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Hepatitis B virus-positive blood donors in the early stages of Hepatitis B virus infection: Significance of nucleic acid amplification testing

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Abstract

Since 1999, 1,387,094 units of serologically negative blood donations to the Hyogo Blood Center have been screened by nucleic acid amplification testing (NAT). Thirty hepatitis B virus (HBV)-DNA positive donors were identified from among all the donors. Twenty-one out of the 30 selected donors were contacted and monitored with their courtesy and consent. Their HBV serological markers, HBV copy number, and alanine transaminase (ALT) levels were analyzed. They were then classified into three groups according to the pattern of increase in their ALT levels. Nine cases in Group I showed more than 1,000 IU/L ALT and 9 cases in Group II showed slightly higher levels. Because the monitoring started during the very early stage of the serological negative period, all the phenomena associated with the development of typical hepatitis symptoms became evident in Groups I and II. On the other hand, the ALT levels of three cases in Group III remained within the normal range or only slightly increased, and these three were asymptomatic for hepatitis B. In two cases, a single nucleotide substitution, G1896A, was observed in the precore region but not in the core promoter region; this substitution result in the tryptophan codon (TGG) being converted to a stop codon (TAG). Another case showed a mixed type mutation involving the wild type and G1896A substitution. Although many reports on fulminant or subfulminant hepatitis patients associated with

precore-defective HBV mutants have been published, asymptomatic hepatitis B patient with precore-defective HBV mutants have not been reported. Thus, it is suggested that a mutation in the core promoter (T1754C/G and/or A1762T/G1764A) might cause an increase in the viral growth rate or suppression for viral increase.

Key words: Nucleic acid amplification testing, NAT, Asymptomatic B hepatitis, Hepatitis B virus, Precore-defective HBV mutants

Introduction

Approximately 5% of the global population is infected with the hepatitis B virus (HBV) that causes liver disease of variable duration and severity. Most clinical reports on the time course of HBV infection focused on the late stage of the HBV infection, because the patients could only be identified when the symptoms have developed. Therefore, reports focusing on the very early stage of infection or on asymptomatic cases are few.

To eradicate post-transfusion infection, nationwide nucleic acid amplification testing (NAT) for hepatitis B virus, hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) of voluntary blood donors was implemented in July 1999 for both transfusion blood and plasma fractionation by the JRC blood transfusion services. This NAT system consisted of a strict transport system, a computerized network system and an automated analytical system, as previously reported by Mine *et al.*[1]. With this NAT system, HBV DNA-positive donors from serologically negative donors could be identified and examined at the early pre-seroconversion stage. In a typical case of acute hepatitis, the HBsAg appears in serum in 1 to 10 weeks after an acute exposure to HBV and in approximately 2 to 6 weeks before the onset of hepatitis symptoms or the increase of alanine aminotransferase (ALT) level[2].

Thus, we can deduce the development of acute hepatitis, because the

HBV serological markers, HBV DNA copy number, HBV growth rate, and ALT level can be determined. Moreover the moment and route of infection can be speculated on the basis of the HBV growth rate from the blood donation day. This is important to prevent the HBV infection and improve the health care of voluntary blood donors. Indeed, from the samples that are considered negative by the conventional serological method, HBV-positive samples as determined by NAT can be identified, and further examinations such as the determination of the subtype, genotype and mutation sites in HBV can be carried out. Then, the asymptomatic hepatitis B individual with a precore-defective HBV mutant could be identified. This research might serve as an opportunity to solve the correlation between precore/core promoter mutations and the severity of hepatitis.

Materials and Methods

Serological tests for donated blood

All blood units voluntarily donated to the Hyogo Red Cross Blood Center were screened for HBsAg by reverse passive hemagglutination, for antibody to HBV core antigen (anti-HBc) by hemagglutination inhibition, and for antibody to HBsAg (anti-HBs) by the passive hemagglutination method[3,4]. These agglutination tests were carried out using the automatic PK7200 (Olympus Co., Ltd.) analyzer and were confirmed by the specific inhibition test. The end point of the two-fold dilution of test samples was expressed as an exponent of 2^n vs. the end titer of the specific inhibition test. Serologically positive and ALT-elevated (> 60 IU/L) samples were excluded from further NAT screening.

Characterization of positive samples determined by NAT

Blood samples of 5 ml volume in EDTA were shipped for NAT. In the JRC Center for NAT and Quarantine, each 0.1 ml of serum from serologically negative samples was automatically pooled for the first NAT screening for HBV/HCV/HIV-1 (MPX). In an MPX-positive pool, the HBV-positive unit was identified using the HBV control from the pooled sera. Furthermore, the subtype, genotype and mutation sites in the precore region of HBV were determined in the Research Laboratory of the JRC Central Blood Center[4,5,6]. To detect HBV DNA, the primers derived from the HBsAg region were

applied as previously reported[7]. The genotypes of HBV and the precore or core promoter mutation were identified and characterized according to the method of Okamoto and coworkers[7,8]. The HBV DNA copy number was calculated using the internal standards provided by the National Institute for Biological Standards and Control. The calculation was carried out using the Sequence Detector version 1.7 (PE Applied Biosystems). The HBV DNA data represents the average of quadruplicate tests[9].

Follow-up study of HBV DNA-positive donors

HBV DNA-positive donors were contacted and a medical doctor had a talk with them. Twenty-one out of 30 donors were informed of the follow-up study and gave their consent. At the same time, blood was taken from the donors and placed in 5ml PET tubes contaning EDTA . Then, the samples were tested for ALT activity and HBV DNA copy numbers, followed by tests for HBsAg by overnight enzyme immunoassay using Auszyme II (Abbott Lab.,U.S.A.), and for IgM type anti HBcAb (IgMAb) using Corzyme-M (Abbott Lab.) at the Hyogo Blood Center or the Research Laboratory of the JRC Central Blood Center. The donors were monitored with their courtesy and consent through a collaboration between the JRC and the medical institutions.

Estimation of HBV growth rate

The log time of HBV growth (10-fold increase) was calculated from the slope of the exponential phase of HBV DNA accumulation.

Estimated duration

The estimated duration was defined as the period between the moment of infection and the HBV DNA peak. The moment of infection was calculated from the viral loads in the HBV-positive sample and the log time of HBV DNA growth.

Results

After the preliminary serological screening test, DNA samples were collected from 1,387,094 hepatitis-negative donors of the Hyogo Red Cross Blood Center since 1999. First, NAT by the JRC Center for NAT and Quarantine was performed for each of the combined specimens of 500, 50 and 20, respectively, as a mini-pool. The rate of positive samples was not significant compared with the their

pooled samples as shown in Table 1. Among them, 30 HBV DNA-positive donors, 2 HCV RNA-positive donors and 2 HIV RNA-positive donors were identified. HBV DNA-positive donors did not doubly infect of HCV or HIV.

When these 30 HBV DNA-positive donors were examined according to genotype, the genotypes A, B and C were 3, 2 and 25 units, respectively, as shown in Table 1. Genotype A has appeared since 2005. As shown in Table 2, the subtypes of HBV DNA, namely adr, adw, and dr, were found in 22, 7 and 1 units, respectively. Among the 30 HBV DNA-positive donors, 25 were wild type, 4 were precore mutants and 1 was mixed type (wild type/precore mutant).

Finally, the 21 donors as summarized in Table 3 were moniterd with their courtesy and consent through a collaboration between the JRC and the medical institutions. The ALT level, HBV DNA copy number and various serological indexes were monitored approximately once every 2 weeks. On

the basis of the increase in ALT level, the 21 donors were classified into three groups. In group I, their ALT levels increased to more than approximately 1,000 IU/L, in group II, only a slight increase was observed, and in group III, no change within the normal range or only a small increase in ALT level was found, as shown in Table 3. The numbers of donors in each group were 9 (43%), 9 (43%) and 3 (14%), respectively.

On the donation day, the HBV DNA copy number was 10^2-10^5 copies/ml. The interval between the donation day and the peaking of the HBV DNA copy number was 10-40 days (average of 25.7 days) in group I and 14-47 days (average of 29.4 days) in group II. In groups I and II, the maximum copy number was in the range of 10^5-10^8 copies/ml (average of approximately 10^6 copies/ml). The log time required for the HBV growth to increase 10-fold was 6.6-28.0 days (average of 12.2 days) in group I and 8.0-19.0 days (average of 12.8 days) in group II. There were 4 cases in group I and 1 case in group II whose log time of HBV growth was approximately 6 days. The interval between the moment of viral infection and HBV DNA peak (estimated duration) of group I was 53 –168 days (average of 77.3 days), and that of group II was 39-95 days (average of 74.9 days).

On the other hand, 2 cases (#2 and #11) in group III showed 10^2 -10^3 copies/ml on the donation day. Their maximum copy number was 10^4 copies/ml and their log time was 38.5-74 days. The interval between the moment of viral infection and HBV DNA peak (estimated duration) was

154-296 days. The log time of HBV growth and the estimated duration were longer than those of groups I and II. They did not show signs of hepatitis.

The increase in HBV DNA copy number, ALT level and some serological data of groups I, II and III are shown in Figures 1, 2 and 3, respectively. In Figure 1, a typical case (#21: C, adw, wild type) in group I showed acute hepatitis B. The HBV DNA copy number on donation day was $1.2x10^3$ copies/ml. HBsAg was detected after 2 weeks by the EIA method, and then HBeAg was detected after more 2 weeks. During the follow-up observation period, HBcAb, HBsAb, and HBeAg/Ab were detected. The HBV DNA copy number became $5.6x10^8$ copies/ml. Then, the serum ALT level increased to 6,942 IU/L 5 days after the peaking of the HBV DNA copy. HBsAb was detected 6 weeks after the donation day and #21 donor showed a recovery. In Figure 2, a case (#27: C, adr, wild type) presented with mild hepatitis B. His HBV DNA copy number was $1.8x10^3$ copies/ml on the donation day; HBsAg was detected by EIA after 2 weeks. However, his serum ALT level increased to 132 IU/L after more than 2 weeks. Thus, his HBV DNA copy number became $5.8x10^6$ copies/ml. HBeAg seroconversion was observed. After 8 weeks from the donation date, his ALT level became abnormal and he recovered. In Figure 3, a case (#2: C, adr, mutant type) presented with asymptomatic hepatitis B. His HBV DNA copy number showed a slight increase and no change in ALT level was observed. Moreover, HBsAg, HBsAb and HBeAg/Ab could not be detected by ordinary EIA

methods during the follow-up period, and HBcAb was detected 15 weeks after blood donation.γ-GTP values were observed within the range of 81-99 IU/L. This case presented with mild hepatic disorder.

The nucleotide sequences of the HBV core promoter (Nt. 1699-1780) and the precore (Nt. 1885-1919) in group III donors are shown in Figure 4. Two cases (#2 and #11) in group III had a point mutation from guanine to adenine (G1896A), which converted codon 28 from tryptophan (TGG) to a stop codon (TAG). However, other reported mutations such as T1753A/C, T1754C/G, A1762T and G1764A were not recognized in the core promoter site. Another case (#12) in group III was of mixed type (wild type/G1896A).

Discussion

The number of samples for NAT decreased in 500, 50 or 20 units in chronological order. It was expected that the NAT sensitivity would gradually increase as the sample size was decreased. However, the HBV DNA positivity rate remained unchanged. For eight years from 1999, of the 1,387,094 so-called HBV negative donors who donated blood as determined by the usual serological method, 30 HBV DNA positive donors were identified by NAT. Twenty-five of the 30 cases carried the HBV genotype C. The HBV genotype A in Northern Europe and North America was confirmed primarily from 2005. The same trend was observed in the data of the JRC Center for NAT and Quarantine[10]. In the near future, the genotypes A and D in blood donors may increase [11]. Mayerat *et al.* [12] reported the prevalence of genotype A in acute hepatitis, and Joh *et al.* [13,] reported that acute hepatitis B with genotype A increased in the Tokyo area.

Twenty-one HBV DNA-positive donors were monitored to observe some very early aspects of HBV infection, later progress of the hepatitis onset and the recovery phase. Because the advanced degree of hepatitis is indicated by the ALT level, their ALT levels were monitored, and the donors were classified into three groups in this study. As a typical result, the log time for a 10-fold increase in HBV growth was a short period of 6-7 days. The serious cases classified under group I were exemplified in 4 cases and their

estimated duration became shorter. From these data, it would appear that the increase in the ALT level is dependent on HBV growth rate. Therefore, the follow-up interval was 6-7 days, and these donors should be referred to a medical institution. Thus, it was speculated that the estimated duration calculated using the log time of Groups I and II were 77.3 and 74.9 days, respectively. Moreover, for the donor (#12) with a mixed type in Group III, it was 75 days. These results agreed with the 72 days previously reported by Yoshikawa *et al.* [14]. Because the moment of viral infection can be speculated using the slope of the exponential phase, the infection pathway can be clarified. It was suggested that the quick referral to a medical institution is extremely important.

All the cases in groups I and II carried the wild type HBV, but 3 cases in group III presented with asymptomatic hepatitis B and carried two variants and a mixed type. Although there are previous reports[15-19] showing that precore-defective mutants are associated with fulminant or subfulminant hepatitis B, recent reports have shown that the mutations in the precore (G1896A caused termination as a stop codon) and core promoter (T1753A/C and/or T1754C/G and/or A1762T/G1764A) are associated with fulminant hepatitis. This predominant G1896A mutation leads to premature termination of the precore/core protein, thus, preventing the production of HBeAg. HBeAg might be expressed on the hepatocyte membrane and may serve as a target for immune attack. Recent studies suggest that the G1896A

mutation may also be preferred because of its ability to enhance HBV replication [20, 21].

Sainokami *et al* .[22,23] reported that mutation in the core promoter (T1753A/C and/or T1754C/G and/or A1762T/G1764A) is associated with fulminant hepatitis. Dixit *et al.* [24] reported a case of only asymptomatic chronic hepatitis B without reference to the virus. Furthermore, Suzuki *et al.* [25] and Fang *et al* .[26] reported the clinical knowledge of asymptomatic carriers of hepatitis B virus. However, asymptomatic hepatitis B patients with precore-defective HBV mutants have not been reported. A stop codon at codon 28 was found in donor #2 and #11, but their ALT levels remained within the normal range or slightly increase; moreover, only HBcAb (total) was detected. Indeed, other mutations such as T1753A/C, T1754C/G, A1762T and G1764A were not recognized in the core promoter site.

In the case of a mixed type of mutation such as that found in donor #12, an obstruction of the hepatitis onset was observed. It was important that the HBV copy number increased to 5.6 x 10^6 and that the ALT level remained unchanged. A model experiment on these differences between HBV growth and ALT level was conducted using transgenic mice. Upon infection with HBV, the cytotoxic T cells recognized the infected hepatocytes and killed them, and the HBV were confined and inactivated by some cytokines [27]. It is important to determine the presence of both precore and core promoter mutations in HBV DNA-positive blood donors. Our study confirmed that the

NAT system could prevent the transfusion of the blood and in plasma derivatives of a donor with a specific mutation type.

This study was able to show that information on the moment of viral infection and infection pathway, and the subsequent monitoring are absolutely important in the health care of a blood donor.

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Legends for Figures

Figure 1, Clinical course of donor #21. The donor #21 was a genotype, a subtype and a mutation at precore region (A, adw, wild) in Table 3. A schematic depiction of the immune response in acute infection with wild type HBV, followed by clinical recovery, is shown.

Figure 2, Clinical course of donor #27 (C, adr, wild) as shown in Table 3 and Figure 1.

Figure 3, Clinical course of donor #2 (C, adr mutant) as shown in Table 3.

Figure 4, The nucleotide sequences of HBV core promoter (nucleotides 1699-1780) and precore (nucleotides 1885-1919) regions of donors #2, #11 and #12 in Group III. The HBV of all the donors were of genotype C subtype adr. Donors #2 and #11 have a precore mutation (nucleotide 1896, precore codon 28). Donor #12 has a mixture of A and G nucleotide 1896.

Table 1. Donors of positive HBV DNA, HCV RNA and HIV RNA after NAT.

Donation period	Pool size	No. for NAT	HBV DNA-positive donors	Genotype			HCV RNA	HIV RNA
				A	B	C		
1999.7-2000.1	500 samples	75,893	1	0	0	1	0	0
2000.2-2004.8	50 samples	846,870	19	0	1	18	1	0
2004.9-2007.1	20 samples	464,331	10	3	1	6	1	2
Total		1,387,094	30	3	2	25	2	2

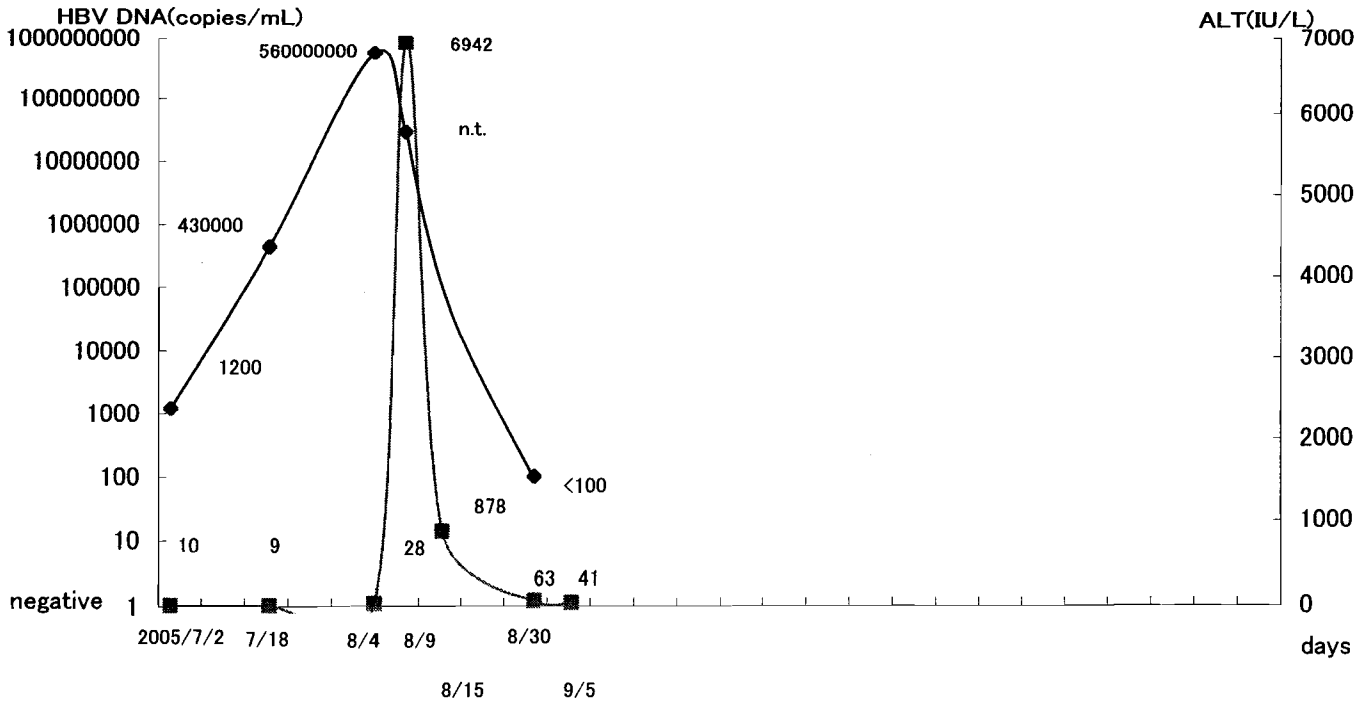
Table 2. Distribution of HBV genotypes among HBV DNA-positive donors.

Genotype		Subtype			Precore		
		adr	adw	dr	wild	mutant	mix
A	3(2)	0	3(2)	0	3(2)	0	0
B	2(1)	0	2(1)	0	2(1)	0	0
C	25(18)	22(15)	2(2)	1(1)	20(15)	4(2)	1(1)
Total	30(21)	22(15)	7(5)	1(1)	25(18)	4(2)	1(1)

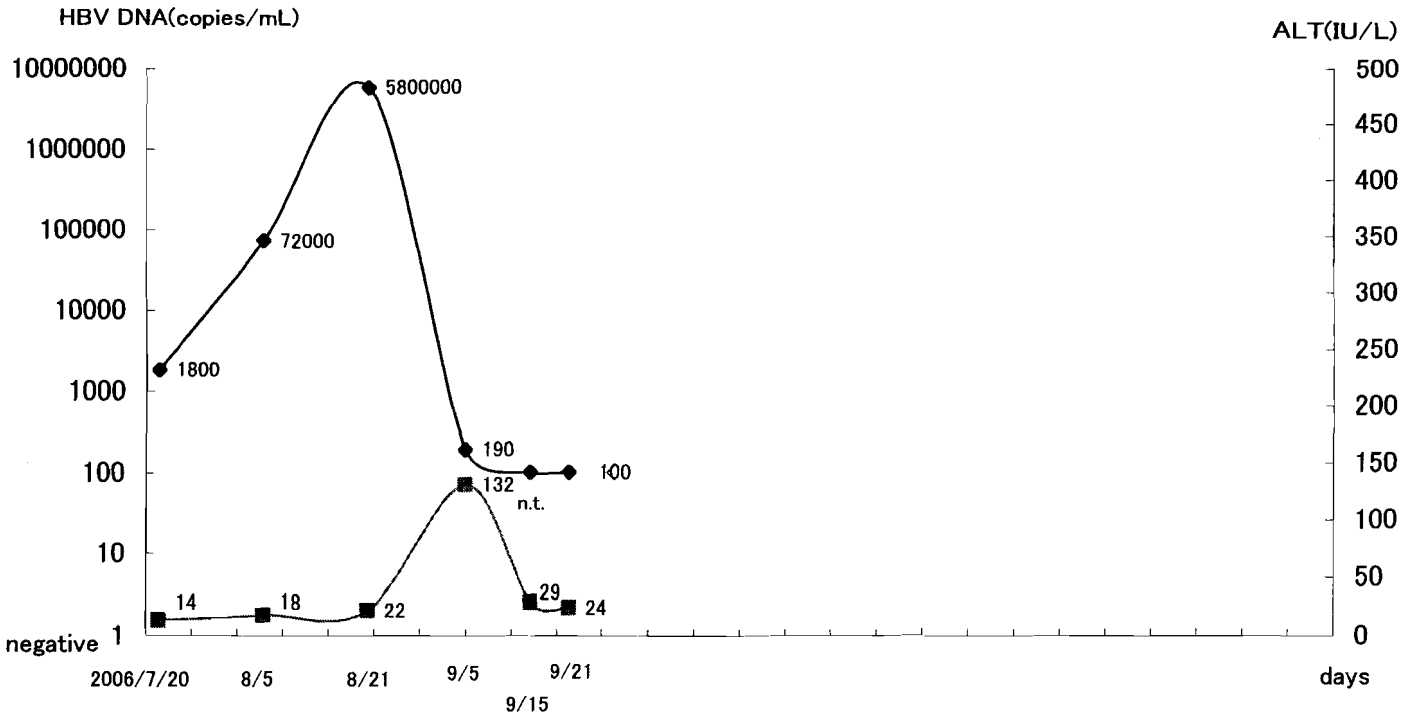
( ) : follow-up donors

Table 3. Distribution of ALT levels and HBV DNA copy number in 21 follow-up cases.

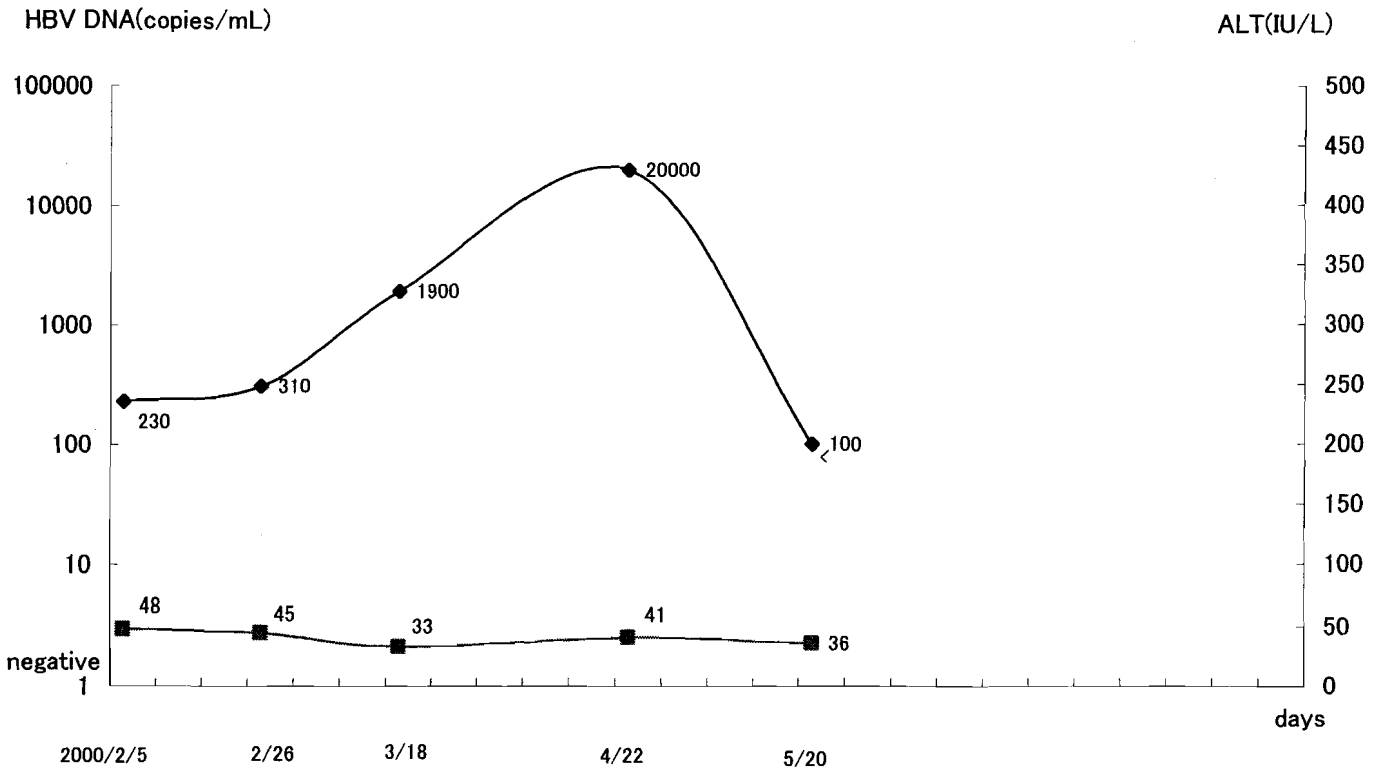
Group	Follow – up No.	Date of donation	Genotype	Subtype	Precore	Viral copies upon blood donation (copies/ml)	Maximum number of copies (copies/ml)	Interval between blood donation and HBV DNA (days)	Log times of HBV DNA (days)	ALT peak IU/L	Estimated duration. (days)
	21	2005/7/2	A	adw	wild	1.2×10^{3}	5.6×10^{8}	33	6.6	6,942	53
	9	2001/1/28	C	adw	wild	2.4×10^{3}	1.7×10^{5}	38	19.0	6,940	95
	30	2007/1/17	C	adr	wild	2.9×10^{4}	3.6×10^{5}	12	12.0	2,500	60
I	22	2005/11/1	B	adw	wild	6.0×10^{4}	6.2×10^{5}	10	10.0	1,837	50
	20	2003/11/2	C	adr	wild	1.8×10^{5}	7.6×10^{8}	20	6.7	1,430	54
	13	2002/5/8	C	adr	wild	2.7×10^{3}	1.2×10^{7}	27	6.8	1,395	48
	14	2002/12/28	C	adw	wild	1.1×10^{5}	4.6×10^{6}	28	28.0	1,223	168
	1	1999/11/30	C	adr	wild	1.1×10^{5}	7.1×10^{8}	40	13.3	1,029	106
	26	2006/6/12	A	adw	wild	1.4×10^{5}	3.2×10^{8}	23	7.7	964	62
		Average					$\times10^{6.7}$	25.7	12.2		77.3
	4	2000/5/14	C	adr	wild	1.0×10^{2}	5.5×10^{5}	32	10.7	459	54
	10	2001/8/9	C	adr	wild	1.3×10^{4}	2.5×10^{8}	41	10.3	265	82
	25	2006/4/29	C	adr	wild	2.8×10^{4}	3.2×10^{8}	32	8.0	202	64
II	27	2006/7/20	C	adr	wild	1.8×10^{3}	5.8×10^{6}	47	15.7	132	94
	29	2006/11/15	C	dr	wild	8.8×10^{2}	1.1×10^{5}	23	7.7	101	39
	3	2000/2/12	C	adr	wild	6.3×10^{4}	3.0×10^{5}	14	14.0	77	70
	5	2000/7/28	C	adr	wild	1.6×10^{5}	9.8×10^{5}	19	19.0	71	95
	24	2006/4/20	C	adr	wild	5.4×10^{4}	1.3×10^{7}	41	13.7	69	96
	23	2006/3/6	C	adr	wild	1.7×10^{4}	1.2×10^{5}	16	16.0	55	80
		Average					$\times10^{6.0}$	29.4	12.8		74.9
III	2	2000/2/5	C	adr	Mutant	2.3×10^{2}	2.0×10^{4}	77	38.5	48	154
	11	2001/11/30	C	adr	Mutant	1.4×10^{3}	1.2×10^{4}	74	74.0	27	296
	12	2001/2/27	C	adr	Mix	7.4×10^{4}	5.6×10^{6}	15	7.5	23	75



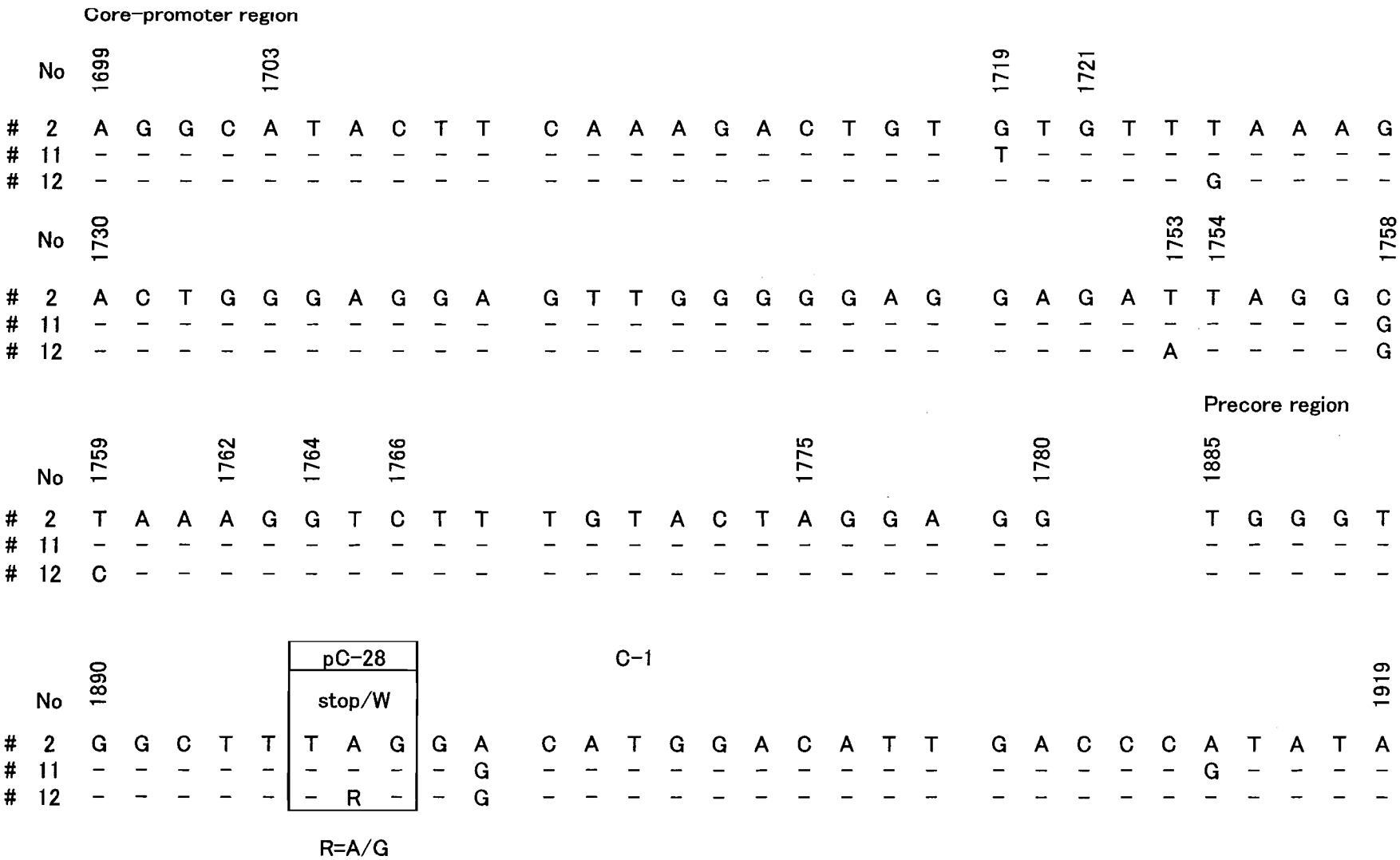
	2005/7/2	7/18	8/4	8/9	8/15	8/30	9/5
days	0	16	33	38	44	59	65
HBsAg(EIA)	−	+	+	+	−	−	−
HBsAb(PHA)	−	−	−	−	+	+	+
HBcAb(EIA) (IgM)	−	−	−	n.t.	n.t.	+	n.t.
HBcAb(EIA) (Total)	−	−	+	+	+	+	+
HBeAg/Ab(EIA)	−/−	−/−	+/−	+/−	+/+	n.t./n.t.	+/+



	2006/7/20	8/5	8/21	9/5	9/15	9/21	
days	0	16	32	47	57	63	
HBsAg(EIA)	−	+	+	−	n.t.	−	
HBsAb(PHA)	−	−	−	−	n.t.	−	
HBcAb(EIA) (IgM)	−	−	−	−	n.t.	*	
HBcAb(EIA) (Total)	−	−	−	+	n.t.	+	
HBeAg/Ab(EIA)	−/−	+/−	+/−	−/+	n.t.	−/+	*: Indeterminate



	2000/2/5	2/26	3/18	4/22	5/20	2003/3/31	
days	0	21	42	77	105	after 3years	
HBsAg(EIA)	−	−	−	−	−	−	
HBsAb(PHA)	−	−	−	−	−	−	
HBcAb(EIA) (IgM)	−	−	−	−	−	n.t.	
HBcAb(EIA) (Total)	−	−	−	−	+	−*	*: HI method
HBeAg/Ab(EIA)	n.t./n.t.	−/−	−/−	−/−	−/−	n.t./n.t.	


Core-promoter region
No
1699
1703
1719
1721
2 A G G C A T A C T T C A A A G A C T G T G T G T T T A A A G
11 - T - - - - - - - -
12 - G - - - -
No
1730
1753
1754
1758
2 A C T G G G A G G A G T T G G G G A G G A G A T T A G G C
11 - G
12 - A - - - G
Precore region
No
1759
1762
1764
1766
1775
1780
1885
2 T A A A G G T C T T T G T A C T A G G A G G T G G G T
11 -
12 C -
pC-28
stop/W
C-1
No
1890
1919
# 2 G G C T T T A G G A C A T G G A C A T T G A C C C A T A T A
# 11 - - - - - - - - G - - - - - - - - - - - - - - - G - - - -
# 12 - - - - - - R - G -
R=A/G