Hepatology Research

Hepatology Research 2012; 42: 139–149 doi: 10.1111/j.1872-034X.2011.00910.x

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

Akihiro Matsumoto,[1] Eiji Tanaka,[1] Yoshiyuki Suzuki,[2] Mariko Kobayashi,[2] Yasuhito Tanaka,[4] Noboru Shinkai,[4] Shuhei Hige,[6] Hiroshi Yatsuhashi,[8] Shinya Nagaoka,[8] Kazuaki Chayama,[9] Masataka Tsuge,[9] Osamu Yokosuka,[10] Fumio Imazeki,[10] Shuhei Nishiguchi,[11] Masaki Saito,[11] Kei Fujiwara,[5] Nobuyuki Torii,[3] Naoki Hiramatsu,[12] Yoshiyasu Karino[7] and Hiromitsu Kumada[2]

[1]Department of Medicine, Shinshu University School of Medicine, Matsumoto, [2]Department of Hepatology, Toranomon Hospital, [3]Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo, [4]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, [5]Gastroenterology Section, Nagoya Daini Red Cross Hospital, Nagoya, [6]Department of Gastroenterology and Hepatology, Graduate School of Medicine, Hokkaido University, [7]Department of Gastroenterology, Sapporo Kosei General Hospital, Sapporo, [8]The Clinical Research Center, NHO Nagasaki Medical Center, Omura, [9]Program for Biomedical Research, Division of Frontier Medical Science, Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, [10]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, [11]Division of Hepatobiliary and Pancreatic Diseases, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, and [12]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan

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

Correspondence: Professor Eiji Tanaka, Department of Medicine, Gastroenterology Division, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan. Email: etanaka@shinshu-u.ac.jp

Financial support
This research was supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan.
Received 7 August 2011; revision 31 August 2011; accepted 5 September 2011.

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

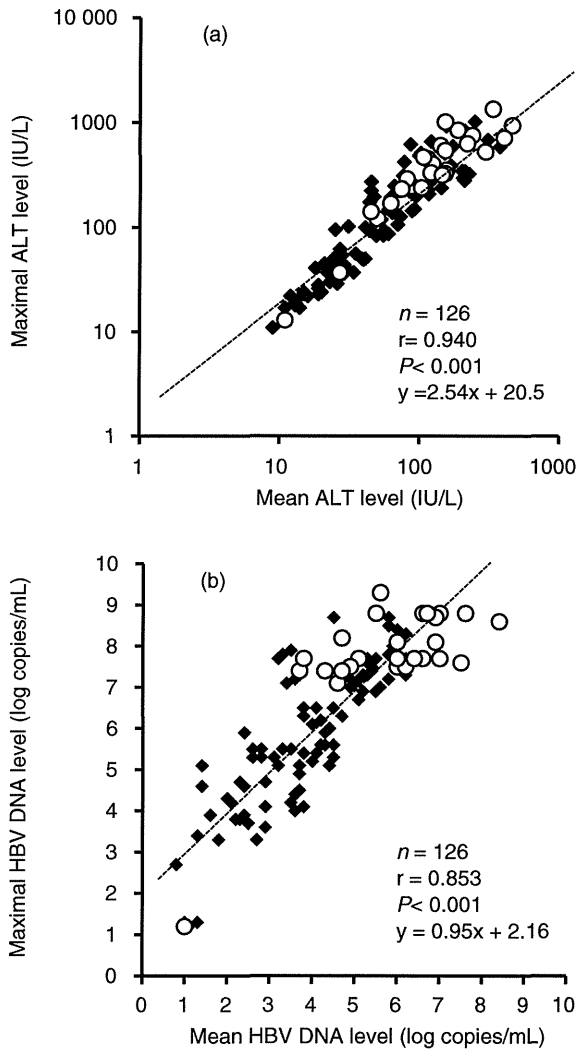

Figure 1 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (a) and hepatitis B virus (HBV) DNA (b) after discontinuation of nucleos(t)ide analogs (NAs). Open circles indicate patients with detectable hepatitis B e antigen (HBeAg) and closed squares indicate patients without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

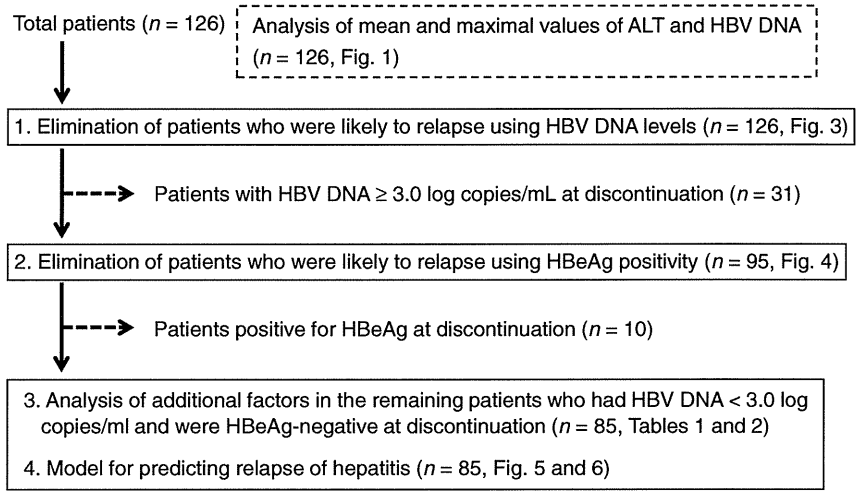

Figure 2 The progression of analyses in the present study and population structure of each analysis.

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

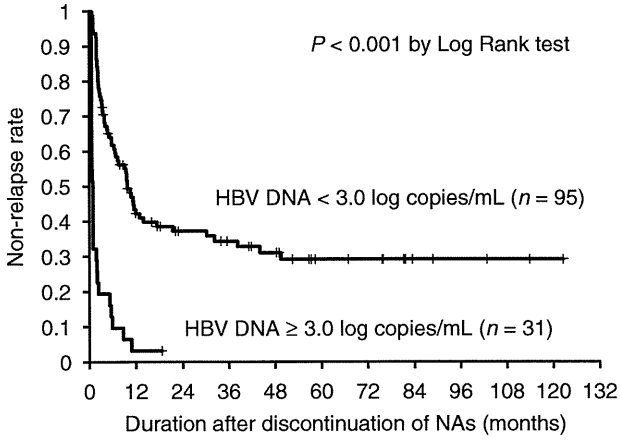

Figure 3 Comparison of non-relapse rates using the Kaplan–Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

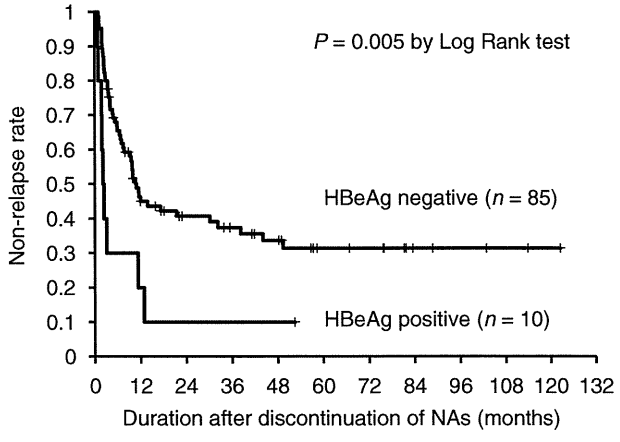

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

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.

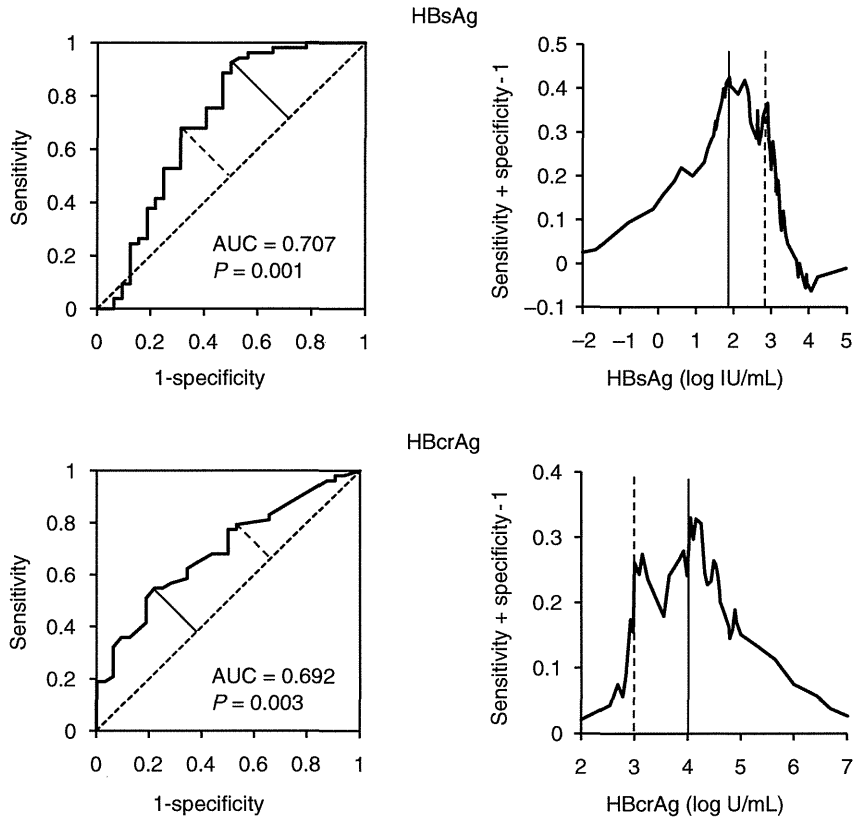

Figure 5 Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short 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

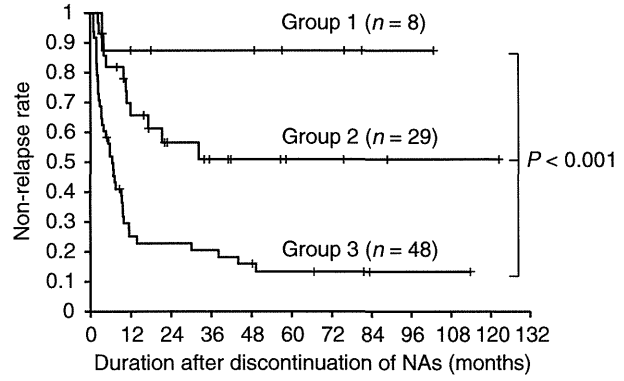

Figure 6 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleo-s(t)ide analog (NA) discontinuation.

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.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan.

We thank Ms. Hiroe Banno for her secretarial assistance and thank Ms. Nozomi Kamijo and Ms. Etsuko Iigahama for their technical assistance. We also thank Mr Trevor Ralph for his English editorial assistance.

REFERENCES

1 Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056–75.
2 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
3 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733–45.
4 Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. *Gastroenterology* 2007; **132**: 1574–85.
5 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521–31.
6 Matsumoto A, Tanaka E, Rokuhara A *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic

hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; **32**: 173–84.
7 Suzuki Y, Kumada H, Ikeda K *et al.* Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. *J Hepatol* 1999; **30**: 743–8.
8 Lok AS, Zoulim F, Locarnini S *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; **46**: 254–65.
9 Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW. Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. *Hepatology* 2000; **32**: 635–9.
10 Honkoop P, de Man RA, Heijtink RA, Schalm SW. Hepatitis B reactivation after lamivudine. *Lancet* 1995; **346**: 1156–7.
11 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.
12 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; **354**: 1011–20.
13 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61–8.
14 Liu F, Wang L, Li XY *et al.* Poor durability of lamivudine effectiveness despite stringent cessation criteria: a prospective clinical study in hepatitis B e antigen-negative chronic hepatitis B patients. *J Gastroenterol Hepatol* 2011; **26**: 456–60.
15 Reijnders JG, Perquin MJ, Zhang N, Hansen BE, Janssen HL. Nucleos(t)ide analogues only induce temporary hepatitis B e antigen seroconversion in most patients with chronic hepatitis B. *Gastroenterology* 2010; **139**: 491–8.
16 Liaw YF, Leung N, Kao JH *et al.* Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263–83.
17 Leung N. Recent data on treatment of chronic hepatitis B with nucleos(t)ide analogues. *Hepatol Int* 2008; **2**: 163–78.
18 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 1–7.
19 Schuttler CG, Wend UC, Faupel FM, Lelie PN, Gerlich WH. Antigenic and physicochemical characterization of the 2nd International Standard for hepatitis B virus surface antigen (HBsAg). *J Clin Virol* 2010; **47**: 238–42.
20 Dai CY, Yu ML, Chen SC *et al.* Clinical evaluation of the COBAS Amplicor HBV monitor test for measuring serum HBV DNA and comparison with the Quantiplex branched DNA signal amplification assay in Taiwan. *J Clin Pathol* 2004; **57**: 141–5.
21 Ronsin C, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 2006; **44**: 1390–9.
22 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450**: 66–71.
23 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
24 Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27–33.
25 European Association for the Study of the Liver. EASL Clinical Practice Guidelines: management of chronic hepatitis B. *J Hepatol* 2009; **50**: 227–42.
26 Gish RG, Lok AS, Chang TT *et al.* Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; **133**: 1437–44.
27 Marcellin P. Hepatitis B and hepatitis C in 2009. *Liver Int* 2009; **29** (Suppl 1): 1–8.
28 Moucari R, Lada O, Marcellin P. Chronic hepatitis B: back to the future with HBsAg. *Expert Rev Anti Infect Ther* 2009; **7**: 633–6.
29 Yokosuka O, Kurosaki M, Imazeki F *et al.* Management of hepatitis B: consensus of the Japan Society of Hepatology 2009. *Hepatol Res* 2011; **41**: 1–21.
30 Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; **130**: 678–86.
31 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65–73.
32 Werle-Lapostolle B, Bowden S, Locarnini S *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; **126**: 1750–8.
33 Sung JJ, Wong ML, Bowden S *et al.* Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. *Gastroenterology* 2005; **128**: 1890–7.
34 Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; **45**: 3942–7.
35 Brunetto MR, Moriconi F, Bonino F *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009; **49**: 1141–50.
36 Moucari R, Mackiewicz V, Lada O *et al.* Early serum HBsAg drop: a strong predictor of sustained virological response

to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; **49**: 1151–7.
37 Tanaka E, Matsumoto A, Yoshizawa K, Maki N. Hepatitis B core-related antigen assay is useful for monitoring the antiviral effects of nucleoside analogue therapy. *Intervirology* 2008; **51** (Suppl 1): 3–6.
38 Hosaka T, Suzuki F, Kobayashi M *et al.* HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy. *Liver Int* 2010; **30**: 1461–70.
39 Matsumoto A, Tanaka E, Minami M *et al.* Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. *Hepatol Res* 2007; **37**: 661–6.
40 Shinkai N, Tanaka Y, Orito E *et al.* Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. *Hepatol Res* 2006; **36**: 272–6.

VoxSanguinis
The International Journal of Transfusion Medicine

ISBT International Society of Blood Transfusion

Vox Sanguinis (2012) **102**, 285–293

ORIGINAL PAPER

DOI: 10.1111/j.1423-0410.2011.01561.x

Significant background rates of HBV and HCV infections in patients and risks of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres in Japan: a prospective, individual NAT study of transfusion-transmitted HBV, HCV and HIV infections

Y. Tani,[1] H. Aso,[1] H. Matsukura,[1] K. Tadokoro,[2] A. Tamori,[3] S. Nishiguchi,[4] H. Yoshizawa[5], H. Shibata,[1] & JRC NAT Screening Research Group

[1]*Japanese Red Cross Osaka Blood Center, Osaka, Japan*
[2]*Japanese Red Cross Central Blood Institute, Tokyo, Japan*
[3]*Osaka City University Hospital, Osaka, Japan*
[4]*Hyogo College of Medicine, Hyogo, Japan*
[5]*Hiroshima University, Graduate School of Biomedical Sciences, Hiroshima, Japan*

Vox Sanguinis

Background The Japanese Red Cross (JRC) conducted a prospective study to evaluate the frequency of transfusion-transmitted HBV, HCV and HIV infections to assess the risk of transfusion of blood components routinely supplied to hospitals.

Study Design and Methods Post-transfusion specimens from patients at eight medical institutes were examined for evidence of infection with HBV (2139 cases), HCV (2091) and HIV (2040) using individual nucleic acid amplification testing (NAT). If these specimens were reactive, pre-transfusion specimens were also examined for the virus concerned by individual NAT. In the event that the pre-transfusion specimen was non-reactive, then all repository specimens from implicated donors were tested for the viruses by individual donation NAT. In addition, a further study was carried out to evaluate the risk of transfusion of components from donors with low anti-HBc titres or high anti-HBc with high anti-HBs titres.

Results Transfusion-transmitted HCV and HIV infections were not observed. One case of post-transfusion HBV infection was identified (rate, 0·0004675; 95% CI for the risk of transmission, 1 in 451–41 841). The background rates of HBV, HCV and HIV infections in patients prior to transfusion were 3·4% (72/2139), 7·2% (150/2091) and 0% (0/2040), respectively. Sixty-four anti-HBc- and/or anti-HBs-reactive blood components were transfused to 52 patients non-reactive for anti-HBc or anti-HBs before and after transfusion (rate, 0; 95% CI for the risk of transmission, <1 in 22).

Conclusion This study demonstrated that the current criteria employed by JRC have a low risk, but the background rates of HBV and HCV infections in Japanese patients are significant.

Received: 25 January 2011,
revised 4 July 2011,
accepted 15 July 2011,
published online 14 November 2011

Key words: nucleic acid amplification testing, occult HBV infection, transfusion-transmitted viral infection.

Correspondence: Yoshihiko Tani, MD, PhD, Japanese Red Cross Osaka Blood Center, 2-4-43 Morinomiya, Joto-Ku, Osaka 536-8505, Japan
E-mail: tani@osaka.bc.jrc.or.jp

Introduction

In Japan, all blood components are collected from non-remunerated voluntary donors by the Japanese Red Cross (JRC). Subsequently, the components are screened by serological testing for syphilis, HBV, HCV, HIV, HTLV-1 and human parvovirus B19, and serologically non-reactive components (the criteria to determine the suitability of blood for supply, which are based on infectious markers, are shown in Table 1) are then subjected to nucleic acid amplification testing (NAT) for HBV DNA, HCV RNA, and HIV-1 RNA in a mini-pool (MP) using an automated multiplex assay system (AMPLINAT MPX; Roche Diagnostics) that can amplify these three viruses simultaneously [1]. When all the above-mentioned tests are non-reactive, the blood components are supplied to hospitals. An important point is that unlike some other countries [2], blood components from donors non-reactive for HBsAg and HBV DNA but reactive for anti-HBc titres of <1:32 or anti-HBc titres of ≥1:32 with anti-HBs ≥200 mIU/ml are considered suitable for transfusion in Japan on the basis of a study of the correlation between anti-HBc titres and HBV DNA levels in blood units without detectable HBsAg [3, 4].

Since the introduction of the automated NAT multiplex assay system by the JRC in October 1999, the risk of transmission of HBV, HIV and HCV via transfusion has reduced significantly in Japan [5, 6]. However, several cases of transfusion-transmitted viral infections, especially of HBV, continue to occur each year [7]. This is partly because the doubling time of HBV is longer than that of HCV or HIV [8, 9], and thus, the NAT window period is also longer. The other reason is that occult HBV-infected donors [3] with low anti-HBc titres and low levels of HBV DNA that are not detected by MP-NAT may not be identified [10].

In this study, we randomly selected five JRC blood centres (Hokkaido, Iwate, Osaka, Ehime, and Fukuoka) and eight hospitals within the jurisdiction of these centres and prospectively investigated the risk involved in routine blood transfusion to patients in these hospitals. In addition, we also examined the safety of blood transfusion from anti-HBc-positive donors with anti-HBc titres of <1:32 or anti-HBc titres of ≥1:32 with anti-HBs ≥200 mIU/ml.

Materials and methods

Serological tests on donated blood

All donated blood samples were serologically screened as shown in Table 1.

NAT

The NAT screening system used in Japan has been reported previously by Mine *et al.* [12]. In brief, NAT screening is performed using a multiplex system capable of simultaneous detection of HBV DNA, HCV RNA and HIV-1 RNA to reduce the cost and ensure that the test is completed within 72 h. Samples are tested in MPs of 50 with the ability to detect 185–550 IU/ml for HBV, 3050–5600 IU/ml for HCV

Table 1 Criteria for infectious and other markers

	Serological tests		
Pathogens	**Contents**	**Methods**	**Criteria**
Syphilis	Serodiagnosis	Treponema pallidum particle agglutination (TPPA)[a]	Non-reactive
HIV	Anti-HIV-1/2	Agglutination of gelatin particles coated with recombinant HIV-1/2 proteins[a]	Non-reactive
HCV	Anti-HCV	Passive hemagglutination (PHA[b]) or particle agglutination (PA[a])	Non-reactive
HBV	HBsAg	Reverse passive hemagglutination (RPHA[c])	Non-reactive
	Anti-HBs	PHA[c]	–[d]
	Anti-HBc	Haemagglutination inhibition (HI[c])	
HTLV-1	Anti-HTLV-1	PA[a]	Non-reactive
B19	Anti-B19	Receptor-mediated hemagglutination (RHA[e])	
Others	Serum ALT	Method of Wroblewski and LaDue (11)	≤60 IU/ml

[a]Fujirebio Inc., Tokyo, Japan.

[b]Dainabot Co. Ltd., Tokyo, Japan.

[c]Reagents prepared by JRC.

[d]Blood units with the following profile were excluded from being transfused: 1. Specimen reactive for HBsAg on RPHA, with the result subsequently confirmed by enzyme immune assay (EIA). 2. Specimen reactive for anti-HBc at a dilution of 1:32 or higher on HI and in which anti-HBs is either absent or at a level of not more than 200 mIU/ml.

[e]RHA using reagents prepared by JRC.

and 1650–3300 IU/ml for HIV in donations contained within the pool.

In this study, HBV DNA, HCV RNA and HIV-1 RNA from patients were individually tested using the modified methods of Iizuka *et al.* [4], Okamoto *et al.* [13] and Matsumoto *et al.* [14], respectively, at the JRC NAT centres in Hokkaido and Kyoto. The analytical sensitivity cut-off of ID-NAT was 3·7–11 IU/ml for HBV, 61–112 IU/ml for HCV and 33–66 IU/ml for HIV [1].

Criteria for blood transfusion

The serological test criteria for the release of blood donations in Japan are shown in Table 1. Donations must also be non-reactive for HBV DNA, HCV RNA and HIV RNA on 50-MP-NAT.

Study design

Informed consent was obtained from each patient before transfusion between November 2003 and December 2006 at eight hospitals [Asahikawa Medical College Hospital (Hokkaido); Iwate Medical University Hospital (Iwate); Osaka City University Hospital, Osaka City General Hospital, Osaka Red Cross Hospital (Osaka); National Hospital Organization Shikoku Cancer Center, Ehime Red Cross Hospital (Ehime); and Fukuoka University Hospital (Fukuoka)]. In total, 2139 patients who survived 3 months after transfusion (approximately 40% of patients died of their original disease or complications within 3 months) were enrolled in this study. Their pre-transfusion blood specimens had been collected and cryopreserved in these hospitals (Fig. 1).

Approximately 3 months after blood transfusion, post-transfusion specimens were collected from the patients and individually tested for HBV DNA, HCV RNA and HIV-1 RNA at the JRC NAT centres. In the case of neonates and elderly patients, when the specimen volume was insufficient to perform NAT for all the three viruses, the priority of examination was HBV DNA >HCV RNA >HIV-1 RNA.

If the post-transfusion specimen was non-reactive for all the three viruses, the study was terminated for the patient concerned. However, if the specimen was reactive, the patient's cryopreserved pre-transfusion specimen was tested for the virus concerned by NAT. If the pre-transfusion specimen was reactive, it was concluded that the patient was infected before transfusion. However, if the pre-transfusion specimen was non-reactive, all repository specimens from the implicated donors, which were drawn at the time of blood donation and cryopreserved at the JRC NAT centres, were also tested for the virus concerned by ID-NAT, as reported by Satake *et al.* [15]. If these specimens were non-reactive and the case was restricted to HBV, the remaining pre-transfusion specimen of the patient was serologically tested for anti-HBc, anti-HBs and/or HBsAg using an enzyme immunoassay

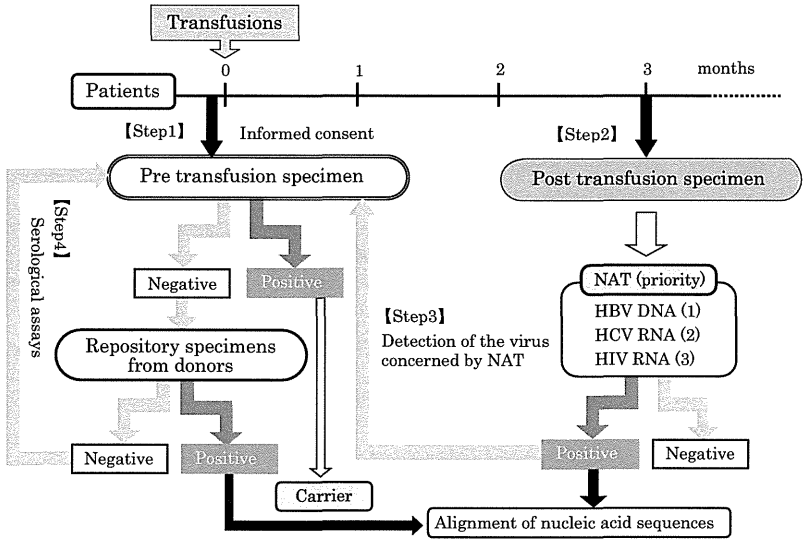

Fig. 1 Study design. Step 1: After obtaining informed consent from patients, pre-transfusion specimens were collected and cryopreserved. Step 2: Approximately 3 months after blood transfusion, post-transfusion specimens were collected from the patients at the eight hospitals and individually tested for HBV DNA, HCV RNA and HIV-1 RNA at the JRC nucleic acid amplification testing (NAT) centres. Step 3: If the post-transfusion specimen was reactive, the patient's pre-transfusion specimen was tested for the virus concerned by NAT. Step 4: If the pre-transfusion specimen was non-reactive (this was restricted to HBV alone), all repository specimens from the donors concerned were also tested for HBV DNA by ID-NAT. If these specimens were non-reactive, the remaining pre-transfusion specimen of the patient was then serologically tested for anti-HBc, anti-HBs and/or HBsAg.

(EIA) system in addition to the methods shown in Table 1.

Assessing the risk of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres

We randomly selected 247 patients at the Osaka City University Hospital whose post-transfusion specimens were non-reactive for all the three viruses on NAT and 1009 blood components were transfused to these patients. Their pre- and post-transfusion specimens were tested for anti-HBs and anti-HBc. The repository specimens from the implicated donors were also examined to determine anti-HBs and anti-HBc titres and HBV DNA levels.

Results

The risk of transfusion-transmitted HBV, HCV and HIV infections

We examined 2139, 2091 and 2040 post-transfusion specimens for HBV DNA, HCV RNA and HIV-1 RNA, respectively, by NAT. The 2040 post-transfusion specimens were non-reactive for HIV-1 RNA (Table 2). Of the 2091 post-transfusion specimens, 150 specimens (7·2%) were reactive for HCV RNA (Table 2). However, the pre-transfusion specimens from the same 150 patients were also reactive for HCV RNA, indicating that the patients were already infected with HCV prior to the transfusion. Of the 2139 post-transfusion specimens, 73 (3·4%) specimens were reactive for HBV DNA (Table 2). Among these 73 patients, pre-transfusion specimens from 56 patients were reactive for HBV DNA, indicating that these patients were already infected with HBV prior to the transfusion. Pre-transfusion specimens from the remaining 17 patients were non-reactive for HBV DNA. Among these 17 patients, one patient who received 115 units of blood was judged to have transfusion-transmitted HBV infection on the basis of a donor-triggered look-back investigation on a donor, who was found to be reactive for HBV DNA at his next donation. The HBV DNA sequence of this donor was consistent with that of the patient. The repository specimens from the remaining 114 donors were non-reactive for HBV DNA.

Fourteen of the sixteen remaining patients were considered to have late-stage HBV infection because their pre-transfusion specimens were reactive for anti-HBc, and none of the repository specimens from the donors were reactive for HBV DNA. The other two patients were also considered to have late-stage infection because their HBsAg levels were relatively low (Table 3). According to additional information obtained from the hospital, one patient (No. 16 in Table 3) became infected with HBV several years ago and then periodically visited the hospital, and hospital records identified him as being HBsAg positive (AxSYM; Abbott Japan Co., Ltd, Tokyo, Japan). Considering their ages, diseases, therapies [16] administered to the patients, and follow-up observations by the hospitals, these 16 patients were strongly suggested to have occult hepatitis B infection (OBI).

The risk of transfusion of blood components from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres

None of the 1009 repository specimens were reactive for HBV DNA, but 86 of these specimens were reactive in the anti-HBc test at a titre of <1:32 (75 specimens) or ≥1:32

Table 2 Patient ages and HIV-1 RNA, HCV RNA and HBV DNA results

	Age		≤9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	≥90	Total
HIV-1 RNA	Post-transfusion	Non-reactive	49	38	56	125	137	345	548	577	157	8	2040
		Reactive	0	0	0	0	0	0	0	0	0	0	0
	Total number		49	38	56	125	137	345	548	577	157	8	2040
HCV RNA	Post-transfusion	Non-reactive	55	38	59	128	134	330	517	518	154	8	1941
		Reactive	0	0	0	1	6	26	40	69	8	0	150
	Pre-transfusion	Non-reactive	0	0	0	0	0	0	0	0	0	0	0
		Reactive	0	0	0	1	6	26	40	69	8	0	150
	Total number		55	38	59	129	140	356	557	587	162	8	2091
HBV DNA	Post-transfusion	Non-reactive	79	43	61	129	135	334	546	574	156	9	2066
		Reactive	0	0	0	1	5	24	18	19	6	0	73
	Pre-transfusion	Non-reactive	0	0	0	0	0	5	4	5	3	0	17
		Reactive	0	0	0	1	5	19	14	14	3	0	56
	Total number		79	43	61	130	140	358	564	593	162	9	2139

Table 3 The details of 16 patients considered to have late-stage HBV infection

				Pre-transfusion				Post-transfusion			
No.	Age	Disease	Therapy	HBsAg	Anti-HBs (mIU/ml)	Anti-HBc	HBV DNA	HBsAg	Anti-HBs	Anti-HBc	HBV DNA
1	64	Heart disease	Operation	+	−	+	−	+	−	NT	+
2	54	Haematologic malignancy	HSCT	−	−	+	−	−	−	+	+
3	77	Gastric cancer	Chemotherapy	−	−	+	−	−	−	NT	+
4	60	AML	Chemotherapy	−	−	+	−	−	−	NT	+
5	56	Haematologic malignancy	HSCT	−	+	+	−	NT	−	+	+
6	76	Macroglobulinemia	HSCT	−	+ 2100	+	−	−	+	+	+
7	72	Oesophageal cancer	Chemotherapy	−	+ 134·2	+	−	−	+	+	+
8	57	Aplastic anaemia	HSCT	−	+ 5·2	+	−	−	+	+	+
9	89	Orthopaedic disorder	Operation	−	+ 34·9	+	−	−	+	+	+
10	70	Heart disease	Operation	−	+ 42·1	+	−	−	+	NT	+
11	77	Intracerebral haemorrhage	Operation	−	+ 1·4	+	−	−	+	NT	+
12	58	Gastric cancer	Chemotherapy	NT	+ 7·5	+	−	−	+	NT	+
13	58	Haematologic malignancy	HSCT	−	+	+	−	NT	NT	NT	+
14	82	Heart disease	Operation	NT	NT	+	−	−	+	+	+
15	80	Cancer	Chemotherapy	+2·52	−	−48·8	−	−1·69	−	+56·8	+
16	67	Gynaecological cancer	Chemotherapy	−1·87	−	−	−	+2·35	−	−	+

+, reactive or positive; −, non-reactive or negative; NT, not tested; HSCT, haematopoietic stem cell transplantation; AML, acute myelocytic leukaemia; HBsAg, anti-HBs and anti-HBc (Nos. 15 and 16) measured by EIA (AxSYM) in the hospital (normal range = HBsAg, S/N of <2·00; anti-HBc, % INH (inhibition) of <50·0) because the specimen volume was not sufficient to perform RPHA, PHA and HI.

Table 4 Analysis of blood components (n = 1009) transfused to 247 randomly selected patients negative for all three viruses on NAT

		Anti-HBc (HI) 2^n									
		0	1	2	3	4	5	6	7	8	Total
Anti-HBs (PHA) 2^n	0	896	14	13	6	3	Excluded from blood transfusion				932
	1	5	2		1	1					9
	2	8		2	3	1					14
	3	3	1	1	2	1					8
	4	3		3		3					9
	5			2	1		1	1		1	6
	6	3		1	1		1	1	1		8
	7	2		2	4	3				1	12
	8	1			1	2					4
	9	1				1			2	1	5
	10	1									1
	11							1			1
	Total	923	17	24	19	15	2	3	3	3	1009
			75				11				

NAT, nucleic acid amplification testing.

Values indicate the number of blood components with titres (2^n) of anti-HBc and anti-HBs transfused.

For example, '14' blood components with titres of anti-HBc and anti-HBs of 2^1 and 2^0, respectively, were transfused to patients.

with an anti-HBs titre of >1:32 that corresponds to 200 mIU/ml (11 specimens) (Table 4). All of the 86 donations met criteria for release for transfusion in Japan (Table 1). Of the 247 patients tested, neither pre- nor post-transfusion specimens from 165 patients were reactive for anti-HBs or anti-HBc, although 52 of these patients received blood components (total of 64) that were serologically reactive for anti-HBs and/or anti-HBc (Fig. 2). In

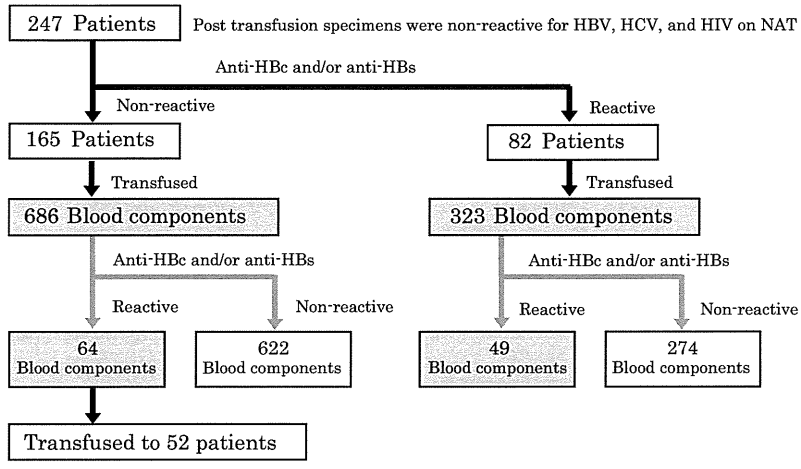

Fig. 2 The risk of transfusion of blood components with low anti-HBc and/or high anti-HBs titres. A total of 247 patients whose post-transfusion specimens were non-reactive for HBV, HCV and HIV on nucleic acid amplification testing were randomly selected, and their pre- and post-transfusion specimens were tested for anti-HBc and anti-HBs. One hundred and sixty-five patients showed non-reactivity for anti-HBc and anti-HBs in both their pre- and post-transfusion specimens, and they were transfused 686 blood components. The remaining 82 patients were reactive for anti-HBc or anti-HBs, and they were transfused 323 blood components. The repository specimens from the donors concerned were examined to determine the anti-HBc and anti-HBs titres. Of the 686 blood components, 64 were reactive for anti-HBc and/or anti-HBs and were transfused to 52 patients whose pre- and post-transfusion specimens were non-reactive for HBV DNA, anti-HBc and anti-HBs.

other words, 64 blood components that were serologically reactive for anti-HBs and/or anti-HBc were transfused to 52 patients, but no reactivity was observed for any of the HBV markers (HBsAg, anti-HBs, anti-HBc and HBV DNA) before and after transfusion.

Discussion

We commenced this study in November 2003 when the tests for post-transfusion hepatitis were not routinely performed in hospitals in Japan, including most of the hospitals that participated in this study. Medical insurance covered the cost of the tests for post-transfusion hepatitis only when a doctor suspected its possibility, and thus, a number of transmissions might have been missed. Similarly, anti-HBc and anti-HBs tests were also not performed before transfusions. Therefore, the JRC conducted this study to try to identify more accurately the transmission rate of infections in all patients receiving blood transfusions in the specified hospitals. The number of patients participating in this study was, however, insufficient to develop statistically significant rates.

Immediately after this study started, a post-transfusion specimen from a patient was found to be reactive for HBV DNA. However, neither the pre-transfusion specimen from the patient nor the repository specimen from the donor concerned was reactive for HBV DNA. Consequently, the remaining pre-transfusion specimen was tested and found to be reactive for anti-HBc. Therefore, it was concluded that the patient had been infected with HBV before transfusion–i.e., he had so-called OBI [17–20]. This demonstrates that OBI is an important issue among patients in Japan, especially in older patients [15] and patients receiving immunosuppressive therapies such as chemotherapy [21–23]. Since the completion of this study, medical insurance has been available to cover the cost of laboratory tests performed to evaluate viral markers of HBV, HCV and HIV (including anti-HBc) in all patients receiving blood transfusions. Furthermore, considering the significant background rates of HCV (7·2%) and HBV (3·4%) infections seen in Japanese patients, the Ministry of Health, Labor and Welfare has developed guidelines for the timing and testing required to support early detection of transfusion-transmitted HBV, HCV and HIV infections (Table 5). Pre-transfusion specimens can be tested for HBsAg, anti-HBs, anti-HBc, HCV core antigen, anti-HCV and anti-HIV. If these specimens are non-reactive for all the viral markers, post-transfusion specimens are tested for HBV DNA, HCV core antigen and anti-HIV. If any of the viral markers are reactive in pre-transfusion specimens, there is no requirement to undertake further testing for the viruses concerned in post-transfusion specimens. Of course, we can cryopreserve pre-transfusion specimens as performed in this study, and if the post-transfusion specimens are reactive for HBV DNA, HCV core antigen or anti-HIV, the cryopreserved pre-transfusion specimens can then be tested for the relevant viral markers.

The reasons for the high background rates of HBV and HCV infections, especially among older patients, are unclear; however, these rates might partly be the result of the reuse of needles and syringes for vaccination during

Table 5 The guideline of test markers for early detection of transfusion associated HBV, HCV and HIV infections in Japan

	Pre-transfusion	Post-transfusion	
Virus	**Test markers**	**When to test**	**Test markers**
HBV	HBsAg Anti-HBs Anti-HBc	Approximately 3 months later	HBV DNA
HCV	HCV core antigen Anti-HCV	1–3 months later	HCV core antigen
HIV	Anti-HIV	2–3 months later	Anti-HIV

childhood to save costs, a practice that lasted until the 1980s, or to the use of plasma anticoagulant products such as fibrinogen and factor VIII, which were not pathogen inactivated in the 1980s and 1990s. In fact, according to a report by Tanaka *et al.* [24], the prevalence of HBV and HCV in first-time blood donors was 0·63% (1·5% estimated for donors above 50 years) and 0·49% (2% estimated for donors above 50 years and 3% for donors above 60 years), respectively.

Transfusion-transmitted HCV or HIV infection was not observed in this study. The patient with confirmed transfusion-transmitted HBV infection was a 61-year-old man with acute myeloid leukaemia, and he underwent hematopoietic stem cell transplantation. On 29 November 2003, the patient received a platelet transfusion. The platelet component was derived from a donor on November 27. The donor's next donation was on 30 December 30 2003, and his blood sample was found to be reactive for HBV DNA using the 50 donation MP-NAT. A look-back study of the donor revealed that HBV DNA was detectable by ID-NAT in the repository specimen collected on 27 November 2003. Serum drawn from the patient on 26 January 2004 (on the same day when the result of look-back was obtained) was also reactive for HBV DNA, but the DNA level was too low (30 copies/ml) to sequence. His pre-transfusion specimen was non-reactive for HBsAg, anti-HBc, anti-HBs and HBV DNA. A total of 115 units of blood including this platelet component transfused to him were implicated, and the repository specimens from the donations were tested for HBV by ID-NAT. All specimens except the one identified previously were non-reactive for HBV DNA. Approximately 4 months later (1 June 2004), the patient's HBV DNA level was elevated (≥1000 copies/ml) along with leukaemia recurrence, and the specified HBV DNA sequences were consistent with those of the original donor. As the HBV DNA level in the patient was monitored, immediate administration of lamivudine (when the HBV DNA level was >1000 copies/ml), a nucleoside analogue reverse transcriptase inhibitor, prevented the development of acute hepatitis.

Despite the implementation of NAT screening, several cases of transfusion-transmitted HBV infection continue to occur each year in Japan [25]. One reason may be that only a few patients are immunized with a hepatitis B vaccine because only selective vaccination against HBV is carried out in Japan (medical staff, coworkers and babies born to HBV carrier mothers). In addition, donors in the early and late stages of HBV infection may have low HBV DNA levels that are detectable in ID-NAT but not by 50-NAT [26]. The patient discussed earlier is a typical case of transfusion of a blood component from a donor with an early acute HBV infection. The risk of HBV transmission identified in this study was 0·0004675 (95% CI for the risk of transmission, 1 in 451–41 841). However, data from donor-triggered look-back studies involving more than 10 000 cases between 2000 and 2004 [15] have been used to assess the residual risks of transfusion–transmission of these three viruses. On the basis of data reported by transfusion monitoring hospitals in Tokyo, the number of patients receiving blood transfusions was calculated to be 1·2 million per year in Japan [27]. The risks of transfusion-transmitted HBV, HCV and HIV infections were estimated at 13–17 cases per year (1 in 70 588–92 307), 1 case every 2–4 years (1 in 2 400 000–4 800 000), and 1 case in 4 years (1 in 4 800 000), respectively. In fact, 74, 41 and 0 cases of HBV, HCV and HIV infections, respectively, associated with transfusion were reported to the JRC in 2007. Investigation of these confirmed transfusion as the cause of 13 cases of HBV and 1 case of HCV [28].

Hollinger [29] has indicated that the reagents used in Japan to test blood donations and the criteria used by the JRC for the release of donations are different from those used in Western countries [30–33]. In Japan, if a specimen is reactive for anti-HBc at a titre of <1:32 based on a hemagglutination inhibition test or is ≥1:32 along with a passive hemagglutination inhibition assay revealing an anti-HBs titre of ≥200 mIU/ml, the blood components can be transfused to patients [3, 4]. We attempted to improve our understanding of the risk of routine transfusion of blood components to patients when these criteria are used. Pre-transfusion specimens, cryopreserved in Osaka City University hospitals, were tested for anti-HBs and anti-HBc. The specimens were non-reactive for HBsAg in all 247 patients tested but were serologically reactive for anti-HBs and/or anti-HBc in 82 patients. The remaining 165 patients whose pre-transfusion specimens were non-reactive for HBsAg, anti-HBc, anti-HBs and HBV DNA were transfused with 686 blood components. The repository specimens of the 686 donors concerned were tested for HBsAg, anti-HBs, anti-HBc and HBV DNA. Specimens of 64 of the donors were reactive for anti-HBs and/or anti-HBc, and their blood components (64) were transfused to 52 patients. None of

the HBV markers changed in those patients receiving these components indicating that the blood components with low anti-HBc and/or high anti-HBs titres and with non reactive results for HBV DNA by MP-NAT have a low risk (rate, 0; 95% confidence interval for the risk of transmission, <1 in 22) (Fig. 2).

The JRC implemented a chemiluminescent EIA system (Fujirebio Inc., Tokyo, Japan) in 2008 replacing the earlier agglutination method. We have continued the same strategy of using blood from donors with low anti-HBc titres (cut-off index <12) or high anti-HBc titres (cut-off index≥12) with high anti-HBs titres (≥200 mIU/ml) for transfusion because discarding these blood components (86/1009, 8·5% in Table 4) would have a huge influence on our ability to maintain a stable blood supply to hospitals. However, most of these donors are ≥50 years in most cases [34], and it is likely that we will be able to review this approach and adopt a policy of only issuing anti-HBc-negative blood components in the future. Meanwhile, we will continue to evaluate the residual risk of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres.

Collaborating institutes

Asahikawa Medical College Hospital (Hokkaido).
Iwate Medical University Hospital (Iwate).
Osaka City University Hospital (Osaka).
Osaka Red Cross Hospital (Osaka).
Osaka City General Hospital (Osaka).
Ehime Red Cross Hospital (Ehime).
National Hospital Organization Shikoku Cancer Center (Ehime).
Fukuoka University Hospital (Fukuoka).

Acknowledgements

We thank the doctors and medical staff of the collaborating institutes and members of the five JRC blood centres and NAT centres for conducting this study.

Conflict of interest

The authors have no conflict of interest to declare regarding this manuscript.

References

1 Meng Q, Wong C, Rangachari A, *et al.*: Automated multiplex assay system for simultaneous detection of hepatitis B virus DNA, hepatitis C virus RNA, and human immunodeficiency virus type1 RNA. *Vox Sang* 2001; 39:2937–2945
2 Yotsuyanagi H, Yasuda K, Moriya K, *et al.*: Frequent presence of HBV in the sera of HBsAg-negative, anti-HBc-positive blood donors. *Transfusion* 2001; 41:1093–1099
3 Japanese Red Cross Non-A, Non-B Hepatitis Research Group: Effect of screening for hepatitis C virus antibody and hepatitis B virus core antibody on incidence of post-transfusion hepatitis. *Lancet* 1991; 338:1040–1041
4 Iizuka H, Ohmura K, Ishijima A, *et al.*: Correlation between anti-HBc titers and HBV DNA in blood units without detectable HBsAg. *Vox Sang* 1992; 63:107–111
5 Japanese Red Cross NAT Screening Research Group: Nationwide nucleic acid amplification testing of hepatitis B virus, hepatitis C virus and human immunodeficiency virus type 1 for blood transfusion and follow-up study of nucleic acid amplification positive donors. *Jpn J Infect Dis* 2000; 56:116–123
6 Ohnuma H, Tanaka T, Yoshikawa A, *et al.*: The first large-scale nucleic acid amplification testing (NAT) of donated blood using multiplex reagent for simultaneous detection of HBV, HCV, and HIV-1 and significance of NAT for HBV. *Microbiol Immunol* 2001; 45:667–672
7 Japanese Red Cross Blood Programme: Drug information; Haemovigilance Information. http://www.jrc.or.jp/mr/english/index.html
8 Busch MP: Donor screening using nucleic acid amplification assays short topic #219: AABB 51st Annual Meeting, 1998;164–169
9 Busch MP, Kleinman SH: Nucleic acid amplification testing of blood donors for transfusion-transmitted infectious diseases. *Transfusion* 2000; 40:143–159
10 Yoshikawa A, Gotanda Y, Minegishi K, *et al.*: Lengths of hepatitis B viremia and antigenemia in blood donors: preliminary evidence of occult (hepatitis B surface antigen-negative) infection in the acute stage. *Transfusion* 2007; 47:1162–1171
11 Wroblewski F, LaDue JS: Serum glutamic pyruvic transaminase in cardiac and hepatic disease. *Proc Soc Exp Bio Med* 1956; 91:569–571
12 Mine H, Emura H, Miyamoto M, *et al.*: High throughput screening of 16 million serologically negative blood donors for hepatitis B virus, hepatitis C virus and human immunodeficiency virus type-1 by nucleic acid amplification testing with specific and sensitive multiplex reagent in Japan. *J Virol Methods* 2003; 112:145–151
13 Okamoto H, Okada S, Sugiyama Y, *et al.*: Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5 non-coding region. *Jpn J Exp Med* 1990; 60:215–222
14 Matsumoto C, Nishioka K, Oguchi T, *et al.*: Detection and quantitation of HBV DNA by semi-nested PCR in donated blood: comparison with HBV serological markers. *J Virol Methods* 1997; 66:61–69
15 Satake M, Taira R, Yugi H, *et al.*: Infectivity of blood components with low hepatitis B virus DNA levels identified in a lookback program. *Transfusion* 2007; 47:1197–1205
16 Burrows L, Tartter P: Effect of blood transfusions on colonic malignancy recurrent rate. *Lancet* 1982; 18:662

17 Brechot C, Thiers V, Kremsdorf D, *et al.*: Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen:clinically significant or purely "occult"?. *Hepatology* 2001; **34**:194–203

18 Torbenson M, Thomas DL: Occult hepatitis B. *Lancet Infect Dis* 2002; **2**:479–486

19 Allain JP: Occult hepatitis B virus infection: implications in transfusion. *Vox Sang* 2004; **86**:83–91

20 Liu CJ, Chen DS, Chen PJ: Epidemiology of HBV infection in Asian blood donors:emphasis on occult HBV infection and the role of NAT. *J Clin Virol* 2006; **36**:S33–S44

21 Power JP, El Chaar M, Temple J, *et al.*: HBV reactivation after fludarabine chemotherapy identified on investigation of suspected transfusion-transmitted Hepatitis B virus. *J Hepatol* 2010; **53**:780–787

22 Ng HJ, Lim LC: Fulminant hepatitis B virus reactivation with concomitant listeriosis after fludarabine and rituximab therapy: case report. *Ann Hematol* 2001; **80**:549–552

23 Dervitte I, Hober D, Morel P: Acute hepatitis B in a patient with antibodies to hepatitis B surface antigen who has receiving rituximab. *N Engl J Med* 2001; **344**:68–69

24 Tanaka J, Kumagai J, Katayama K, *et al.*: Sex-and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3,485,648 first-time blood donors during 1995–2000. *Intervirology* 2004; **47**:32–40

25 Japanese Red Cross Blood Programme: Drug information; Haemovigilance Annual Report 2001 and 1993–2001. http://www.jrc.or.jp/vcms_lf/iyakuhin_shiryou080925-05.pdf

26 Kuhns MC, Busch MP: New strategies for blood donor screening for hepatitis B virus:nucleic acid testing versus immunoassay methods. *Mol Diagn Ther* 2006; **10**:77–91

27 Bureau of Social Welfare and Public Health, Tokyo Metropolitan Government: http://www.fukushihoken.metro.tokyo.jp/iryo/k_isyoku/kakokekka/index.html

28 Japanese Red Cross Blood Programme: Drug information; Haemovigilance by JRCS 2007. http://www.jrc.or.jp/vcms_lf/iyakuhin_english_benefit_material_100428.pdf

29 Hollinger FB: Hepatitis B virus infection and transfusion medicine: science and the occult. *Transfusion* 2008; **48**:1001–1026

30 Kleinman SH, Strong DM, Tegtmeier GG, *et al.*: Hepatitis B virus (HBV) DNA screening of blood donations in mini-pools with the COBAS AmpliScreen HBV test. *Transfusion* 2005; **45**:1247–1257

31 Brojer E, Grabarczyk P, Liszewski G, *et al.*: Characterization of HBV DNA+/HBsAg-blood donors in Poland identified by triplex NAT. *Hepatology* 2006; **44**:l666–l1674

32 Linauts S, Saldanha J, Strong DM: PRISM HBsAg detection of HBV mini-pool NAT yield samples. *Transfusion* 2008; **48**:1376–1382

33 Hourfar MK, Schmidt M, Roth W, *et al.*: Experience of German Red Cross blood donor services with NAT-testing: results of screening more than 30 million blood donations for HIV-1, HCV, and HBV. *Transfusion* 2008; **48**:1558–1566

34 Hoshi M, Shibagaki K, Hosoda Y, *et al.*: HBV DNA reactivity and age distribution in the anti-HBc positive blood donors in Japan. *J Jpn B Prgm* 2006; **29**:354