evaluated among the studies. In our previous study [13], the rate of response (defined as alanine aminotransferase [ALT] normalization, an HBeAg loss, and an HBV DNA level $< 10^5$ copies/ml at 24 weeks post-treatment) to sequential therapy using lamivudine and IFN-β in 24 Japanese HBeAg-positive patients with chronic HBV genotype C infection was 29%, lower than the rate reported by Serfaty *et al.* [7]. However, the long-term outcome of sequential therapy is unknown.

In this study, we investigated the long-term outcomes in 24 genotype C patients who received sequential therapy with lamivudine and IFN-β. Since the initial study was published in 2007 [13], substantial advances have been made in clinical studies on HBV. First, since the large REVEAL-HBV study showed that serum HBV DNA level of 10^4 copies/mL or more was a strong risk factor for disease progression [25,26], a sustained response to IFN is now defined as an HBV DNA level below 2,000 IU/mL (equivalent to 10^4 copies/mL) post-treatment [27–29]. Second, with the development of assays for hepatitis B surface antigen (HBsAg) [30,31] and core-related antigen (HBcrAg) [32,33], quantification of viral antigens has become an indispensable tool in clinical practice. Herein, we applied the new definition of therapeutic response to investigate the long-term outcomes of sequential therapy, and we also determined the changes in serum HBsAg and HBcrAg levels.

Methods

Patients

This study included 24 HBeAg-positive patients with chronic HBV genotype C infection (21 males and 3 females; mean age, 37 ± 11 [range, 21–65] years) who had received sequential therapy with lamivudine and IFN-β between 2002 and 2005. The inclusion criteria have been described previously [13]. In short, patients were eligible if they had been both HBsAg- and HBeAg-positive for more than 6 months, had persistent or fluctuating elevations of serum ALT levels, and had a genotype C HBV DNA level $>10^5$ copies/ml. Major exclusion criteria were use of corticosteroids or immunomodulatory drugs within 1 year before the start of therapy, previous use of nucleos(t)ide analogues, decompensated liver disease, or other likely causes of chronic liver disease. The procedures of this study were in accordance with the Helsinki Declaration of 1964 guidelines (2008 revision) and were approved by our hospital ethics committee. All patients provided written informed consent.

Treatment

Patients were treated with lamivudine alone for 16–32 weeks, then with both lamivudine and IFN-β for 4 weeks, and lastly with IFN-β alone for 20 weeks. Lamivudine (GlaxoSmithKline K.K., Tokyo, Japan) was administered orally at a dose of 100 mg once daily. Natural IFN-β (Mochida Pharmaceuticals, Tokyo) was given by intravenous injection at a dosage of 6 MU every day for the first 2 weeks, followed by 6 MU three times a week for the remaining 22 weeks. All patients were followed up for 7.1 ± 2.8 (range, 0.6–9.5) years after the end of sequential therapy.

In the initial study [13], responses to therapy were defined as ALT normalization, HBeAg loss, and HBV DNA levels $< 10^5$ copies/ml at 24 weeks post-treatment, referred

to here as the *initial response*. In the present study, treatment responses were assessed according to the updated EASL Clinical Practice Guidelines [28]. *Biochemical response* was defined as ALT normalization, *serological response* as an HBeAg loss, and *virological response* as an HBV DNA level < 10^4 copies/mL (equivalent to 2000 IU/ml). *Sustained off-treatment response* was defined as fulfillment of the criteria for biochemical, serological, and virological responses for at least 12 months after the end of therapy. When relapse occurred post-treatment, the treating physician considered retreatment based on the guidelines, and a decision was finally reached by discussion with the patient. Patients who were re-treated after the initial therapy were regarded as *non-responders*.

Assays

At baseline, the following variables were determined for all enrolled patients as described previously [13]: serum ALT activity, HBeAg, HBV genotypes, proportion of mutants in the precore and basal core promoter regions of HBV DNA, and HBV DNA concentrations. After obtaining informed consent, liver biopsy was performed before starting therapy to assess histopathological findings such as the grade of inflammatory activity and stage of fibrosis according to the METAVIR scoring system [34].

Serum samples collected at baseline, at the start and end of IFN-β therapy, and at 24–48 weeks post-treatment were stored. When HBV DNA was not detected by transcription-mediated amplification assay (Chugai Diagnostics, Tokyo, Japan) [35] or the Amplicor Monitor test (Roche Diagnostics K.K., Tokyo) [36], we re-evaluated the stored sera for HBV DNA using a real-time polymerase chain reaction (PCR) assay (COBAS TaqMan HBV Test v2.0, Roche Diagnostics K.K.) [37]; the detection range of the assay was between 2.1 and 9.0 $\log_{10}$ copies/mL. The stored sera were also used to

measure HBsAg using a chemiluminescent microparticle immunoassay (Architect HBsAg QT, Abbott Japan Corp., Tokyo) [38] and HBcrAg was detected using a chemiluminescence enzyme immunoassay (Fuji-Rebio, Tokyo), as described elsewhere [32].

In patients who consented to genomic analysis, we examined a genetic polymorphism in the interleukin-28B (*IL28B*) gene [39,40]. Genomic DNA was extracted from the buffy coat obtained from whole blood samples. The presence of the genetic polymorphism located upstream of the *IL28B* gene, rs8099917, was determined using TaqMan SNP Genotyping Assay on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed using the SAS program, version 9.2 for Windows (SAS Institute, Cary, NC, USA). Distributions of continuous variables were analyzed using the Mann-Whitney *U* test. Differences in proportions were assessed using Fisher's exact test. Cumulative incidences were plotted using the Kaplan-Meier method, and the significance of the differences was determined using the log-rank test. A two-tailed *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Characteristics of patients with short-term responses

As described previously [13], an initial response (defined as ALT normalization, HBeAg loss, and an HBV DNA < 10^5 copies/ml) was seen in seven of the 24 (29%) patients 24 weeks after the end of sequential therapy. In two of the seven initial responders, the level of HBV DNA was 10^4–10^5 copies/ml at 24 weeks post-treatment. Since an off-treatment response is herein defined as ALT normalization, HBeAg loss, and an HBV DNA level less than 10^4 copies/ml, the rate of response at 24 weeks post-treatment was 5/24 (21%) in this study.

The baseline demographic, biochemical, and virological characteristics of the patients at the start of lamivudine treatment are classified according to the short-term response at 24 weeks post-treatment in **Table 1**. The host *IL28B* genotype was not determined in six patients, and liver histology was not determined in nine, because informed consent was not obtained. The patients with the short-term response were significantly younger than the patients with no response ($P = 0.039$), as in our previous study [13]. In the present study, we added the data of HBsAg and HBcrAg levels in the analyses, but there were no significant differences in the viral antigen levels between the two groups.

The characteristics of the patients at the start of IFN-β treatment in the initial sequential therapy are given according to the short-term response 24 weeks post-treatment in **Table 2**. The HBcrAg level at the start of IFN-β treatment was lower in the short-term responders than in the non-responders, but not significantly ($P = 0.091$). The proportion of patients with HBV DNA detected by PCR at the start of IFN-β treatment was significantly lower among the short-term responders than among non-responders ($P = 0.0059$).

Rate of long-term responses

Among the five (21%) patients with a short-term response 24 weeks post-treatment, two maintained the response but were lost to follow-up 0.71 years and 2.3 years post-treatment, respectively. Another two patients maintained the response at the most recent visit of 5.3 years and 8.6 years post-treatment, respectively, and the remaining patient relapsed and began retreatment with lamivudine at 1.8 years post-treatment. The rates of response at 24 weeks and 1, 2, 3, and 5 years post-treatment are shown in **FIG. 1.** In the majority of patients with an off-treatment response at 1 year post-treatment, the response was sustainable during subsequent long-term follow-up. None of the 19 patients without a short-term response 24 weeks post-treatment experienced a sustained off-treatment response 1 year post-treatment or later.

During the long-term follow-up, four of the five (80%) short-term responders remained free of the need for further drug treatment (referred to here as *drug-free*) for 4.2 ± 3.5 years post-treatment. Among the 19 short-term non-responders, 16 started lamivudine or entecavir within 5 years post-treatment, two refused retreatment despite persistent elevation of ALT and/or HBV DNA levels, and one was lost to follow-up 0.6 years post-treatment. The proportion of drug-free patients was significantly higher among the short-term responders than among the non-responders ($P = 0.035$, **FIG. 2**).

Changes in serum HBsAg and HBcrAg levels

As shown in **Supplementary FIG. S1**, serum HBsAg levels did not differ between the short-term responders and non-responders during sequential therapy and subsequent long-term follow-up. Serum HBcrAg levels were significantly higher in the short-term non-responders than in the responders at the end of sequential therapy ($P =$

0.017) and 24–48 weeks post-treatment ($P = 0.033$), but decreased in response to subsequent therapy to levels comparable to those in responders at the most recent hospital visit.

To evaluate the suppressive effects of subsequent therapy on HBsAg and HBcrAg levels, we classified patients into three groups (**Supplementary FIG. S2**): Patients 1–7 were drug-free (drug-free group), Patients 8–15 were placed on nucleos(t)ide analogues only (NUC group), and Patients 16–24 were also given pegylated or non-pegylated IFN sequentially (IFN group) to attain a drug-free state. The baseline characteristics of the patients in the treatment groups were similar (**Supplementary Table S1**). **FIG. 3** shows the changes in serum HBsAg and HBcrAg levels during and after sequential therapy between the two groups according to the subsequently used therapy agents. Although not a significant difference, the median decrease in HBsAg from baseline was larger in the IFN group than in the NUC group (1.4 vs. 0.32 log, $P = 0.10$) at the most recent visit; this was also true for HBcrAg (3.2 vs. 1.7 log, $P = 0.12$), despite a shorter total treatment duration in the IFN group than in the NUC group (5.3 vs. 7.5 years, $P = 0.39$).

In Patient 19 (**FIG. 4**), HBsAg seroclearance was achieved through repeated use of IFN after the initial sequential therapy was completed. Since the 41-year-old man relapsed following initial sequential therapy, sequential therapy was reintroduced. However, as acute exacerbation occurred during IFN-β administration, lamivudine was started for the third time, and adefovir dipivoxil was added to treat breakthrough hepatitis. As the HBsAg titer was reduced after approximately 5 years of lamivudine and adefovir therapy, the combination was switched to pegylated IFN-α, which resulted in HBsAg seroclearance.

Unfortunately, hepatocellular carcinoma developed in Patients 8 and 21 at 4.2 and 7.0 years after the end of sequential therapy, respectively.

Discussion

In the initial study [13], the rate of response to sequential therapy with lamivudine and IFN-β in HBeAg-positive patients with chronic HBV genotype C infection was limited (29%). However, we found that patients who were young or who lost HBeAg and HBV DNA during lamivudine therapy were more likely to have a sustained response to sequential therapy. These findings have been confirmed by other authors [14,15]. The present study showed that the response to sequential therapy with lamivudine and IFN 1 year post-treatment was sustainable thereafter, as was the response to IFN monotherapy [41–44]. The guidelines [28], which recommended to assess a sustained response 12 months post-treatment or later, seem to be valid. Taken together, the initial and present studies suggest that in patients with chronic hepatitis B who responded favorably to a nucleos(t)ide analogue, sequential therapy followed by IFN might be beneficial in the long-term.

Reijnders *et al.* [45] reported that pegylated IFN-α induced a larger HBsAg decline than did entecavir, whereas entecavir induced a larger HBV DNA decline than did IFN-α. Combining a nucleos(t)ide analogue and IFN with different mechanisms of action is theoretically a reasonable approach for treating chronic hepatitis B. In large randomized controlled trials [5,6], the 1-year concomitant combination of lamivudine with IFN-α had little clinical benefit compared with IFN-α monotherapy in terms of the rates of off-treatment response 24 weeks post-treatment. However, in a long-term follow-up study, patients treated with IFN-α and lamivudine were significantly more likely to have undetectable HBV DNA than those treated with IFN-α alone [43]; the long-term efficacy of combination therapy is still controversial.

One objective of sequential therapy starting with a nucleos(t)ide analogue is to lower the viral load before beginning IFN therapy, thereby restoring treatment

sensitivity, since low HBV DNA levels are associated with a favorable response to IFN. Basic studies have suggested that a high viral load is associated with an inefficient T cell response [46], and treatment with nucleos(t)ide analogues can restore CD4 followed by a CD8 cellular immune response against HBV [47].

Another objective of sequential therapy is to prevent biochemical and virological relapse after discontinuing nucleos(t)ide analogues through the use of IFN. The high risk of relapse after treatment may be attributed to the persistence of covalently closed circular DNA in the liver, which is correlated with HBsAg and HBcrAg levels in the serum. Since seroclearance of HBsAg is associated with a reduced risk of disease progression, it is the ideal end point of anti-HBV therapies. However, the rate of HBsAg seroclearance in Asian patients is much lower than that of non-Asian patients, probably due to perinatal infection with predominantly genotype C. In Japan, the annual incidence of spontaneous HBsAg seroclearance was reported to be 0.97–1.65% in chronic HBV carriers [48,49]. In this study, HBsAg seroclearance was achieved in only one patient (**FIG. 4**), who was given IFN repeatedly following nucleos(t)ide analogues.

The HBcrAg assay measures the serum levels of all antigens transcribed from the pre-core/core gene, including a 22kDa precore protein, hepatitis B core and e-antigens, using monoclonal antibodies that recognize the common epitopes of the denatured antigens [32,33]. By scoring the HBsAg and HBcrAg levels, Matsumoto *et al.* [50] proposed a model for predicting relapse of hepatitis after discontinuation of nucleos(t)ide analogue therapy. In the present study, among patients who relapsed after sequential therapy and received retreatment with nucleos(t)ide analogues, the decreases in HBsAg and HBcrAg levels from baseline were larger in patients sequentially treated with IFN than in those who were not. The results suggested that repeated use of IFN may effectively lower HBsAg and HBcrAg levels to attain a drug-free state.

In this study, we used IFN-β for 24 weeks following approximately 24 weeks of lamivudine administration during the initial sequential therapy. During the study period, IFN-β was commonly used in Japan as well as IFN-α to treat chronic hepatitis B and C. Both IFN-α and -β are type I IFNs that bind to a common receptor and initiate the Janus kinase/signal transducer, activating the transcription signaling cascade leading to the transcription of IFN-stimulated genes [51,52]. The efficacy of IFN-β is clinically comparable to that of IFN-α, although the efficacy of the non-pegylated IFNs is inferior to that of pegylated IFNs [53].

In general, younger age, female sex, higher ALT levels, lower HBV DNA levels, and HBV genotypes A and B are associated with a higher probability of sustained response to IFN therapy [54,55]. Additionally, single nucleotide polymorphisms located near the *IL28B* gene have recently attracted interest. Large genome-wide association studies revealed an association between *IL28B* gene polymorphisms and responses to IFN and ribavirin in patients with chronic hepatitis C [39,40]. Some groups have evaluated the association between the *IL28B* genotype and IFN response in chronic hepatitis B, but the results have been conflicting [56–58]. Further studies are needed to clarify the significance of the *IL28B* genotype in sequential therapy.

This study has some limitations. First, it was not a randomized controlled trial. In previous studies comparing the lamivudine/IFN combination and IFN monotherapy [5,6], combination therapy showed greater on-treatment viral suppression, but no difference in the off-treatment response was observed. Controlled trials are necessary to confirm whether sequential therapy confers an additional benefit compared with monotherapy for treating chronic hepatitis B. Second, the sample size was relatively small. However, our patients were followed for up to 9.5 years, and over 80% of them (20/24) underwent follow-up for longer than 5 years after the end of sequential therapy.

To our knowledge, this is the first report of the long-term outcomes achieved using sequential therapy with a nucleos(t)ide analogue and IFN in patients with chronic hepatitis B.

In summary, the rate of response (defined as ALT normalization, HBeAg loss and an HBV DNA level < 10^4 copies/ml) to sequential therapy was limited (21%) in HBeAg-positive patients with chronic HBV genotype C infection 24 weeks post-treatment, but patients who were young or who had a favorable virological response to lamivudine therapy were more likely to have a short-term response. Also, the majority of patients with a short-term response 24 weeks post-treatment sustained the response and remained drug free 1 year post-treatment and thereafter. At the most recent visit, serum HBsAg and HBcrAg levels were suppressed more effectively in patients subsequently given IFN than in patients given nucleos(t)ide analogues only. Repeated use of IFN following nucleos(t)ide analogues may be beneficial for lowering HBsAg and HBcrAg levels to attain a drug-free state.

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TABLE 1 Baseline Characteristics of the Patients Classified According to the Short-term Response to the Initial Therapy 24 Weeks Post-treatment

Variables	*Responders* (n = *5)*	*Non-responders* (n = *19)*	P *value*
Age (years)[b]	29 ± 7	39 ± 11	0.039
Sex (M/F)[a]	5/0	16/3	0.99
ALT (IU/l)[c]	226 (77, 453)	123 (68, 154)	0.15
HBsAg (log_{10} IU/ml)[b]	3.62 ± 0.54	3.66 ± 0.58	0.92
HBcrAg (log_{10} IU/ml)[b]	7.04 ± 0.84	7.40 ± 1.33	0.29
HBV DNA (log_{10} copies/ml)[b]	7.2 ± 1.2	7.8 ± 0.9	0.24
Precore (wild/mixed/mutant)[a]	4/1/0	8/10/1	0.31
Basal core promoter (wild/mixed/mutant)[a]	1/1/3	3/3/13	0.94
IL28B genotype at rs8099917 (TT/TG/GG)[a*]	3/0/0	13/2/0	0.99
Grade of inflammation (mild/moderate/severe)[a*]	0/0/2	1/5/7	0.46
Stage of fibrosis (mild/moderate/severe/cirrhosis)[a*]	0/0/1/1	4/8/1/0	0.014

[a]Numbers of patients; [b]Mean ± SD; [c]Median (interquartile range).

ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; HBV, hepatitis B virus; *IL28B*, interleukin-28B.

[*]The *IL28B* genotype was not determined in six patients, and liver histology was not determined in nine, because informed consent was not obtained.