available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Quantitative measurement of HBsAg was done using an HISCL® HBsAg assay based on the chemiluminescence enzyme immunoassay (CLEIA) (Sysmex Co. Ltd., Kobe, Japan), which had a quantitative range from −1.5 to 3.3 log IU/ml. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. Changes in HBsAg levels during the natural course of HBV infection were calculated as: difference in HBsAg level at baseline and at last visit (not undergoing NA treatment) divided by the corresponding follow-up time. Results were expressed as log change per year. Points when patients were negative for HBsAg were omitted in calculations; thus, three patients who had cleared HBsAg by the first follow-up were excluded from the study. Changes in HBsAg levels during NA treatment were calculated similarly using the differences in HBsAg levels between the start and either the end of NA treatment or the last visit.

Serum HBcrAg levels were measured using a CLEIA-based HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously [11]. Briefly, 150 µl of serum was incubated with 150 µl of pretreatment solution containing 15% sodium dodecyl sulphate at 60°C for 30 min. After heat treatment, 120 µl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture (HB44, HB61, and HB114) against denatured HBcAg, HBeAg, and the 22 kDa precore protein. [12] After 10 min of incubation at 37°C and washing, further incubation was carried out for 10 min at 37°C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After washing, 200 µl of substrate solution was added to the test cartridge, which was then incubated for 5 min at 37°C. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids −10 to 183 of the precore/core gene product). The immunoreactivity of pro-HBeAg at 10 fg/ml was defined as 1 U/ml. HBcrAg was expressed in terms of log U/ml, and the quantitative range was set at 3.0–6.8 log U/ml.

Serum concentration of HBV DNA was determined using an AccuGene m-HBV kit (Abbott Japan Co., Ltd.) with a quantitative range of 1.7–9.5 log copies/ml when tested in a sample volume of 0.2 ml. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami et al. [13].

Statistical analyses

Correlations between variables were calculated using the Spearman correlation coefficient test. The Fisher's exact and Pearson's Chi-square tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann–Whitney *U* test was employed. To compare paired continuous data, the Wilcoxon signed-rank test for matched pairs was used. The Kaplan–Meier method was used to estimate positive rates of HBsAg and the occurrence rate of HCC. Multivariate analyses were performed using the Cox regression model. Variables associated with a P value of <0.2 in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with clearance of HBsAg. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

Results

Follow-up of patients

Twenty three (23%) of the 101 patients enrolled dropped out of the study for reasons of changing addresses (11 patients) or halting hospital visits (12 patients). Among the remaining 78 patients, six died (four from HCC, one from hepatic failure, and one from old age) and one underwent liver transplantation due to hepatic failure. Thus, 71 patients completed the full follow-up period of 10 years.

Long term treatment with NAs, such lamivudine, was introduced in 23 patients (23%) during the study period.

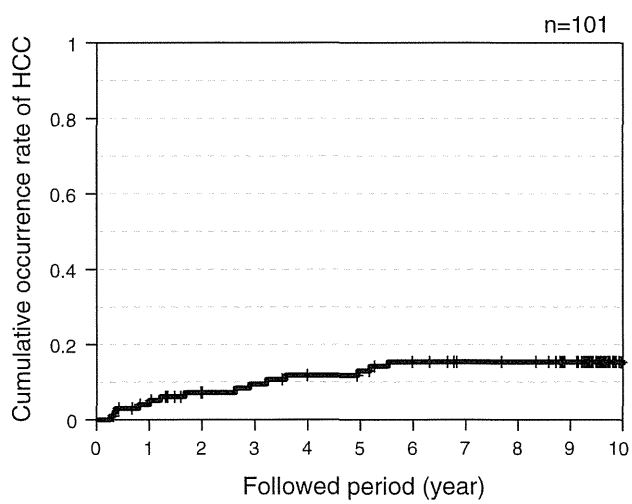

Fig. 1 Changes in the cumulative occurrence rate of HCC during the follow-up period

These patients commenced treatment after showing clinical and/or histological features of chronic active hepatitis B. The treatment period with NAs was excluded from our analysis of HBsAg level changes during the natural disease course.

Complicating HCC was seen in 14 patients within 6 years of their first visit (Fig. 1), leading to an annual rate of HCC occurrence of 2.3% per year for the first 6 years of follow-up. HCC was seen after the disappearance of HBsAg in a 90-year-old woman with negative HBeAg and HBV DNA at the time of diagnosis.

HBsAg levels at baseline and during clinical course

Baseline HBsAg levels ranged from −1.4 to 5.32 log IU/ml, with a median value of 3.2 log IU/ml (Fig. 2). Table 2 shows a comparison of clinical and virological characteristics between patients with lower and higher HBsAg levels divided at the median level. Older patients were significantly more prevalent in the lower level group than in the higher level group. Genotype B was only seen in the lower level group. Detection of HBeAg and median levels of both HBV DNA and HBcrAg were significantly lower in the lower level group. Clearance of HBsAg during the follow-up period was more frequent in the lower level group, but the occurrence of HCC was comparable between the two groups. Of the 52 patients with higher HBsAg levels at baseline, five lost HBsAg positivity and the remaining 47 did not. Median levels of HBV DNA (3.2 vs. 6.0 log copies/ml, $P = 0.023$), HBsAg (3.5 vs. 3.8 log IU/ml, $P = 0.091$), and HBcrAg (3.2 vs. 5.5 log U/ml, $P = 0.095$) tended to be lower in the former than in the latter group of patients at baseline, but the difference was statistically significant for HBV DNA level only. Median age (49 vs. 46 years, $P > 0.2$), male gender (80 vs. 45%, $P = 0.183$), and median ALT level (32 vs. 40 IU/L, $P > 0.2$) did not differ between the two groups at baseline.

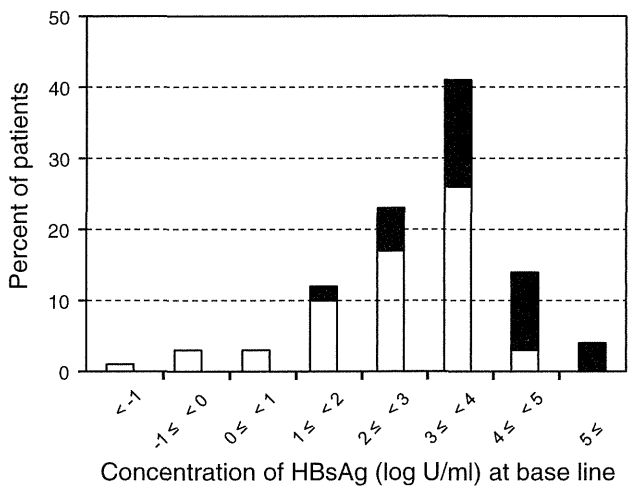

Fig. 2 Distribution of HBsAg concentration at baseline. *Closed bars* indicate patients with detectable HBeAg and *open bars* indicate those without

HBsAg levels at baseline were further analyzed according to age and HBeAg status, and the trend of HBsAg distribution was compared to those of HBcrAg and HBV DNA (Fig. 3). HBsAg levels in HBeAg-positive patients were distributed in a higher range, and the association of HBsAg with age was faint ($r = -0.291$, $P = 0.076$). On the other hand, HBsAg levels were distributed in a higher range in patients younger than 50 years of age, but were distributed more widely in patients 50 years or older. HBsAg levels in HBeAg-negative patients decreased significantly ($r = -0.453$, $P < 0.001$) with age. Furthermore, whereas HBcrAg levels in HBeAg-positive patients ($r = -0.260$, $P = 0.115$) were distributed in a higher range among all

Table 2 Comparison of clinical and virological characteristics between patients with serum HBsAg levels less than 3.2 log IU/ml and those with levels equal to or higher than 3.2 log IU/ml

Characteristic	HBsAg level at baseline		*P*
	<3.2 log IU/ml ($n = 49$)	≥3.2 log IU/ml ($n = 52$)	
At baseline			
Age (years)[a]	55 (32 to 83)	45 (15 to 72)	<0.001
Male[b]	32 (65%)	25 (48%)	0.108
With cirrhosis[b]	13 (27%)	6 (12%)	0.075
ALT (IU/L)[a]	28 (10 to 119)	39 (12 to 447)	0.089
HBV genotype (A:B:C:UD)	1:9:38:1	2:0:49:1	0.018
HBeAg[b]	11 (22%)	27 (52%)	0.004
HBcrAg (log U/ml)[a]	3.3 (<3.0 to >6.8)	5.5 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	3.7 (<1.7 to 8.3)	6.0 (neg. to >9.5)	0.001
During follow-up			
Followed period (years)[a]	4 (1 to 10)	8 (1 to 10)	0.001
Clearance of HBsAg[b]	15 (31%)	5 (10%)	0.012
Occurrence of HCC[b]	9 (18%)	5 (10%)	>0.2
Introduction of NAs[b]	9 (18%)	14 (27%)	>0.2

UD undetermined

[a] Data are expressed as median (range)

[b] Data are expressed as positive number (%)

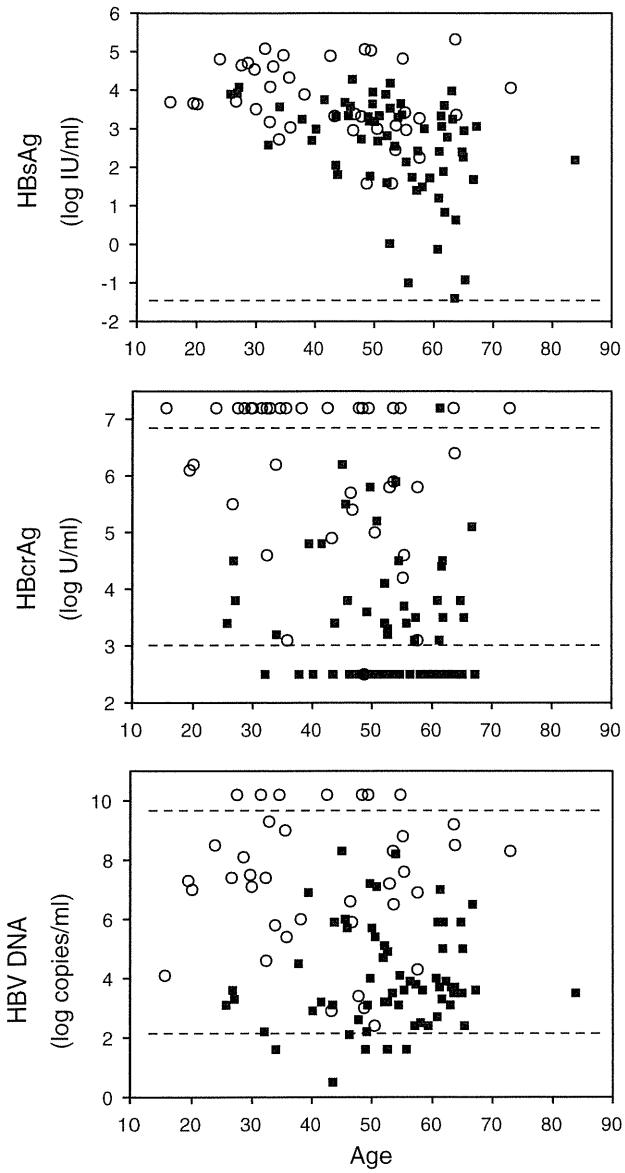

Fig. 3 HBsAg, HBcrAg, and HBV DNA levels analyzed according to HBeAg status and patient age. *Open circles* indicate patients with detectable HBeAg and *closed squares* indicate those without

ages, those in HBeAg-negative patients ($r = -0.103$, $P > 0.2$) were found in a lower range. A similar trend was seen for HBV DNA level distribution ($r = 0.015$, $P > 0.2$ and $r = 0.146$, $P > 0.2$, respectively).

Changes in HBsAg levels during the follow-up period

Positivity for HBsAg decreased gradually over the follow-up period (Fig. 4). A total of 20 patients cleared HBsAg during the follow-up period, for a disappearance rate of 2.1% per year. Clinical and virological backgrounds were compared between patients with and without clearance of HBsAg in Table 3. Patients losing HBsAg positivity were

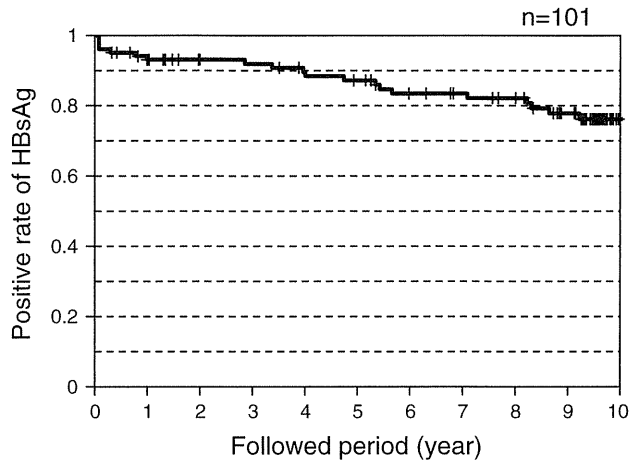

Fig. 4 Changes in HBsAg positivity during the follow-up period

significantly older than those who did not. Baseline levels of HBsAg, HBcrAg, and HBV DNA were significantly lower in these patients as well. Clearance of HBsAg was significantly associated with HBV DNA (HR 3.6, 95% CI 1.1–11.4, $P = 0.033$) and HBcrAg (HR 4.0, 95% CI 1.1–14.9, $P = 0.036$) levels at baseline by multivariate analysis. Of the 20 patients who cleared HBsAg, seven were positive for HBV DNA (range, positive 3.0 log copies/ml) and three were positive for HBcrAg (range 3.0–3.2 U/ml).

Figure 5 shows the distribution of patients according to the rate of change of HBsAg levels. Of the 98 patients analyzed, 79 (81%) showed a decrease in HBsAg. Although this level increased in 19% of patients, such changes were less than 0.2 log IU/year. The rate of change of HBsAg levels peaked at a cut-off value of −0.4 log IU/year. Accordingly, patients were tentatively classified into the rapid decrease group (rate of change <-0.4 log IU/year) and the non-rapid decrease group (rate of change ≥ -0.4 log IU/year). Median age, gender distribution, prevalence of cirrhosis, ALT level, and genotype distribution did not differ between the two groups (Table 4). Levels of HBsAg, HBcrAg, and HBV DNA were significantly lower in the rapid decrease group than in the non-rapid one. Whereas all patients with persistently positive HBeAg were classified into the non-rapid group, patients with persistently negative HBeAg fell more frequently into the rapid decrease group (77%) than into the non-rapid decrease group (54%). In those patients, HBV DNA levels were significantly ($P = 0.028$) lower in the rapid decrease group (median 3.4, range <2.1–5.9 log copies/ml) than in the non-rapid decrease group (median 3.8, range <2.1–8.1 log copies/ml). Complicating HCC was lower in the rapid decrease group, but this difference was not statistically significant.

The median change in HBsAg level before NA treatment (−0.117 log IU/ml/year; range −2.4 to 1.41 log

Table 3 Comparison of clinical and virological characteristics between patients with and without clearance of HBsAg

Characteristic	Clearance of HBsAg		*P*
	Positive ($n = 20$)	Negative ($n = 81$)	
At baseline			
Age (years)[a]	56 (30 to 65)	50 (16 to 84)	0.038
Male[b]	8 (40%)	36 (44%)	>0.2
With cirrhosis[b]	4 (20%)	15 (18%)	1.000
ALT (IU/L)[a]	26 (10 to 108)	35 (13 to 447)	0.057
HBV genotype (A:B:C:UD)	0:2:18:0	3:7:69:2	>0.2
HBeAg[b]	3 (15%)	35 (43%)	0.022
HBsAg (log IU/ml)[a]	1.7 (−1.7 to 4.2)	3.3 (0.83 to 5.3)	<0.001
HBcrAg (log U/ml)[a]	3.0 (3.0 to >6.8)	4.7 (3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	3.0 (<1.7 to 7.6)	5.7 (neg. to >9.5)	<0.001
During follow-up			
Followed period (years)[a]	4.4 (0.31 to 10.0)	5.2 (0.1 to 10.0)	>0.2
Occurrence of HCC[b]	1 (5.0%)	13 (16.0%)	>0.2
Introduction of NAs[b]	0 (0%)	23 (28%)	0.006

UD undetermined

[a] Data are expressed as median (range)

[b] Data are expressed as positive number (%)

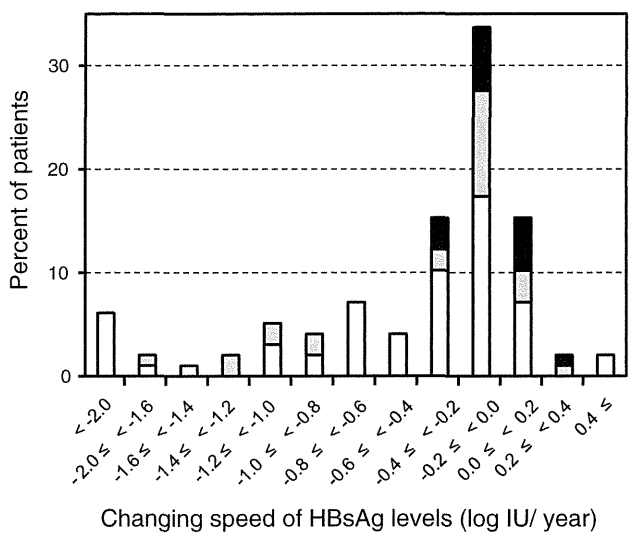

Fig. 5 Distribution of patients classified according to rate of change of HBsAg levels (log IU/year) during follow-up period. *Closed bars* indicate patients with persistent HBeAg-positive status. *Shaded bars* indicate patients who became negative for HBeAg during follow-up period. *Open bars* indicate patients with persistent HBeAg-negative status

IU/ml/year) was similar ($P > 0.2$) to that after starting NA treatment (−0.017 log IU/ml/year; range −5.18 to 0.17 log IU/ml/year) in the 20 patients who commenced therapy with NAs during the study period.

Discussion

During the natural course of HBV infection, HBsAg levels showed almost normal distribution, making a sharp peak at a median value of 3.2 log IU/ml. Lower HBsAg levels were significantly associated with older age and lower viral activity, but not with gender or genotype. A similar trend was observed in patients who cleared HBsAg in our cohort. Chan et al. [10] reported that HBsAg levels were significantly lower in HBeAg-negative patients than in HBeAg-positive ones and tended to fall in accordance with decreases in HBV DNA levels. Simonetti et al. [6] reported that clearance of HBsAg was associated with older age, but not with gender or genotype, in a prospective population-based cohort study. Chu et al. [9] also reported that HBsAg clearance was associated with older age, in which the cumulative probability of clearance increased disproportionally with a longer follow-up period. In light of these results as well as of our own, it appears that lower HBsAg levels are closely associated with older age and lower activity of HBV replication. The HBsAg clearance rate of 2.1% per year in the current study was three times higher than that of the 0.7% per year reported by Simonetti et al. [6]. However, the median age at the start of their follow-up (20 years) was considerably lower than that in our report (50 years). Chu et al. [9] followed 1965 asymptomatic HBV carriers that were positive for HBe antibodies in whom the mean age at baseline was 35.6 years, revealing a HBsAg clearance rate of 0.8% per year after 10 years of follow-up that increased to 1.8% per year over a 25-year observation period. HBsAg clearance appeared to increase as patients aged in that cohort, which may at least partly explain the higher clearance rate found in the present study.

Because HBsAg level is closely associated with age, we analyzed this relationship and compared it with those of HBcrAg and HBV DNA. HBsAg levels decreased in association with age in HBeAg-negative patients. A similar but faint association was also seen in HBeAg-positive patients. On the other hand, HBcrAg and HBV DNA levels

Table 4 Comparison of clinical and virological characteristics between patients with rapid and non-rapid decrease of HBsAg

Characteristic	Rapid decrease ($n = 31$)	Non-rapid decrease ($n = 67$)	P
At baseline			
Age (years)[a]	52 (15 to 65)	49 (19 to 83)	0.338
Male[b]	17 (55%)	38 (57%)	1.000
With cirrhosis[b]	6 (19%)	13 (19%)	1.000
ALT (IU/L)[a]	27 (10 to 108)	36 (13 to 447)	0.230
HBV genotype (A:B:C:UD)	1:4:26:0	2:4:59:2	0.617
HBeAg-positive[b]	7 (23%)	31 (46%)	0.028
HBsAg (log IU/ml)[a]	2.8 (−1.0 to 5.0)	3.3 (0.8 to 5.3)	0.001
HBcrAg (log U/ml)[a]	<3.0 (<3.0 to >6.8)	5.1 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	3.7 (<1.7 to >9.5)	5.9 (neg. to >9.5)	0.002
During follow-up			
Followed period (years)[a]	3 (1 to 9)	6 (1 to 10)	<0.001
Change in HBeAg status			0.012
Persistent positive[b]	0 (0%)	15 (22%)	
Became negative[b]	7 (23%)	16 (24%)	
Persistent negative[b]	24 (77%)	36 (54%)	
Clearance of HBsAg[b]	18 (58%)	0 (0%)	<0.001
Complication of HCC[b]	2 (7%)	12 (18%)	0.214
Introduction of NAs[b]	4 (13%)	19 (28%)	0.125

UD undetermined

[a] Data are expressed as median (range)

[b] Data are expressed as positive number (%)

were more uniformly distributed with age in both HBeAg-positive and -negative patients. Therefore, it can be inferred that HBsAg level is affected by age in the natural course of HBV, even when the factor of viral activity is excluded. The precise mechanism of this trend is at present unclear, but may be attributed to the character of HBsAg itself, and not to that of HBV antigens, because HBcrAg levels showed a similar trend as HBV DNA levels. Chan et al. [10] reported that a stronger correlation between HBV DNA and HBsAg was found in the HBeAg-positive phase than in the HBeAg-negative phase. This observation was clearly confirmed by our results in that the distribution pattern analyzed by age was similar between HBsAg and HBV DNA levels in HBeAg-positive patients but differed in HBeAg-negative ones.

The rate of change of HBsAg in the present study suggested the existence of two groups centered around a value of −0.4 log IU/year. A necessary decline in HBV replication was evident in the rapid decrease group, whose median HBV DNA level was lower than the 4.0 log copy/ml usually seen in inactive carriers of HBV. Since no patient with persistently positive HBeAg was classified into the rapid increase group, we presume that a loss of HBeAg is essential for a rapid decrease in HBsAg. In patients with persistently negative HBeAg, HBV DNA levels were significantly lower in the rapid decrease group than in the non-rapid decrease group. Therefore, not only a loss of HBeAg, but also a decline in HBV replication, appears to be fundamental factors necessary for a rapid decrease in HBsAg. Chan et al. [10] concluded that HBs antigen level remained stable in HBe antigen-positive patients and reduced slowly in HBe antigen-negative patients. Our results are similar, but further imply that a decline in HBV replication is also required. The rate of HBsAg level decrease was similar before and after starting NA treatment in the present study. However, additional studies in larger cohorts will be required to determine this particular relationship.

We analyzed HBcrAg in addition to HBsAg as an HBV-related antigen in the present study to further clarify the characteristics of HBsAg. The HBcrAg assay measures serum levels of HBcAg, HBeAg, and the 22 kDa precore protein [12] simultaneously using monoclonal antibodies that recognize the common epitopes of these denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related [14]. It is possible that levels of HBsAg and HBcrAg have different properties because transcriptions of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome [15]. Recent studies have shown that HBsAg quantification may represent a surrogate marker of cccDNA concentration in the liver and a potential tool to monitor virologic response to interferon treatment [4, 5, 16]. On the other hand, serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during nucleos(t)ide treatment [11, 17, 18], and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs [19, 20] or who had a higher possibility to develop hepatocellular carcinoma even under NA treatment [17]. Our results here suggest that there exists a

difference in natural course changes between HBsAg and HBcrAg levels. We recently reported that the combined use of these two antigens was useful for predicting the occurrence of hepatitis relapse after cessation of NAs [21]. Such results also indicated that levels of HBsAg and HBcrAg had different clinical significance despite the fact that both antigen levels are generally considered to reflect the amount of HBV cccDNA in hepatocytes.

Complicating HCC occurred during the first 6 years of follow-up in our study at an annual occurrence rate of 2.3% per year for that period. This complication was seen at similar frequencies in patients with high and low baseline HBsAg levels as well as in patients who showed rapid and non-rapid decreases in HBsAg. Patients with lower HBsAg levels and those with rapid decreases in HBsAg have been shown to have lower levels of HBV replication, which would indicate a lower risk of complicating HCC. However, such patients also tend to be older and presumably more predisposed to HCC. The similar occurrence of HCC irrespective of HBsAg status may be attributed to the existence of these two contrary factors. Yuen et al. [7] reported that the risk of HCC in patients with HBsAg seroclearance was higher in those older than 50 years of age; indeed, the single patient who developed HCC after HBsAg seroclearance in the present study was a 90 year-old woman.

In conclusion, lower HBsAg levels were significantly associated with older age and lower viral activity, but not with gender or genotype. Both a loss of HBeAg positivity and a decline in HBV replication are suggested to be fundamental factors necessary for a rapid decrease in HBsAg. Furthermore, the clinical significance of HBsAg may be different from that of HBcrAg with regard to age. Future studies are required to clarify the difference between the two antigens.

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Conflict of interest The authors declare that they have no conflict of interest.

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Original Article

Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B

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Aim: The factors associated with hepatitis recurrence after discontinuation of nucleos(t)ide analogs (NAs) in patients with chronic hepatitis B were analyzed to predict the risk of relapse more accurately.

Methods: A total of 126 patients who discontinued NA therapy were recruited retrospectively. The clinical conditions of a successful discontinuation were set as alanine aminotransferase (ALT) below 30 IU/L and serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL.

Results: Relapse of hepatitis B were judged to occur when maximal serum ALT became higher than 79 IU/L or when maximal serum HBV DNA surpassed 5.7 log copies/mL following NA discontinuation since these values corresponded with mean values of ALT (30 IU/L) and HBV DNA (4.0 log copies/mL), respectively. At least 90% of patients with either detectable hepatitis B e antigen or serum HBV DNA higher than 3.0 log copies/mL at the time of NA discontinuation relapsed within one year. In the remaining patients, higher levels of both hepatitis B surface and core-related antigens at the time of discontinuation, as well as a shorter course of NA treatment, were significantly associated with relapse by multivariate analysis.

Conclusions: It appears that negative results for hepatitis B e antigen and serum HBV DNA lower than 3.0 log copies/mL are essential for successful NA discontinuation, which may be attained by a longer treatment period. Levels of hepatitis B surface and core-related antigens are also significant factors independently associated with relapse of hepatitis.

Key words: discontinuation, hepatitis B core-related antigen, hepatitis B surface antigen, nucleos(t)ide analogs, relapse of hepatitis

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, and may eventually develop into liver cirrhosis and hepatocellular carcinoma.[1–3] Over the last decade, major advances in the treatment of chronic hepatitis B have been made with nucleos(t)ide

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analogs (NAs) such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV).[4] NAs are orally administered and are associated with low rates of adverse effects. Treatment with NAs shows strong suppression of HBV replication and consequently rapid improvement of elevated ALT levels. Furthermore, these drugs have been reported to lower the risk of complicating cirrhosis and hepatocellular carcinoma,[5–7] and so NAs are becoming widely used to treat patients with chronic hepatitis B. On the other hand, NAs carry the risk of developing drug-resistance;[8] drug-resistant viruses emerging during treatment may be associated with hepatitis flare-ups. Hepatitis B patients are also required to undergo prolonged treatment with NAs because early discontinuance often leads to relapse of hepatitis and ensuing hepatic failure following rises in alanine aminotransferase (ALT) level.[9,10]

Serum HBV DNA is normally used to monitor the antiviral effect of NAs. HBV DNA decreases rapidly and becomes undetectable in the majority of patients who are treated with NAs,[11–13] but relapse after discontinuation is not rare.[14–17] Since it is also true that favorable virological and biochemical responses to NAs may continue indefinitely in some patients,[9,15] reliable markers that can predict relapse of hepatitis after NA discontinuation are needed. Such markers would benefit not only patients who are considering discontinuation of NA treatment, but also clinicians, hospitals, and the medical economy.

In the present study, we assessed several factors associated with relapse of hepatitis after discontinuation of NAs in patients with chronic hepatitis B, including hepatitis B viral antigens, which have been reported as new and promising markers for monitoring the effect of antiviral agents, such as interferon and NAs.

METHODS

Patients

A TOTAL OF 126 patients with chronic hepatitis B who underwent and completed NA treatment between 2000 and 2010 were enrolled in this study. Patients were recruited retrospectively from 11 hospitals across Japan (Toranomon Hospital, Hokkaido University Hospital, Nagoya City University Hospital, Shinshu University Hospital, Hiroshima University Hospital, National Hospital Organization Nagasaki Medical Center, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Japanese Red Cross Nagoya Daini Hospital, and Tokyo Women's Medical University Hospital, Sapporo Kosei General Hospital) and met the following conditions: (i) serum ALT higher than 30 IU/L and serum HBV DNA higher than 4.0 log copies/mL were observed at least twice within the 6 months prior to administration of NAs; (ii) stored serum samples at initiation and discontinuation of NAs were available for measurements of viral markers; (iii) clinical outcomes were followed for at least 6 months after the discontinuation of NAs; and (iv) tests for hepatitis C and human immunodeficiency virus antibodies were negative. Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions at least 6 months apart in all patients before treatment. Patients complicated with hepatocellular carcinoma or signs of hepatic failure at treatment discontinuation were excluded from the study. Our cohort consisted of 83 men and 43 women with a median age of 46 (range, 19 to 79) years when NA administration was discontinued. Hepatitis B e antigen (HBeAg) was positive in 64 patients (51%) at the initiation of treatment and in 24 patients (19%) at its discontinuation. HBV genotype was A in two (2%) patients, B in five (4%), C in 102 (81%), and undetermined in 17 (13%). Thirty-five of the 126 patients in this study were younger than 35 years old. Although not recommended as the first line treatment for this group by Japanese guidelines,[18] NA treatment was commenced since chronic active hepatitis had been persisting in all cases irrespective of their HBeAg status (26 positive and nine negative) at the initiation of treatment.

The decision to discontinue NAs was made by individual physicians using similar, but not uniform, conditions. Four patients who halted NAs for financial reasons were included. No patient underwent interferon treatment during or after NA treatment. The decision to recommence NA administration was also made by individual physicians, essentially when relapse of hepatitis became obvious. With few exceptions, patients were seen at least once a month during the first year after discontinuation of NAs, and at least once every several months afterwards. Stored serum samples were kept frozen at −20°C or below until assayed. This study was approved by the Ethics Committees of all participating institutions.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[19] was done using a chemiluminescence enzyme immunoassay

(CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche, Tokyo, Japan),[20] which had a quantitative range of 2.6 to 7.6 log copies/mL. Serum HBV DNA was also determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 9.0 log copies/mL in 43 patients whose serum samples were available at the time of NA discontinuation. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was described as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[22]

Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously.[23,24] Briefly, 150 μL of serum was incubated with pretreatment solution and then added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After incubation and washing, further incubation was carried out with alkaline phosphatase conjugated with two kinds of monoclonal antibodies against denatured HBcAg, HBeAg, and the 22 kDa precore protein. Following washing, a substrate solution was added to the test cartridge and then incubated. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0 to 6.8 log U/mL.

Statistical analyses

A linear regression model was used to examine for associations between mean and maximal values of both ALT and HBV DNA. Correlations between variables were calculated using the Spearman's rank correction correlation coefficient test. Each cut-off value was decided using receiver operating characteristic curve (ROC) analysis and results were evaluated by measuring the area under the curve (AUC). The Fisher's exact and Pearson's χ^2 tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann–Whitney *U*-test was used. The Kaplan–Meier method was used to estimate rates of non-relapse observations, and the log-rank test was used to test hypotheses concerning differences in non-relapse observations between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a *P*-value < 0.2 in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with relapse of hepatitis after discontinuation of NAs. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Definition of hepatitis relapse after discontinuation of NAs

THE CLINICAL CONDITIONS of a successful discontinuation of NAs were set at serum HBV DNA below 4.0 log copies/mL and ALT below 30 IU/L according to the Japanese guidelines for the treatment of hepatitis B.[18] However, these criteria could not be directly applied to our cohort as post-therapy fluctuations in ALT and HBV DNA were difficult to evaluate consistently. In total, 26 (76%) of 34 patients with successful discontinuation of NAs showed transient abnormal levels of ALT and/or HBV DNA, especially during the early phase after cessation. We therefore used mean and maximal values of these markers to evaluate relapse of hepatitis B in this study; mean values were used to evaluate relapse of hepatitis as a whole, and maximal values were used to dynamically assess relapse during the follow-up period after NA discontinuation. Both ALT and HBV DNA were measured 11.0 times per year on average during the first year and 4.1 times per year on average thereafter.

The mean values of HBV DNA were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.853. Similarly, the mean values of ALT were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.940 (Fig. 1). The mean HBV DNA value of 4.0 log copies/mL corresponded to a maximal HBV DNA value of 5.7 by ROC analysis (AUC = 0.930, $P < 0.001$), and the mean ALT value of 30 IU/L corresponded to a maximal ALT value of 79 IU/L (AUC = 0.988, $P < 0.001$). These results suggested that patients having serum HBV DNA higher

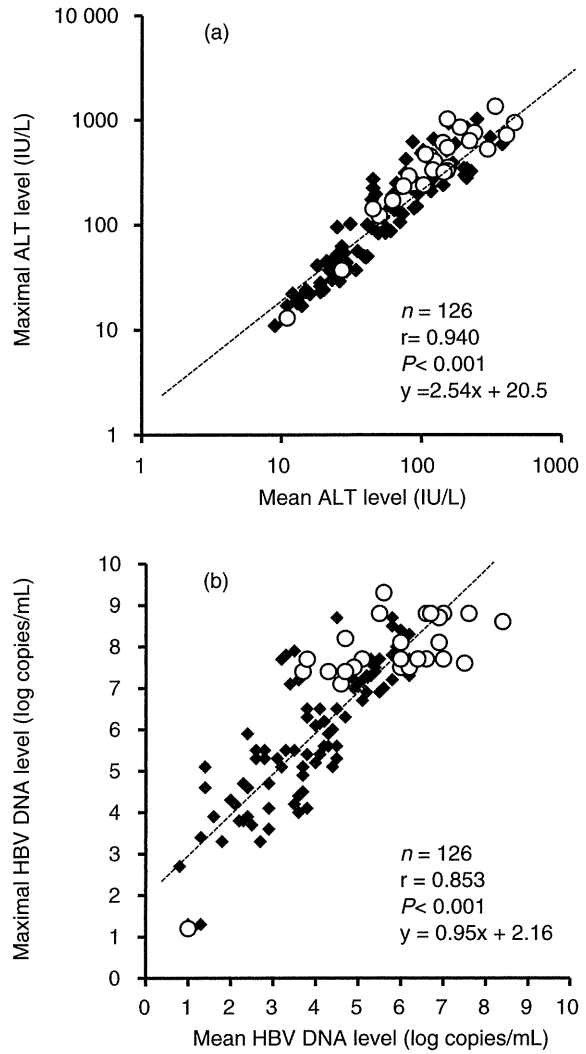

Figure 1 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (a) and hepatitis B virus (HBV) DNA (b) after discontinuation of nucleos(t)ide analogs (NAs). Open circles indicate patients with detectable hepatitis B e antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

than 5.7 log copies/mL during the follow-up period after NA discontinuation were not likely to achieve the HBV DNA criterion of a successful discontinuation of below 4.0 log copies/mL. Similarly, it could be inferred that patients reaching ALT levels higher than 79 IU/L would also not likely achieve the ALT criterion of a successful discontinuation of below 30 IU/L.

Based on our findings, we judged that a relapse of hepatitis B occurred when serum ALT exceeded 79 IU/L or when serum HBV DNA exceeded 5.7 log copies/mL following NA discontinuation. Accordingly, 92 (73%) of the 126 patients enrolled in the present study showed a relapse. We set the follow-up period as discontinuation to relapse for relapse patients and as discontinuation to the last recorded examination for patients without relapse. Whereas re-administration of NAs due to relapse was commenced in 70% of relapse patients in the follow-up period, none was performed in non-relapse patients during that time.

Elimination of cases likely to show relapse of hepatitis

As it is generally believed that patients who are positive for HBeAg and/or have a higher level of HBV DNA at discontinuation of NAs are likely to relapse, these factors were assessed first. The progression of analyses in the present study and the population structure of each analysis are shown in Figure 2.

The non-relapse rate was compared using the Kaplan–Meier method between 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL and 95 patients with levels lower than 3.0 log copies/mL when NAs were discontinued (Fig. 3). The revised cut-off value of 3.0 log copies/mL was determined by ROC analysis (AUC = 0.709, $P < 0.001$). Thirty (97%) of 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL relapsed within one year of discontinuation. On the other hand, approximately 30% of patients with levels lower than 3.0 log copies/mL showed prolonged non-relapse. Thus, the 31 patients with high HBV DNA at the time of discontinuation were eliminated from the following analyses.

In the remaining 95 patients, the non-relapse rate was compared using the Kaplan–Meier method between 10 patients with detectable HBeAg and 85 patients without HBeAg when NAs were discontinued (Fig. 4). Ninety percent of patients with HBeAg experienced relapse within one year, which was significantly ($P = 0.005$) higher than in cases without HBeAg. In patients without HBeAg, the non-relapse rate decreased rapidly during the first year to approximately 45%, and then decreased relatively slowly over the following 3 years to nearly 30%. It is noteworthy that this subgroup did not relapse afterwards. Since the relapse rate was high among patients with detectable HBeAg, they were excluded from the following analyses as well.

Factors associated with relapse of hepatitis after discontinuation of NAs

Additional factors associated with relapse of hepatitis were analyzed in the remaining 85 patients who were

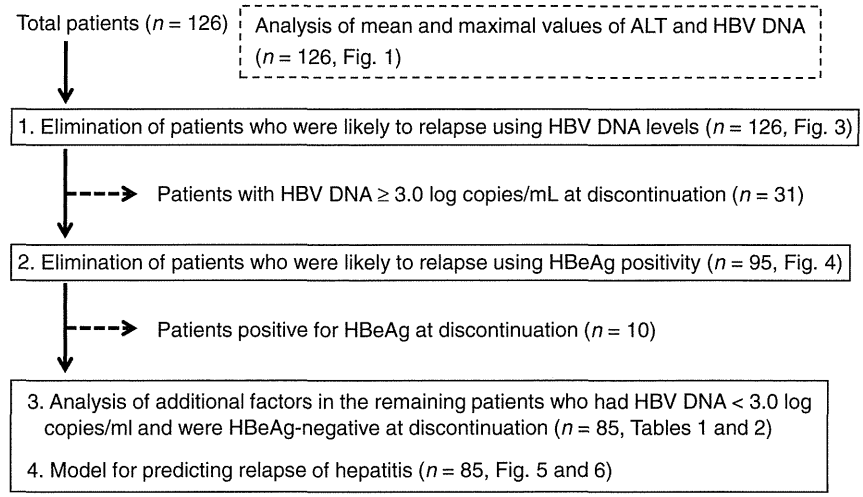

Figure 2 The progression of analyses in the present study and population structure of each analysis.

both negative for HBeAg and whose serum HBV DNA was lower than 3.0 log copies/mL at NA cessation. Table 1 shows the comparison of clinical and virological backgrounds between the 53 relapse and 32 non-relapse patients using univariate analysis. Age and gender distributions were similar between the groups. Approximately 75% of the 85 patients had HBV genotype C, but the distribution of genotypes did not differ between the groups. Approximately 90% of patients were being treated with LVD alone at the time of discontinuation, compared with 6% of patients being given ETV. The median duration of NA treatment was about two times longer in patients without relapse. Levels of both HBsAg and HBcrAg were significantly lower in non-relapse patients than in relapse patients at the time of NA discontinuation. The difference between serum HBsAg was also significant at the initiation of NAs, but not that of HBcrAg. As only patients with HBV DNA lower than 3.0 log copies/mL were analyzed, the majority of these cases showed levels below the 2.6 log copies/mL lower detection limit of the Amplicor assay at NA discontinuation. We therefore also tested HBV DNA with a TaqMan assay, which had a higher sensitivity than the Amplicor assay, in 43 patients whose serum samples were available. The prevalence of patients having a negative detection signal did not differ between the two groups. The number of

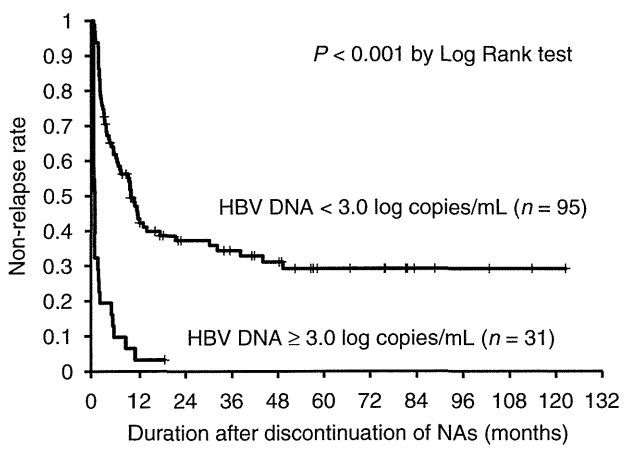

Figure 3 Comparison of non-relapse rates using the Kaplan–Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

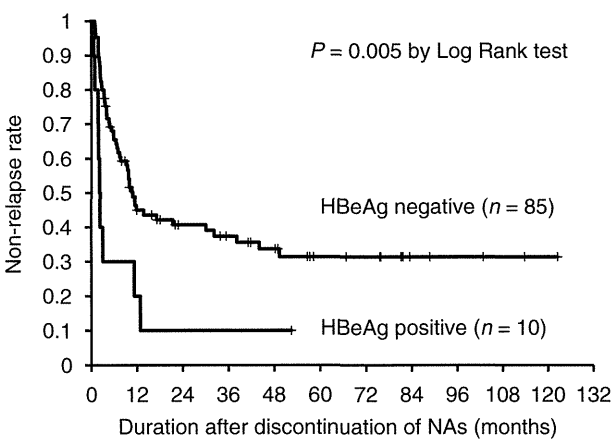

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

Table 1 Comparison of clinical and virological backgrounds between patients with and without relapse of hepatitis at initiation and discontinuation of nucleos(t)ide analogs (NAs)

Background	Non-relapse patients ($n = 32$)	Relapse patients ($n = 53$)	*P*-value
At initiation of NAs			
Age (years)†	47 (17–75)	48 (26–74)	>0.2
Gender (M : F)	23:9	32:21	>0.2
ALT (IU/L)†	183 (9–1182)	187 (20–2052)	>0.2
Genotype (A : B : C : UD)	1:2:21:8	0:3:44:6	0.193
HBeAg (positive)‡	11 (34%)	16 (30%)	>0.2
HBV DNA			
Amplicor assay (log copies/mL)†	6.2 (<2.6->7.6)	6.5 (<2.6->7.6)	0.099
HBsAg (log IU/mL)†	2.7 (0.1–4.3)	3.3 (1.6–3.9)	0.018
HBcrAg (log U/mL)†	5.2 (<3.0->6.8)	5.6 (<3.0->6.8)	>0.2
At discontinuation of NAs			
Age (years)†	50 (21–78)	49 (26–79)	>0.2
NAs (LVD : LVD+ADV : ETV : ADV)	28:1:3:0	50:0:2:1	>0.2
Duration of NA treatment (months)†	36 (4–129)	17 (4–84)	0.007
Follow-up period after discontinuation of NAs (months)†	45 (6–123)	12 (1–111)	0.002
ALT (IU/L)†	16 (7–38)	20 (9–65)	0.002
HBV DNA			
Amplicor assay (log copies/mL)†	<2.6 (<2.6–2.9)	<2.6 (<2.6–2.9)	>0.2
TaqMan assay (negative signal)‡	5 (23%) ($n = 22$)	3 (14%) ($n = 21$)	>0.2
TaqMan assay (negative or positive signal)‡	13 (59%) ($n = 22$)	13 (62%) ($n = 21$)	>0.2
HBsAg (log IU/ml)†	2.0 (<–1.5–4.3)	3.1 (0.6–4.0)	0.001
HBcrAg (log IU/mL)†	3.4 (<3.0–4.9)	4.3 (<3.0->6.8)	0.003

†Data are expressed as the median (range)
‡Data are expressed as a positive number (%)
ADV; adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LVD, lamivudine; UD, undetermined.

patients with a negative detection signal or a positive signal also did not vary significantly. The follow-up period after discontinuation of NAs was significantly shorter in patients with relapse than in those without because formal follow-up ended once patients relapsed. The median period of follow-up was 45 months in patients without relapse.

Multivariate analyses revealed that a shorter duration of NA treatment and higher levels of HBsAg and HBcrAg at discontinuation were significantly associated with the occurrence of hepatitis relapse (Table 2). The cut-off values that showed the highest significance by ROC analysis were 1.9 log IU/mL for HBsAg (AUC = 0.707, $P = 0.001$), 4.0 log U/mL for HBcrAg (AUC = 0.692, $P = 0.003$), and 16 months (AUC = 0.674, $P = 0.007$) for treatment duration.

Model for predicting relapse of hepatitis using levels of HBsAg and HBcrAg

The existence of a second cut-off value was suggested by ROC analysis for both of HBsAg (2.9 log IU/mL) and HBcrAg (3.0 log IU/mL) to discriminate between

Table 2 Multivariate analysis of factors associated with relapse of hepatitis after discontinuation of nucleos(t)ide analogs (NAs)

Factor	Hazard ratio	95%CI	*P*-value
HBsAg at discontinuation ≥ 1.9 log IU/mL	5.21	1.87–14.55	0.002
HBcrAg at discontinuation ≥ 4.0 log U/mL	2.20	1.25–3.87	0.006
Duration of NA treatment ≥ 16 months	0.54	0.31–0.93	0.027

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen.

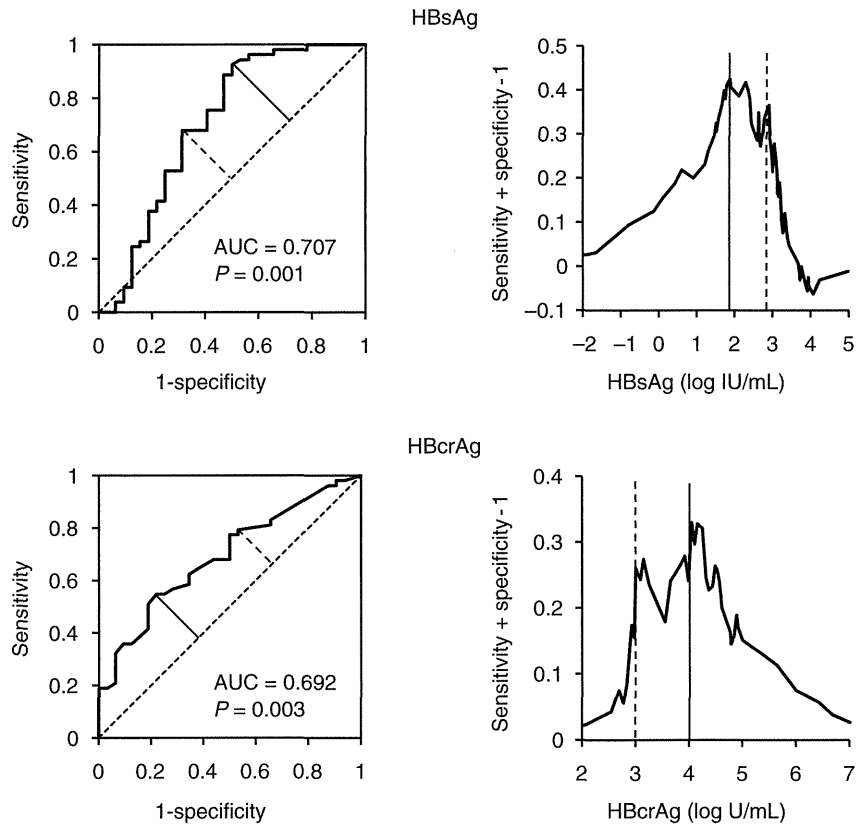

Figure 5 Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points.

patients with and without relapse (Fig. 5). Thus, we set cut-off values as 1.9 and 2.9 log IU/mL for HBsAg and 3.0 and 4.0 log U/mL for HBcrAg in our model for predicting hepatitis relapse.

We tentatively defined three groups using the sum of the scores for HBsAg and HBcrAg levels at the time of NA discontinuation for our model. Conversions were made by assigning a score of 0 for an HBsAg level lower than 1.9 log IU/mL, 1 for a level from 1.9 to 2.8 log IU/mL, and 2 for a level equal to or higher than 2.9 log IU/mL. HBcrAg was scored as 0 for a level lower than 3.0 log U/mL, 1 for a level from 3.0 to 3.9 log U/mL, and 2 for a level equal to or higher than 4.0 log U/mL. Overall, group 1 consisted of patients with a total score of 0, group 2 of patients with a total score of 1 or 2, and group 3 of patients with a total score of 3 or 4.

Patients whose HBV DNA was lower than 3.0 log copies/mL and in whom HBeAg was negative at the time of NA discontinuation were assigned to one of the three groups. Figure 6 shows the comparison of non-relapse rates among the three groups using Kaplan–Meier analysis, which differed significantly. The non-relapse rate was approximately 90% in group 1, as low as 10% in group 3, and intermediate in group 2. When factors associated with relapse were analyzed in group 3 patients, an age of over 40 years at the time of discontinuation was calculated as a significant factor (hazard

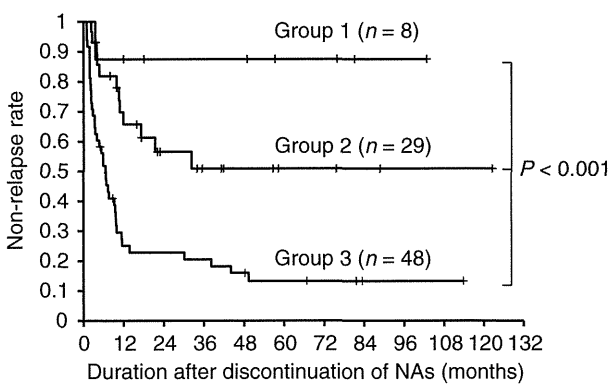

Figure 6 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleos(t)ide analog (NA) discontinuation.

ratio = 5.25, range 2.37–11.65, $P < 0.001$). No significant factors were associated with relapse in group 2 patients.

DISCUSSION

THE EUROPEAN ASSOCIATION for the Study of the Liver recommends continuation of NA treatment until HBsAg is cleared.[25] Liu *et al.* came to a similar conclusion in their study of chronic hepatitis B patients treated with LVD.[14] Indeed, the clearance of HBsAg is a reliable marker for the safe discontinuation of NAs, but the rate of patients who can clear HBsAg is relatively low (1–3%/year).[26–28] Thus, additional factors associated with relapse of hepatitis B after discontinuation of NAs were analyzed in the present study to better identify candidates who could achieve drug-free status. Such studies are relatively few, possibly because patients who discontinue NAs prematurely often experience severe complicating relapse and hepatic failure.[9] Although prospective studies are desirable to obtain accurate results, retrospective studies, such as ours, are also necessary to minimize the risk of adverse complications.

Since HBV cannot be completely eradicated in hosts, the primary goal in treating chronic hepatitis B is to convert symptomatic patients into inactive carriers in whom HBeAg is negative (usually anti-HBe-positive), serum HBV DNA is low, and serum ALT is normal.[1,2,18,29] Thus, we set the clinical conditions of a successful discontinuation of NAs as serum HBV DNA level below 4.0 log copies/mL and ALT below 30 IU/L following NA cessation. Patients who satisfy these conditions are not recommended for treatment by the Japanese guidelines for hepatitis B,[18] and it is also widely accepted that the risk of developing cirrhosis or complicating hepatocellular carcinoma is very low in such patients.[30,31] We used our cohort's mean and maximal values of HBV DNA and ALT for relapse analyses. Mean values were useful for evaluating relapse of hepatitis as a whole since parameter levels often fluctuated after discontinuation, and maximal values were used to evaluate relapse in a real-time fashion during the follow-up period. It is noteworthy that the mean and maximal values correlated very closely for both HBV DNA and ALT. The mean HBV DNA value of 4.0 log copies/mL corresponded to the maximal HBV DNA value of 5.7 by ROC analysis, and similarly the mean ALT value of 30 IU/L corresponded to the maximal ALT value of 79 IU/L. Thus, relapse of hepatitis B was judged to occur when serum ALT became higher than 79 IU/L or when serum HBV DNA surpassed 5.7 log copies/mL after the time of NA discontinuation. Such criteria may also be useful for physicians to detect relapse at an early phase and avoid the occurrence of severe reactivation or unnecessary discontinuation of NAs.

It is generally understood that patients with a higher level of HBV DNA at the time of NA discontinuation are likely to relapse, but this cut-off value has not been analyzed sufficiently. Our findings using ROC analysis showed that patients with levels lower than 3.0 log copies/mL have a good possibility to achieve successful discontinuation. The presence of HBeAg is also generally accepted as a reliable factor to predict relapse of hepatitis. Our study showed that patients with detectable HBeAg at the time of NA discontinuation were likely to relapse, even if their HBV DNA levels were lower than 3.0 log copies/mL. Therefore, we next analyzed additional factors associated with a relapse of hepatitis after discontinuation of NAs by selecting patients who met both of these criteria.

Nucleos(t)ide analog treatment produces a rapid decrease in serum HBV DNA by suppressing reverse transcription of pregenomic HBV RNA. However, the key intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA), tends to remain and is capable of reinitiating replication once NAs are ceased.[32] Measurement of HBV cccDNA has been reported to be useful for monitoring and predicting responses to antiviral treatments.[33] However, its measurement is difficult in the clinical setting as it requires a liver biopsy. Due to the mechanism of action of NAs mentioned above, serum HBV DNA does not reflect intrahepatic HBV cccDNA in patients undergoing NA treatment.[34] To address this, quantitative measurement of HBV antigens has been reported to be useful for predicting the effect of antiviral treatment in patients with chronic hepatitis B. Although HBsAg is usually used as a serum marker for the diagnosis of HBV infection, several groups have shown that HBsAg levels can also be reflective of the response to peg-interferon in chronic hepatitis B.[28,35,36] The HBcrAg assay measures serum levels of HB core and e antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these two denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related.[37] Serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during NA treatment,[24,34,38] and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs.[39,40] It is possible that levels of HBsAg and HBcrAg have different roles in

monitoring antiviral effects because the transcription of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome.[3] Therefore, we analyzed both of these antigens to elucidate their ability to predict relapse of hepatitis after discontinuation of NAs.

Multivariate analysis demonstrated that levels of HBsAg and HBcrAg at the time of NA discontinuation were independent factors significantly associated with relapse of hepatitis. Thus, we believe these factors can also be applied for predicting relapse in patients whose HBV DNA is lower than 3.0 log copies/mL and whose HBeAg is negative at NA discontinuation. HBV DNA levels were further analyzed using a highly sensitive assay based on real-time polymerase chain reaction (PCR). However, even the level of a negative signal did not ensure successful discontinuation of NAs. The results obtained here indicate that the combined use of HBV-related antigens are useful makers for monitoring the effect of anti-viral treatment in ways different from HBV DNA. Finally, since prolonged NA administration was also a significant factor associated with safe discontinuation, physicians are advised to continue patient treatment for at least 16 months for the best possible outcome.

From our data, a tentative model for predicting relapse of hepatitis after discontinuation of NAs was constructed using levels of HBsAg and HBcrAg at discontinuation. A negative result for HBeAg and HBV DNA lower than 3.0 log copies/mL at the time of NA discontinuation are the essential conditions in this system. Levels of HBsAg and HBcrAg were each converted into scores from 0 to 2 partly because two cut-off values were needed for each antigen and partly because a scoring system may be more convenient for clinical use. The sum of the two scores, which ranged from 0 to 4, was used to prospect relapse. We found that group 1 patients who had a low score (0) could be recommended to discontinue NAs because nearly 90% of this group achieved successful discontinuation. Further analysis of factors associated with relapse are needed for group 2 patients who had middle range scores (1 or 2), since the odds of achieving successful discontinuation were approximately 50%. Continuation of NA treatment is recommended for group 3 patients having high scores (3 or 4) because nearly 90% of this group relapsed. However, this recommendation may be reconsidered in patients younger than 40 years; such cases tended to have a lower relapse rate in group 3. It is also noteworthy that relapse occurred mainly during the first and second years following NA discontinuation in all groups, similarly to a report by Liu *et al.*[14] Thus, clinicians should be vigilant in the early phase after discontinuation.

This study has several limitations. The patients who discontinued NAs were recruited retrospectively, and thus the decision to halt NA treatment was made by individual physicians without uniformly established criteria. Based on this, prospective studies are required to confirm our results. Furthermore, as over 90% of the patients we enrolled had genotype C and over 90% of cases were treated with LVD until discontinuation, the results obtained here can not be applied directly to other HBV genotypes or other types of NAs.

In conclusion, the present study showed that maximal levels of serum ALT and HBV DNA were useful for defining relapse patients after discontinuation of NAs. Along with serum HBV DNA of less than 3.0 log copies/mL and negative serum HBeAg, serum levels of HBsAg and HBcrAg at the time of NA discontinuation were able to predict relapse of hepatitis B and should therefore be considered when establishing uniform guidelines regarding the safe withdrawal of NA treatment. To this end, NA administration of more than 16 months is advisable to achieve successful discontinuation.

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Review Article

Risk of hepatitis B reactivation in patients treated with tumor necrosis factor-α inhibitors

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The use of tumor necrosis factor-α (TNF-α) inhibitors has been increasing especially in patients with rheumatoid arthritis (RA). As TNF-α inhibitors are strongly immunosuppressive, the occurrence of hepatitis B virus (HBV) reactivation has recently been observed. Reports suggest a higher risk of complicating HBV reactivation in carriers who are treated with TNF-α inhibitors. Therefore, HBV carriers are recommended to undergo prophylactic administration of nucleos(t)ide analogs (NAs). Our literary analysis uncovered several characteristics of de novo hepatitis B due to TNF-α inhibitors. First, the time between the start of TNF-α inhibitors and the occurrence of de novo hepatitis was longer than one year. Second, patients were usually treated with additional non-biologic agents, which also had immunosuppressive effects. Third, the disease could be fatal. Fourth, several types of TNF-α inhibitors exhibited a risk of developing de novo hepatitis. Although the incidence of de novo hepatitis B varied among reports (0–5%/year), it is suggested that patients with prior HBV infection are at risk of developing de novo hepatitis due to TNF-α inhibitors. Many reports maintain that regular measurement of HBV DNA is effective in preventing de novo hepatitis. Prophylactic administration of NAs is also considered useful to avoid de novo hepatitis, although the issue of cost-effectiveness needs to be addressed. Lastly, whereas maintenance of circulating anti-HBs titer using HB vaccines may be effective in responders to prevent de novo hepatitis, further studies are required to clarify the utility of HB vaccination.

Key words: hepatitis B, nucleos(t)ide analog, de novo hepatitis B, reactivation, rheumatoid arthritis, tumor necrosis factor-α inhibitor

INTRODUCTION

APPROXIMATELY 3 BILLION people have been exposed to the hepatitis B virus (HBV), and there are an estimated 350 million chronic carriers worldwide.[1,2] HBV infection is usually detected by the presence of hepatitis B surface antigen (HBsAg) in the serum, and clearance of HBsAg is generally considered as an indication of hepatitis B resolution. However, recent studies have shown that HBV replication persists at low levels in the liver and peripheral blood mononuclear cells for decades, even in HBsAg-negative patients with resolved HBV infection.[3–5] In such patients, HBV replication is suppressed by immune responses to HBV, for instance specific cytotoxic T lymphocyte-mediated responses.[3]

Hepatitis B virus reactivation in patients with resolved HBV infection has been reported in increasing numbers as the number of patients undergoing strong immunosuppressive therapy grows worldwide for malignant neoplasms, autoimmune disorders, and following transplantation for prevention of rejection. In patients like these with resolved HBV infection, reactivation of hepatitis B is recognized as de novo hepatitis B, which can lead to fulminant hepatic failure and often death.[6,7] Thus, de novo hepatitis B is becoming a well-recognized severe complication of immunosuppressive therapy that should be prevented.[6,8]

The risk of developing de novo hepatitis B varies among immunosuppressive therapies; it is as high as 14–20% in patients who receive hematopoietic stem cell transplantation and as low as 1–3% in those who undergo conventional chemotherapies.[9–13] The introduction of rituximab, a genetically engineered chimeric anti-CD20 monoclonal antibody,[14,15] in the treatment of

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CD20+ B-cell non-Hodgkin's lymphoma increased the risk of de novo hepatitis B. Hui *et al.*[16] analyzed the occurrence of de novo hepatitis B in patients who were treated for lymphoma and reported that its risk was significantly higher in patients who received rituximab and steroids (12%) than in other patients (1%). Similarly, Yeo *et al.*[17] reported that the risk of de novo hepatitis B was significantly higher in patients who were treated with chemotherapy including rituximab (24%) than in those treated with chemotherapy only (0%). Because the introduction of rituximab increased the risk of de novo hepatitis B considerably in lymphoma patients, the need to examine the occurrence of HBV reactivation has emerged when a new agent that suppresses host immune responses is introduced.

Tumor necrosis factor-α is a crucial pro-inflammatory and immunoregulatory cytokine in the pathogenesis of various inflammatory and autoimmune conditions. Inhibitors of TNF-α have recently been introduced in treatments for various kinds of autoimmune and inflammatory disorders, including rheumatoid arthritis (RA), ankylosing spondylitis, psoriatic arthritis, and Crohn's disease. TNF-α inhibitors have revolutionized the therapeutic approaches and treatment paradigms for these patients. However, their optimal use requires consideration of possible adverse effects; increased risks of tuberculosis and other infections are a major concern in TNF-α treatment.[18] Complicating tuberculosis is considered to be caused by reactivation of latent tuberculosis.[19] A similar reactivation of HBV has also been reported, which leads to de novo hepatitis B and possibly fulminant hepatic failure and death. In the present review article, we summarize reports regarding reactivation of hepatitis B due to TNF-α inhibitors to clarify its characteristics and occurrence (Table 1).

Table 1 Summary of references regarding reactivation hepatitis due to tumor necrosis factor-α inhibitors

Category/Reference	Publication type
Case report and review in HBV carriers	
25. Ostuni P, *et al.* Ann Rheum Dis. 2003	Case report
26. Carroll MB, *et al.* Clin Rheumatol. 2010	Review
27. Kuroda T, *et al.* Rheumatol Int. 2010	Case report & review
28. Verhelst X, *et al.* Eur J Gastroenterol Hepatol. 2010	Case report & review
29. Pyrpasopoulou A, *et al.* Rheumatol Int. 2011	Case report
30. Esteve M, *et al.* Gut. 2004	Case report
31. Ojiro K, *et al.* J Gastroenterol. 2008	Case report
34. Wendling D, *et al.* Joint Bone Spine. 2009	Case report
Risk and prevention in HBV carriers	
32. Zingarelli S, *et al.* Reumatismo. 2008	Original
33. Kalyoncu U, *et al.* Rheumatol Int. 2009	Original
35. Vassilopoulos D, *et al.* Ann Rheum Dis. 2010	Original
36. Lan JL, *et al.* Ann Rheum Dis. 2011	Original
37. Calabrese LH, *et al.* Ann Rheum Dis. 2006	Review
Case report of de novo hepatitis B	
40. Madonia S, *et al.* Inflamm Bowel Dis. 2007	Case report
41. Matsumoto T, *et al.* Liver Int. 2010	Case report
42. Montiel PM, *et al.* Liver Int. 2008	Case report
43. Zingarelli S, *et al.* J Rheumatol. 2009	Case report
Risk of de novo hepatitis B	
18. Takeuchi T, *et al.* Ann Rheum Dis. 2008	Original
44. Charpin C, *et al.* Arthritis Res Ther. 2009	Original
45. Caporali R, *et al.* Arthritis Care Res (Hoboken). 2010	Original
46. Tamori A, *et al.* J Gastroenterol. 2011	Original
47. Mori S. Mod Rheumatol. 2011	Original
48. Kim YJ, *et al.* J Rheumatol. 2010	Original
49. Urata Y, *et al.* Mod Rheumatol. 2011	Original

REACTIVATION OF HEPATITIS IN HBV CARRIERS

THE MAJORITY OF patients with a confirmed diagnosis of RA use disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, but the rate of biologic agent use is rising rapidly.[20,21] Since both methotrexate[22–24] and biologic agents carry the danger of HBV reactivation, the advent of new biologic agents, such as TNF-α inhibitors, has increased this risk. Patients with RA who developed reactivation of hepatitis B due to TNF-α inhibitors were first reported in 2003.[25–29] Because these cases had been HBV carriers prior to starting TNF-α inhibitors, the authors recommended preliminary serological tests for HBV infection.

Caroll *et al.* conducted a systemic literature review on HBV reactivation in carriers who were treated with TNF-α inhibitors for RA and reported that reactivation was seen in six (17%) of 35 patients.[26] They concluded that clinicians prescribing TNF-α inhibitors to HBsAg-positive patients should consider prophylactic antiviral therapy and close monitoring for any clinical or sero-

logical evidence of hepatitis. Reactivation of hepatitis B was also reported in patients with Crohn's disease who were treated with TNF-α inhibitors,[30,31] and thus reactivation became considered to be drug dependent and not disease dependent.

Prophylaxis using nucleos(t)ide analogs (NAs) has been reported to be effective in preventing the occurrence of hepatitis reactivation in HBV carriers.[32–36] Vassilopoulos *et al.*[35] administered lamivudine in 14 HBV carriers with RA who were treated with TNF-α inhibitors and showed that reactivation of hepatitis B did not occur in any patient except one. The appearance of lamivudine resistance was considered to be the cause of reactivation in this exceptional patient, and so the authors concluded that TNF-α inhibitors represented a safe option for patients with chronic HBV infection when combined with NAs. Zingarelli *et al.*[32] reported 20 patients with RA who were treated with DMARDs and/or TNF-α inhibitors. Prophylaxis and therapy with lamivudine were performed in patients with a high risk of HBV reactivation, and no cases of viral reactivation were observed. Thus, it is likely that prophylaxis using NAs may prevent the occurrence of hepatitis reactivation in HBV carriers who are treated with TNF-α inhibitors. Indeed, Calabrese *et al.*[37] recommended that all HBsAg-positive patients be started on prophylactic anti-viral drugs before receiving immunosuppressive therapy. However, long-term follow-up studies in large groups of patients are required to ensure the safety of prophylaxis with NAs.

Descriptions of HBV reactivation due to TNF-α inhibitors in the guidelines of rheumatologist associations several years ago tended to be brief and passive. It was described that TNF-α inhibitor therapy should be avoided in patients with hepatitis B infection until more definitive data were available in the 2005 guidelines of The British Society for Rheumatology.[38] In the 2007 Japanese guidelines,[39] it was advised that TNF-α inhibitors should be avoided in patients with HBV infection. However, if the potential benefits of treatment with TNF-α inhibitors exceeded the risk of reactivation, such therapy could be pursued provided that patients were pre-treated with lamivudine.

RISK OF DE NOVO HEPATITIS B

ALTHOUGH IT HAS become clear that HBsAg-positive patients are prone to developing HBV reactivation during TNF-α inhibitor therapy, little is known about the occurrence of de novo hepatitis B. Several cases of de novo hepatitis B due to TNF-α inhibitors have been reported recently.[40–43] Mondonia *et al.*[40] reported a 41-year-old woman with Crohn's disease who developed de novo hepatitis B after having been treated with prednisolone for 13 years and infliximab for 3 years. The hepatitis subsided with lamivudine administration. Montiel *et al.*[42] described a 73-year-old man with ankylosing spondylitis who developed de novo hepatitis 15 months after starting etanercept. The patient had also undergone treatment with prednisolone for 23 years. Although etanercept was discontinued when the hepatitis occurred, it could be re-started with concurrent lamivudine administration. Matsumoto *et al.*[41] reported a 71-year-old woman with RA who developed de novo hepatitis 22 months after starting treatment with infliximab, methotrexate, and prednisolone. Although entecavir was given when hepatitis occurred, the patient died of hepatic failure. Such case reports reveal several characteristics of de novo hepatitis B due to TNF-α inhibitors. First, the duration between the start of the drugs and the occurrence of de novo hepatitis was at least one year. Second, patients were treated not only with TNF-α inhibitors, but also with DMARDs and prednisolone, which themselves had immunosuppressive effects. Third, there was a risk of death from de novo hepatitis. Fourth, several kinds of TNF-α inhibitors appeared able to cause de novo hepatitis.

The incidence of HBV reactivation from occult HBV infection and ensuing de novo hepatitis B due to TNF-α inhibitor therapy in patients with RA has been reported by several groups. Charpin *et al.*[44] followed 21 patients with RA who were HBsAg-negative and hepatitis B core antibody (HBcAb)-positive before starting TNF-α inhibitors, and found that no patient developed HBV reactivation during a mean follow-up period of 27.2 months. They concluded that TNF-α inhibitor therapy was likely safe in patients with a past hepatitis B serological pattern. However, they also suggested that such patients required HBV virological follow-up, especially those with a low HBs antibody (HBsAb) titer at baseline because HBsAb decreased significantly during therapy. Caporali *et al.*[45] followed 67 patients with RA who also had HBV markers of past HBV infection, and found no elevations of HBV DNA in sera or appearances of HBsAg during a mean follow-up period of 42.5 months. Of the 67 patients, 23 were treated with infliximab, 23 with etanercept, and 19 with adalimumab. Almost all patients underwent methotrexate (51 patients) and/or prednisolone (43 patients) administration in addition to TNF-α inhibitors. Tamori *et al.*[46] followed 50 patients with RA who were positive for HBcAb for a mean period of 23 months. All patients were treated with immunosuppressive agents such as