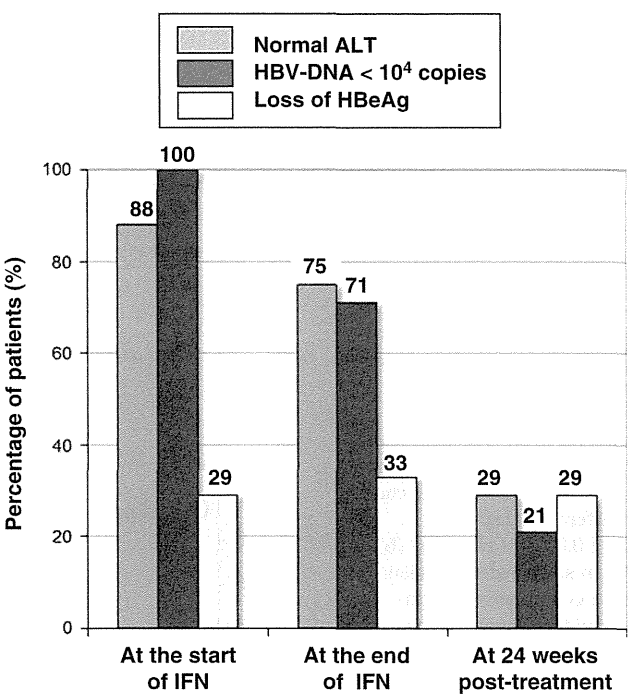

Fig. 1 Rate of biochemical, virological, and serological responses during and after sequential therapy with entecavir and interferon-α. Combined sustained biochemical, virological, and serological response was achieved in 5 (21 %) of the 24 enrolled patients 24 weeks after completion of the sequential therapy. *ALT* Alanine aminotransferase, *HBeAg* hepatitis B envelope antigen, *HBV* hepatitis B virus, *IFN* interferon

(88 and 100 %, respectively). However, loss of HBeAg was achieved in a minority of patients (29 %) during the entecavir treatment.

In most patients without HBeAg loss at the end of the entecavir treatment, serum ALT and HBV DNA levels increased even during the interferon-α treatment. Hepatitis flare (defined as a rise in ALT equivalent to 10 times higher than the upper limit of normal and more than twice the baseline value) occurred in 3 patients after the withdrawal of entecavir. Although peak ALT levels in these patients were 693, 721, and 876 IU/L, respectively, none had jaundice or hepatic decompensation. At the end of the interferon-α treatment, the percentages of patients with normal ALT, HBV DNA $<10^4$ copies/mL, and loss of HBeAg were 75, 71, and 33 %, respectively.

Lastly, 24 weeks after the completion of the sequential therapy, a sustained biochemical, virological, and serological response was achieved in 5 (21 %) of the 24 patients. No patient had loss of serum HBsAg in response to the sequential therapy.

Changes in HBsAg and HBcrAg during and after sequential therapy

Changes in serum HBsAg and HBcrAg levels during and after the sequential therapy with entecavir and interferon-α are shown in Fig. 2. The serum HBsAg level did not change significantly during or after the therapy (Fig. 2a). The serum HBcrAg levels were significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$), but returned to baseline levels after completion of the sequential treatment (Fig. 2b). The serum HBsAg level did not differ significantly between patients with a sustained response and those with no response (Fig. 2c). In contrast, the serum HBcrAg level was significantly lower in patients with a sustained response than in those with no response at the end of the interferon-α therapy ($P = 0.013$) and 24 weeks post-treatment ($P = 0.031$) (Fig. 2d).

Characteristics of patients at the start of entecavir treatment

The baseline demographic, biochemical, virological, and histological characteristics of patients at the start of entecavir treatment, classified according to the response to sequential therapy, are listed in Table 1. The mean age of patients with a sustained response was more than 10 years less than that of the patients with no response, but this difference did not reach statistical significance ($P = 0.102$). There were no significant differences between the two groups with respect to sex ratio, proportion of patients with a history of interferon treatment, ALT level, HBV DNA level, ratios of HBV genotypes, ratios of precore or basal core promoter mutants, or histopathological findings in the liver.

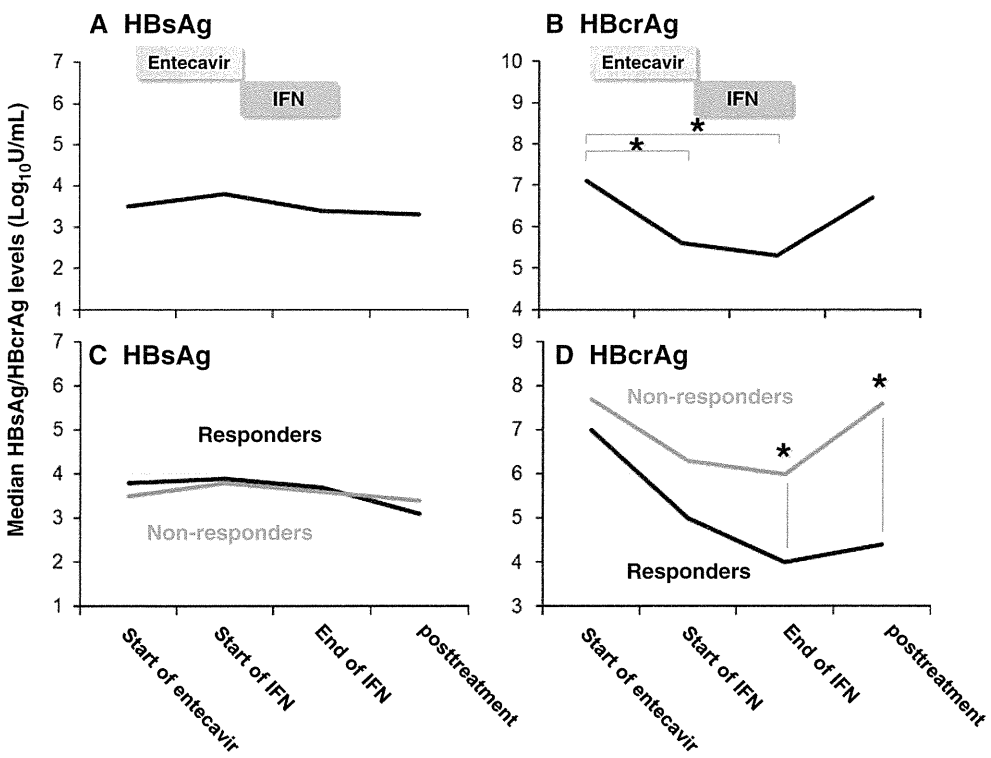

Fig. 2 Changes in serum levels of hepatitis B surface antigen (*HBsAg*) and hepatitis B core-related antigen (*HBcrAg*) during and after sequential therapy with entecavir and interferon-α. Serum HBsAg levels did not change during or after therapy (**a**). As compared with the baseline value, the serum HBcrAg level was significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$) (*asterisks*) (**b**). When sustained responders were compared with non-responders, there was no significant difference in the serum HBsAg level (**c**). In contrast, the serum HBcrAg level was significantly lower in sustained responders than in non-responders at the end of interferon-α therapy ($P = 0.013$) and 24 weeks post-treatment ($P = 0.031$) (*asterisks*) (**d**)

Table 1 Baseline characteristics of patients at the start of entecavir treatment

Characteristics	Sustained responders ($n = 5$)	Non-responders ($n = 19$)	P values
Age (years)	29 ± 6	41 ± 5	0.10
Male sex (%)	5 (100 %)	18 (95 %)	0.99
History of interferon treatment (%)	3 (60 %)	12 (63 %)	0.99
ALT (IU/L)	85 (65, 322)	210 (79, 531)	0.37
HBV DNA ($\log_{10}$ copies/mL)	7.7 ± 0.4	7.8 ± 0.8	0.31
Genotype (A/B/C/D)	0/0/5/0	1/0/18/0	0.99
Precore (wild/mixed/mutant)	0/4/1	9/9/1	0.12
Basal core promoter (wild/mixed/mutant)	1/0/4	5/8/6	0.070
Grade of inflammation (mild/moderate/severe)	2/3/0	9/7/2	0.60
Stage of fibrosis (mild/moderate/severe/cirrhosis)	2/2/0/1	10/5/3/0	0.19

Values are means ± SDs for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables

ALT alanine aminotransferase, *HBV* hepatitis B virus

Table 2 Characteristics of patients at the start of interferon-α treatment

Characteristics	Sustained responders ($n = 5$)	Non-responders ($n = 19$)	P values
ALT (IU/L)	24 (23, 35)	20 (15, 32)	0.27
ALT normal (%)	5 (100 %)	16 (84 %)	0.99
HBV DNA ($\log_{10}$ copies/mL)	2.1 ± 0.3	2.3 ± 0.4	0.18
HBV DNA negative (%)	3 (60 %)	6 (32 %)	0.33
HBeAg loss (%)	4 (80 %)	3 (16 %)	0.015

Values are means ± SDs for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables

HBeAg hepatitis B envelope antigen

Characteristics of patients at the start of interferon-α treatment

The characteristics of the patients at the start of interferon-α treatment, classified according to the response to sequential therapy, are shown in Table 2. The responders and non-responders did not differ significantly with respect to ALT level or HBV DNA level at the start of interferon-α treatment. The proportion of patients in whom HBeAg was lost during entecavir treatment was significantly higher among those with a sustained response than among those with no response ($P = 0.015$). In another comparison, a sustained response was achieved in 4 (57 %) of the 7 patients with loss of HBeAg during entecavir treatment, as compared with 1 (5.9 %) of the 17 patients without loss of HBeAg during treatment; this difference was also statistically significant ($P = 0.015$).

Case presentation

A 24-year-old man with no response to previous treatment with interferon-α was referred to us (Fig. 3). His ALT level

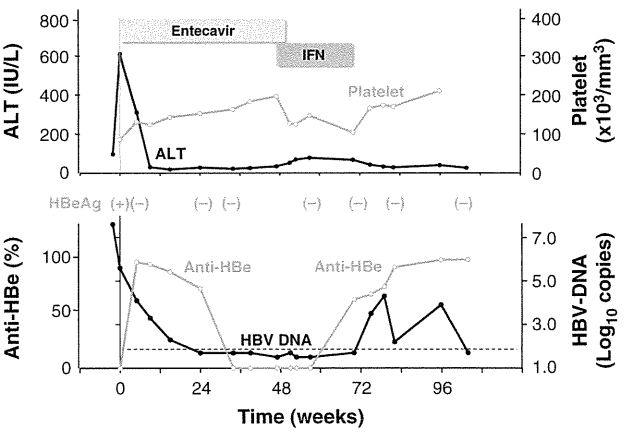

Fig. 3 Changes in platelet count, ALT, HBeAg, anti-HBe, and HBV DNA in a 24-year-old man with sustained response to sequential therapy with entecavir and interferon-α. In the *upper panel*, the changes in ALT levels (*filled circles*) and platelet counts (*open circles*) are shown. In the *lower panel*, the changes in HBV DNA (*filled circles*) and anti-HBe (*open circles*) titers are shown. During 1 year of entecavir treatment, the platelet count rose from 87,000 to 199,000/mm^3. After the patient was switched to interferon-α, his anti-HBe antibody titer increased. At the most recent hospital visit, the patient's ALT level was normal, HBeAg was negative, and HBV DNA was negative; the patient has remained drug-free since the completion of treatment

was 617 IU/L, HBV DNA level was 7.6 $\log_{10}$ copies/mL, and HBV genotype was C. A precore stop codon mutation at *nt* 1896 and basal core promoter mutations at *nt* 1762 and *nt* 1764 were detected. A liver biopsy showed moderate inflammation and cirrhosis. Although the patient was young, interferon-α was not indicated because of a low platelet count and concern about exacerbation of hepatitis. However, during 1 year of entecavir treatment, his ALT level became normal, and his platelet count rose from 87,000 to 199,000/mm^3. After switching to interferon-α, his HBV DNA rose transiently, but his anti-HBe antibody titer increased. At the most recent hospital visit (up to 35 weeks after the completion of treatment), his ALT level was normal and HBeAg and HBV DNA were negative; the

patient has remained drug-free since the completion of treatment.

Discussion

Several groups have evaluated protocols for sequential therapy with lamivudine and interferon-α, and their protocols were similar to that originally described by Serfaty et al. [10]. Manesis et al. [11], from Greece, where HBV genotype D is predominant, found that in HBeAg-*negative* patients, the rate of sustained biochemical and virological response was 22 %, which did not differ from that obtained in an age/sex-matched historical control group treated with interferon-α alone. In another report from Greece [12], sequential combination therapy significantly prevented the emergence of resistance to lamivudine, but the rate of sustained virological response was only 17 % among HBeAg-*negative* patients. A group from China, where genotype B or C is predominant, reported very similar results [13]. To date, only the study by Moucari et al. [17] has used adefovir dipivoxil instead of lamivudine. Sustained virological response was achieved in 50 % of their subjects, although only 20 HBeAg-*negative* patients were included.

In Japan and other countries in East Asia, genotype C is the most prevalent type of HBV [18, 19], and most patients with chronic hepatitis B acquire the virus perinatally or in early childhood [7]. The rates of response to interferon are thus lower than those reported in Europe and the United States. In our previous study [14], using a sequential therapy protocol similar to that described by Serfaty et al. [10], we found that the rate of sustained response was only 29 % among 24 HBeAg-*positive* patients. The patients with a sustained response were significantly younger and had a significantly lower HBV DNA level at the start of interferon than did those with no response. The rate of HBeAg loss during lamivudine treatment was slightly but not significantly higher among sustained responders than among non-responders. Minami and Okanoue [15] also found that patients who lost HBeAg during lamivudine treatment were more likely to have a sustained response to sequential therapy. Okuse et al. [16] reported that sequential therapy was effective for patients with acute exacerbations of chronic hepatitis B, particularly those in whom HBeAg had become negative during lamivudine treatment.

One objective of sequential therapy is to lower the viral load by the use of a nucleos(t)ide analogue, thereby restoring sensitivity to interferon treatment. In clinical studies, a low HBV DNA level is predictive of a favorable response to interferon-α [30, 31]. In basic studies, a high viral load is associated with T-cell hyporesponsiveness [32], and treatment with nucleos(t)ide analogues restores cellular immune response in chronic HBV infection [33]. Although lamivudine had been administered for about half a year before the start of interferon administration in previous studies (including ours) [10–16], we administered entecavir, a more potent antiviral agent, for about 1 year in the present study. Treatment with entecavir was given for a longer period because it has been reported in previous studies that patients in whom HBeAg and HBV DNA levels were lowered by lamivudine were more likely to have a sustained response and because few entecavir-resistant variants emerge within the first few years [34]. However, the use of entecavir for a longer duration did not raise the rate of off-treatment sustained response to sequential therapy in the present study, although the rate of on-treatment biochemical and virological responses was higher with entecavir than that obtained with lamivudine in our previous study [14].

Another objective of sequential therapy is to prevent the relapse of hepatitis after discontinuation of the nucleos(t)ide analogue through the use of interferon-α. Nucleos(t)ide analogues rapidly decrease serum HBV DNA levels by suppressing the reverse transcription of pregenomic HBV RNA, but viral relapse commonly occurs after the cessation of treatment. This high risk of viral relapse may be attributed to the persistence of HBV replicative intermediate covalently closed circular DNA (cccDNA) in the liver even during nucleos(t)ide treatment. The measurement of HBV antigens in serum is thus clinically important as a surrogate marker of intrahepatic cccDNA. In particular, a decline in serum levels of HBsAg is strongly associated with response to interferon-α [35]. The HBcrAg assay measures serum levels of all antigens transcribed from the precore/core gene, including hepatitis B core and e antigens, by using monoclonal antibodies that recognize common epitopes of the denatured antigens [23, 24]. Matsumoto et al. [36] recently proposed a model for predicting relapse of hepatitis after discontinuation of nucleos(t)ide analogue administration, in which cut-off values were set at 1.9–2.9 $\log_{10}$ IU/mL of HBsAg and 3.0–4.0 $\log_{10}$ U/mL of HBcrAg at the withdrawal of treatment. In our study, only one patient had a decrease in HBsAg to between 1.9 and 2.9 $\log_{10}$ IU/mL and another one had a decrease in HBcrAg to between 3.0 and 4.0 $\log_{10}$ U/mL at the withdrawal of entecavir (data not shown), probably because of an insufficient duration of entecavir treatment in our protocol. The finding that at least 21 % of our patients with insufficient HBsAg and HBcrAg decline during entecavir treatment achieved a sustained response to sequential therapy suggests that switching to interferon-α contributes to the safe termination of nucleos(t)ide analogue treatment in some patients.

The major advantages of interferon-α include a finite course of treatment, the opportunity to obtain an off-treatment durable response to therapy, and absence of drug resistance. The advantages of nucleos(t)ide analogues include good tolerance and potent antiviral activity associated with high rates of on-treatment response to therapy. Guidelines proposed by the Japanese Study Group of the Standardization of Treatment of Viral Hepatitis basically recommend interferon-α as the first-line treatment for patients with chronic hepatitis B who are younger than 35 years, to attain a 'drug-free state'; and entecavir for patients who are 35 years or older, to persistently suppress HBV DNA [37]. Consistent with the findings of previous studies [14–16], our results show that sequential therapy is best indicated for patients who have lost HBeAg during nucleoside analogue treatment, because such patients have a higher probability of a sustained response. As shown in Fig. 3, patients who are young but have exacerbation of hepatitis, cirrhosis, or both, were also good candidates for sequential therapy, because interferon-α is generally not recommended for such patients because of concern about hepatic decompensation, and the preceding use of a nucleos(t)ide analogue can reduce such risk.

Our study had several limitations. First, it was not a randomized controlled trial. The reported rate of HBeAg seroconversion obtained by 6-month interferon-α monotherapy among Japanese patients was about 20 % [38], which is similar to the rate obtained by the sequential therapy used in our study (21 %). As compared with our previous study of lamivudine [14], the rate of sustained response in our present study of entecavir did not differ significantly (21 % in the entecavir group vs. 29 % in the lamivudine group). Although the patients were not randomly assigned to treatment, the baseline characteristics of the subjects did not differ between those in our previous study of lamivudine and those in the present study of entecavir with respect to mean age, sex ratio, ALT level, HBV DNA level, ratios of HBV genotypes, ratios of precore or basal core promoter mutants, or histopathological findings (data not shown). Thus, we cannot conclude that sequential therapy with entecavir and interferon-α is more effective than interferon-α monotherapy or sequential therapy with lamivudine and interferon-α. Second, we gave patients non-pegylated interferon-α for 6 months, because pegylated interferon-α had not been approved for the treatment of chronic hepatitis B by the Japanese medical insurance system during the study period. Further studies are thus needed to evaluate the efficacy of sequential therapy with entecavir and pegylated interferon-α.

To our knowledge, this is the first study to report on the response to sequential therapy with entecavir and interferon-α in patients with chronic hepatitis B. In summary, an off-treatment sustained response to sequential therapy with entecavir and interferon-α was achieved in 21 % of HBeAg-*positive* patients with chronic hepatitis B in Japan, where genotype C is predominant. This rate of response was not higher than that in our previous study using lamivudine [14]. Patients who had loss of HBeAg during entecavir treatment were more likely to have a sustained response to sequential therapy.

Acknowledgments The authors are grateful to Ms. Sanae Deguchi, Ms. Rie Yasuda, and Ms. Ami Saito for their technical assistance. This work was supported in part by a grant from the Ministry of Health, Labour and Welfare, Japan.

Conflict of interest Dr. Shuhei Nishiguchi has received research grants from Bristol-Myers K.K. and Otsuka Pharmaceutical Co., Ltd. Dr. Norifumi Kawada has received research grants from Bristol-Myers K.K. and Otsuka Pharmaceutical Co., Ltd.

Appendix

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J Gastroenterol (2013) 48:999–1005
DOI 10.1007/s00535-012-0742-5

REVIEW

Combination therapy with a nucleos(t)ide analogue and interferon for chronic hepatitis B: simultaneous or sequential

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Received: 2 October 2012 / Accepted: 11 December 2012 / Published online: 22 January 2013

Abstract Currently available antiviral treatment for chronic hepatitis B virus infection can be divided into two classes of therapeutic agents: nucleos(t)ide analogues (NAs) and interferon (IFN). The major advantages of NAs are good tolerance and potent antiviral activity associated with high rates of on-treatment response to therapy; the advantages of IFN include a finite course of treatment, absence of drug resistance, and an opportunity to obtain a post-treatment durable response to therapy. The use of these two antiviral agents with different mechanisms of action in combination is theoretically an attractive approach for treatment. Here, we have reviewed previous reports of either simultaneous or sequential combination therapy with NA and IFN for chronic hepatitis B patients. In previous studies comparing the lamivudine/IFN combination and lamivudine monotherapy in a finite course, combination therapy was associated with higher rates of sustained post-treatment response and lower rates of drug resistance than lamivudine monotherapy. However, NAs such as lamivudine are generally administered indefinitely because of high rates of post-treatment relapse. In addition, concern for drug resistance has decreased significantly with newer, high-potency NAs even when administered alone. In previous studies comparing the lamivudine/IFN combination and IFN monotherapy, the combination therapy showed greater on-treatment viral suppression, but no difference was observed in the post-treatment sustained response. Thus, whether combination therapy confers an additional benefit compared to monotherapy for treating chronic hepatitis B remains unclear. The efficacy of IFN in combination with a more potent NA, such as entecavir or tenofovir, remains to be comprehensively evaluated.

Keywords Chronic hepatitis B · Lamivudine · Adefovir · Entecavir · Interferon · Nucleos(t)ide analogue

Abbreviations

ALT	Alanine aminotransferase
CccDNA	Covalently closed circular DNA
HBcrAg	Hepatitis B core-related antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
IFN	Interferon
NA	Nucleos(t)ide analogue

Introduction

More than 350 million people worldwide are infected with hepatitis B virus (HBV) which is a leading cause of liver-related morbidity that accounts for 1 million deaths annually [1, 2]. Currently available antiviral treatment for chronic hepatitis B can be divided into two classes of therapeutic agents: nucleos(t)ide analogues (NAs) and interferon (IFN). Nucleosides include lamivudine [3, 4], telbivudine [5], and entecavir [6, 7]; nucleotides include adefovir [8, 9] and tenofovir [10]. The direct, potent antiviral effects of NAs induce an on-treatment response in

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most patients, but post-treatment relapse commonly occurs after treatment discontinuation. Long-term use of NAs triggers the emergence of drug-resistant variants possessing mutations in the hepatitis B virus (HBV) polymerase gene. Among the NAs currently available, entecavir or tenofovir is recommended as the first-line treatment because of the low rate of drug resistance. In contrast, IFN has both antiviral and immunomodulatory actions [11, 12]. The major advantages of IFN include a finite course of treatment, absence of drug resistance, and an opportunity to obtain a post-treatment durable response to therapy; however, a response to IFN is achieved in only a minority of patients with chronic hepatitis B.

In this article, we have reviewed previous reports on combination therapy with NA and IFN for chronic hepatitis B. Regimens of combination therapy can be classified into two main groups: (1) simultaneous therapy with the drugs in the combination and (2) sequential combination therapy in which treatment with one drug follows that of the previously administered one. To compare the results of previous trials, we noted that age/sex of the included subjects, HBV genotypes, and mode of vial transmission varies among the different studies. These differences may affect the results and their interpretations as older age, male sex, HBV genotypes C and D (vs. A and B), and vertical transmission are associated with a poor response to IFN therapy [13–15]. In particular, HBV genotypes have specific geographic distributions, with genotype A being prevalent in Northwest Europe, North America, and Central Africa, genotypes B and C being common in Southeast Asia, China, Japan, and Oceania, and genotype D being prevalent in Southern Europe, the Middle East, and India, although it has a nearly worldwide distribution.

Theoretical background

Nucleos(t)ide analogues directly inhibit viral replication by targeting at least one of the three replication steps: priming of HBV DNA polymerase, reverse transcription of negative-strand HBV DNA from pregenomic RNA, and synthesis of positive-strand HBV DNA. IFN also possesses antiviral activity but does not act directly on the virus or replication complex. Instead, it acts by inducing IFN-stimulated genes to establish a non-virus-specific antiviral state within the cell. In addition to their role as antivirals, IFNs are important immunomodulators that interact with the adaptive and innate immune responses. Combining NA and IFN, with their different mechanisms of action, in a therapeutic regimen is theoretically an attractive approach for treating chronic hepatitis B.

The action of NAs has little or no effect on the decrease in the intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA). In the experimental woodchuck hepatitis virus system, cccDNA persisted even when viral production was strongly reduced by NA treatment [16, 17]. To reduce the level of intrahepatic cccDNA, the immunomodulatory activity of IFN, which presumably induces cytotoxic T cell activity for immune clearance of infected cells, may be required. However, a high HBV DNA load is associated with an inefficient T cell response to HBV-related antigens, such as hepatitis B surface antigen (HBsAg) [18]. Several studies have shown that a decreased viral load induced by NA treatment can result in the subsequent restoration of CD4 followed by CD8 cellular immune response against HBV [19, 20]. The rationale for combination therapy is based on the concept that suppression of viral replication by NA can decrease viral protein synthesis on the surface of hepatocytes, which may restore the immune response and optimize the immunomodulatory effects of IFN for clearing infected cells.

Simultaneous combination with NA and IFN

Table 1 shows a summary of previous studies examining simultaneous combination therapy with NA and IFN for chronic hepatitis B. The first trial was reported by Mutimer et al. [21] in the UK. Since this study was designed to assess the safety and tolerability of combination treatment, the duration of treatment was only 16 weeks, and few patients showed sustained seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe (antibody to HBe) by this short-term therapy with lamivudine and IFN-α. Barbaro et al. [22] reported the results of a randomized trial conducted in Italy where the 24-week combination with lamivudine and IFN-α increased the rate of sustained HBeAg seroconversion compared to the 52-week lamivudine monotherapy (33 vs. 15 %; $P = 0.014$). Tatulli et al. [23] in Italy found that the 52-week combination with lamivudine and IFN-α resulted in a sustained loss of serum HBV DNA, based on the results of a solution hybridization assay, and normalization of alanine aminotransferase (ALT) in only 14 % of HBeAg-negative patients, but drug-resistant mutation variants did not emerge in any patients. However, from these previous studies, it is still unclear whether combination therapy with lamivudine and IFN confers an additional benefit compared to IFN monotherapy.

Three randomized controlled trials (2 in HBeAg-positive patients [24, 25] and 1 in HBeAg-negative patients [26]) did not show that 1-year combination therapy with lamivudine and pegylated IFN-α was superior to monotherapy with pegylated IFN-α in terms of the rate of sustained response. The results of these globally conducted

Table 1 Simultaneous combination therapy with nucleos(t)ide analogues and interferon

Reference (first author)	HBeAg	*n* (genotype)	Age (years)	Male (%)	Regimens	Biochemical response (%)	Virologic response (%)
Mutimer [21]	+	20 (N.D.)	39 ± 11[a]	95	LAM + IFN for 12–16 weeks	0	5
Barbaro [22]	+	76 (N.D.)	42 (33–50)[b]	84	LAM + IFN for 24 weeks	37	33
Tatulli [23]	−	29 (N.D.)	44 (27–64)[b]	90	LAM + IFN for 52 weeks	14	14
Janssen [24]	+	130 (A43/B11/C18/D52)	34 ± 12[a]	75	LAM + PEG for 52 weeks	35	35
Lau [25]	+	271 (A18/B82/C156/D11)	32 ± 10[a]	77	LAM + PEG for 48 weeks	39	28
Marcellin [26]	−	179 (N.D.)	41 ± 11[a]	82	LAM + PEG for 48 weeks	60	44
Wursthorn [29]	±	26 (A8/B0/C1/D14)	34 (19–55)[b]	77	ADV + PEG for 48 weeks	N.D.	N.D.
Takkenberg [30]	±	40 (A20/B2/C2/D9)	40 ± 10[a]	88	ADV + PEG for 48 weeks	N.D.	50

HBeAg hepatitis B e antigen, *LAM* lamivudine, *ADV* adefovir dipivoxil, *IFN* interferon, *PEG* pegylated interferon, *N.D.* not described

[a] Mean (± standard deviation, SD)

[b] Median (range)

trials, which included many patients with various HBV genotypes, appear to be reliable. All studies found that the combination therapy had greater on-treatment viral suppression and higher rates of sustained post-treatment response than therapy with lamivudine alone, but no difference was observed in the sustained post-treatment virologic response compared to that with pegylated IFN-α alone. Janssen et al. [24], for example, found that more patients in the patient group receiving the 52-week pegylated IFN-α + lamivudine combination than in the group receiving the 52-week pegylated IFN-α monotherapy showed a response, as assessed by serum HBeAg loss at the end of treatment (44 vs. 29 %; $P = 0.01$). However, this difference was not sustained; 35 % of the combination-therapy group and 36 % of the monotherapy group showed a sustained HBeAg loss at the end of follow-up ($P = 0.91$). The trial also showed that pegylated IFN-α therapy improves liver histology, particularly in responders to therapy, but that the addition of lamivudine to therapy with pegylated IFN-α did not further improve histological outcome [27] and that genotypes C and D are associated with a lower rate of response to IFN than genotypes A and B.

To date, few studies have examined the combination of IFN and other NAs that are more potent than lamivudine. The telbivudine + IFN combination is prohibited because of the high risk of severe polyneuropathy [28]. Interestingly, Wursthorn et al. [29] from Germany found that 48-week combination therapy with adefovir dipivoxil and pegylated IFN-α led to marked decreases in cccDNA in the liver, which has been correlated with reduced HBsAg in serum. However, the rate of the post-treatment sustained response was not reported in this study. Another group from the Netherlands [30] showed that intrahepatic cccDNA levels at the end of 48-week treatment with adefovir dipivoxil and pegylated IFN-α were predictive of a sustained response defined as HBV DNA <2,000 IU/mL and normal ALT. The efficacy of combining IFN and other NAs, such as entecavir or tenofovir, remains to be elucidated.

Sequential combination starting with IFN followed by NA

Table 2 shows a summary of previous reports concerning sequential combination therapy starting with IFN followed by NAs for chronic hepatitis B. Hasan et al. reported that the rate of sustained HBeAg seroconversion was only 6.2 % in patients in Kuwait receiving IFN-α alone for 4 weeks, followed by the IFN-α + lamivudine combination for 12 weeks, and lastly by lamivudine alone for 36 weeks; this rate was similar to that observed in patients receiving lamivudine alone for 48 weeks [31].

In contrast, a randomized trial by Chan et al. in China [32] showed that the rate of sustained virologic response, defined as HBeAg seroconversion and a HBV DNA level of <500,000 copies/mL, was 36 % in patients receiving pegylated IFN-α alone for 8 weeks, followed by the pegylated IFN-α + lamivudine combination for 24 weeks, and lastly by lamivudine alone for 28 weeks; this rate was

Table 2 Sequential combination therapy starting with IFN followed by nucleos(t)ide analogues

Reference (first author)	HBeAg	*n* (genotype)	Age (years)	Male (%)	Regimens	Biochemical response (%)	Virologic response (%)
Hasan [31]	+	32 (N.D.)	32 (17–63)[b]	88	IFN for 4 weeks, IFN + LAM for 12 weeks, and then LAM for 36 weeks	9.3	6.2
Chan [32]	+	50 (A0/B18/C35/D0)	32 (19–57)[b]	62	PEG for 8 weeks, PEG + LAM for 24 weeks, and then LAM for 28 weeks	50	36

[a] Mean (range)

[b] Median (range)

significantly higher than that observed in patients receiving lamivudine alone for 52 weeks (14 %; $P = 0.011$). At the end of the treatment period, 21 % of patients in the sequential combination treatment group developed a lamivudine-resistant mutant, compared to 40 % of patients in the lamivudine monotherapy group. Follow-up of this study demonstrated that sequential combination with pegylated IFN-α followed by lamivudine maintained a higher long-term virologic response than lamivudine monotherapy for up to 3 years [33]. However, this study did not include a study arm of pegylated IFN-α alone.

Sequential combination starting with NA followed by IFN

Table 3 shows a summary of previous studies which examined sequential combination therapy starting with NAs followed by IFN for chronic hepatitis B. In a pilot study [34] by Serfaty et al. in France, sustained responses, defined as serum HBV DNA clearance based on the results of a branched DNA assay and ALT normalization, were achieved in 57 % of patients who received lamivudine alone for 20 weeks followed by the lamivudine + IFN-α combination for 4 weeks, and lastly by IFN-α alone for 24 weeks.

Some groups have studied similar protocols for sequential therapy, but the results have been conflicting. Consistent with the results reported by Serfaty et al. [34], Sarin et al. [35] in India reported that the addition of 4-week lamivudine before starting 24-week pegylated IFN-α therapy resulted in a significantly higher rate of sustained HBeAg clearance (39 %) than that with 24-week pegylated IFN-α monotherapy (14 %; $P = 0.05$). In contrast, Manesis et al. [36] found that in HBeAg-negative patients in Greece, where genotype D is predominant, the rate of sustained response to sequential therapy, defined as HBV DNA of ≤30,000 copies/mL and normal ALT, was only 22 %, which did not differ from that obtained in age/sex-matched historical controls treated with IFN-α alone for 12 months (14 %; $P = 0.36$). In another report in Greece [37], sequential therapy did not raise the rate of sustained virologic response, defined as HBV DNA levels of <400 copies/mL, in HBeAg-negative patients compared to lamivudine monotherapy for a median duration of 25 months (33 vs. 17 %; $P = 0.40$), although no patients in the sequential therapy group showed emerging resistance to lamivudine. A group in China, where genotype B or C is predominant, reported very similar results [38].

In Japan and other East Asian countries, genotype C is the most prevalent HBV type [39, 40], and most patients with chronic hepatitis B acquire the virus perinatally [13]. Thus, response rates to IFN-based therapy in these countries are lower than those reported in Europe and the USA. In our previous study [41] using sequential therapy with lamivudine alone for 16–32 weeks, followed by the lamivudine + IFN combination for 4 weeks and lastly by IFN alone for 20 weeks, the rate of sustained loss of HBeAg was only 29 %. The rate of HBeAg loss during lamivudine treatment was higher among sustained responders than that among non-responders. In a multicenter trial, Minami et al. [42] found that patients who lost HBeAg during lamivudine treatment were more likely to show a sustained response to sequential therapy. Okuse et al. [43] reported that sequential therapy was effective for patients with acute exacerbations of chronic hepatitis B, particularly those in whom HBeAg had become negative during lamivudine treatment.

To date, a small study by Moucari et al. [44] from France has been the only one to evaluate the efficacy of sequential therapy with adefovir dipivoxil followed by IFN-α. Sustained virologic response, defined as serum HBV DNA of <10,000 copies/mL, was achieved in 50 % of patients, but only 20 HBeAg-negative patients were included in this study.

We recently reported the outcomes of sequential therapy with entecavir followed by IFN-α [45]. Among the 24 patients receiving entecavir alone for 36–52 weeks, followed by the entecavir + IFN-α combination for 4 weeks, and lastly by IFN-α alone for 20 weeks, the rate of sustained response, defined by HBeAg loss, HBV DNA of <10,000 copies/mL, and normal ALT, was 21 %; this was not higher than the rate found in our previous study using lamivudine [41]. In the study carried out in China, Chen

Table 3 Sequential combination therapy starting with a nucleos(t)ide analogue followed by IFN

Reference (first author)	HBeAg	*n* (genotype)	Age (years)	Male (%)	Regimens	Biochemical response (%)	Virologic response (%)
Serfaty [34]	±	14 (A6/B0/C1/D4)	40 (30–57)[a]	100	LAM for 20 weeks, followed by LAM + IFN for 4 weeks, and then IFN for 24 weeks	57	57
Sarin [35]	+	36 (N.D.)	33 ± 11[b]	93	LAM for 4 weeks, followed by PEG for 24 weeks	36	39
Manesis [36]	−	36 (N.D.)	55 (46–66)[a]	69	LAM for 6 months, followed by LAM + IFN for 6 months, and then IFN for 6 months	39	22
Vassiliadis [37]	−	18 (N.D.)	42 (19–63)[a]	83	LAM for 3 months, followed by LAM + PEG for 3 months, and then by PEG for 9 months	72	33
Shi [38]	−	64 (N.D.)	35 (21–56)[a]	60	LAM for 20 weeks, followed by LAM + IFN for 4 weeks, and then IFN for 24 weeks	53	14
Enomoto [41]	+	24 (C)	37 ± 11[b]	88	LAM for 16–32 weeks, followed by LAM + IFN for 4 weeks, and then IFN for 20 weeks	46	29
Minami [42]	±	37 (N.D.)	N.D.	N.D.	LAM for 20 weeks, followed by LAM + IFN for 4 weeks, and then IFN for 20 weeks	46	35
Okuse [43]	±	12 (C)	32 ± 8[b]	83	LAM for 20 weeks, followed by LAM + IFN for 4 weeks, and then IFN for 20 weeks	N.D.	58
Moucari [44]	−	20 (A5/B3/C1/D9)	44 (41–52)[a]	85	ADV for 20 weeks, followed by ADV + PEG for 4 weeks, and then PEG for 44 weeks	50	50
Enomoto [45]	+	24 (A1/B0/C23/D0)	39 ± 7[b]	96	ETV for 36–52 weeks, followed by ETV + IFN for 4 weeks, and then IFN for 20 weeks	29	21
Chen [46]	±	32 (A0/B23/C9/D0)	35 ± 5[b]	72	ETV for 12–26 days, followed by ETV + PEG for 2 weeks, and then PEG for 22–46 weeks	61	74

[a] Median (range)

[b] Mean (± SD)

ETV Entecavir

et al. [46] included only patients with acute exacerbation (ALT >10-fold the upper limit of normal) who were treated with entecavir alone for 12–26 days before the ALT had declined to five- to ten-fold the upper limit of normal, followed by the entecavir + pegylated IFN-α combination for 2 weeks, and then by pegylated IFN-α alone for 22–46 weeks. Sustained virologic response, defined as HBV DNA of <10,000 copies/mL, was obtained in 69 % of HBeAg-positive and in 80 % of HBeAg-negative patients with acute exacerbation of chronic hepatitis B.

One objective of sequential therapy starting with NA is to lower the viral load before IFN therapy is initiated, thereby restoring treatment sensitivity as low HBV DNA levels are associated with a favorable response to IFN. Another objective of sequential therapy is to prevent the relapse of hepatitis following the discontinuation of NA therapy through the use of IFN. The high risk of viral relapse after treatment may be attributed to the persistence of cccDNA in the liver, which is correlated with HBV antigen levels in the serum. Using HBsAg and hepatitis B core-related antigen (HBcrAg) levels, Matsumoto et al. [47] proposed a model for predicting relapse after the discontinuation of NA therapy. In our study of sequential therapy using entecavir [45], few patients showed a decrease in HBsAg or HBcrAg to the level meeting the criteria of safe discontinuation of NA. Taken together with the fact that at least 21 % of our patients achieved a sustained response, we suggest that the switch to IFN-α contributes to the safe termination of NA therapy in some patients [48].

Combination with NA and IFN in the guidelines

Combination therapy with NA and IFN is not recommended in the guidelines proposed by the Asian–Pacific Association for the Study of the Liver (updated in 2008) [13] and the American Association for the Study of Liver Diseases (updated in 2009) [14] because there has been no large clinical trial to confirm the benefits of combination therapy over monotherapy in inducing a higher rate of sustained response. The most recently updated guidelines proposed by the European Association for the Study of the Liver (updated in July 2012) [15] also does not recommend combination therapy of IFN with lamivudine or telbivudine. However, the limited information currently available on the efficacy and safety of combining IFN with other NAs has raised an unresolved issue of assessing the safety and efficacy of combining IFN with a more potent NA, such as entecavir or tenofovir.