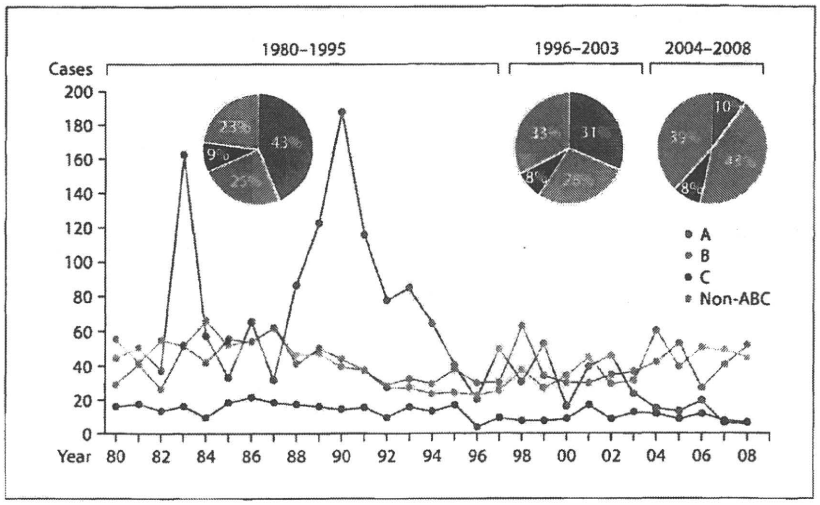

Fig. 1. Trends of AVH in Japan (1980–2008, n = 4,302).

ly enrolled to the study, and sera taken on admission were stored for further analysis. There were some, but only minor changes in the member hospitals due to personnel transfer and/or elimination and consolidation of hospitals during the observation period.

Clinical Case Definition

Acute hepatitis was defined as acute illness with (1) discrete onset of symptoms (e.g. nausea, anorexia, fever or malaise) and (2) jaundice or elevated serum aminotransferase and/or total bilirubin levels.

The laboratory criteria for confirming each type of AVH were as follows: (1) acute hepatitis A (AHA): positivity for immunoglobulin M (IgM) antibody to hepatitis A virus (anti-HAV); (2) acute hepatitis B (AHB): positivity for IgM antibody to hepatitis B core antigen (anti-HBc) or hepatitis B surface antigen (HBsAg); (3) acute hepatitis C (AHC): positivity for RT-PCR for HCV RNA, and either anti-HCV negativity at the onset of disease or significant increase of anti-HCV in the assay 2 weeks after onset.

Exclusion criteria were as follows: (1) involvement of non-hepatitis viruses, including Epstein-Barr virus, cytomegalovirus, parvovirus and herpes virus; (2) involvement of other etiologies such as autoimmune hepatitis, drug-induced hepatitis, and (3) alcoholic liver diseases. After excluding such types of liver dysfunction, the 4th category of non-ABC hepatitis was made.

Data Collection

In this survey, patients' data and serum samples were collected on an annual basis and stored at the National Nagasaki Medical Center until they were used for analysis. After 2005, all patients' written informed consent for enrollment in this study was obtained.

RT-PCR and Sequencing

Samples that were serologically confirmed for AHB or acute hepatitis E (AHE) were further subjected to DNA or RNA testing by means of PCR and RT-PCR, respectively. Amplification and sequencing of appropriate regions were performed by the methods described elsewhere. Briefly, total RNA or DNA was extracted from the patient's serum sample. Detection of hepatitis virus genome was performed by PCR with primers derived from well-conserved genomic areas. Sequences were compared with those from isolates from various origins. Phylogenetic trees were constructed by neighbor-joining method.

Results

Trends of AVH in Japan

A total of 4,302 cases are the subject of this study. In total, throughout the study period (1980–2008) AHA accounted for 1,583 cases (37%), AHB for 1,197 (28%), AHC for 359 (8%) and non-ABC hepatitis for 1,163 (27%). The period between 1980 and 1995 when AHA was apparently predominant among viral hepatitis can be called the AHA dominant phase. During the following 8 years (1996–2003), however, the proportions of AHA, AHB, AHC and non-ABC hepatitis changed to 31, 28, 8 and 33%, respectively. This phase is characterized by even distribution of AHA, AHB and non-ABC hepatitis. Furthermore, the proportions of AHA, AHB, AHC and non-ABC hepatitis in the last 5 years (2004–2008) were 10, 43, 8 and 39%, respectively. Apparently, these changes in trend of hepatitis were caused by a major decrease of AHA incidence (fig. 1).

Genotype Shifting of AHB

Genotype analyses for AHB cases were done on 498 samples (1991–2008). Figure 2a shows the proportions of genotypes. Respectively, genotypes A, B and C accounted for 23% (115), 8.6% (43) and 67.5% (336) of AHB during

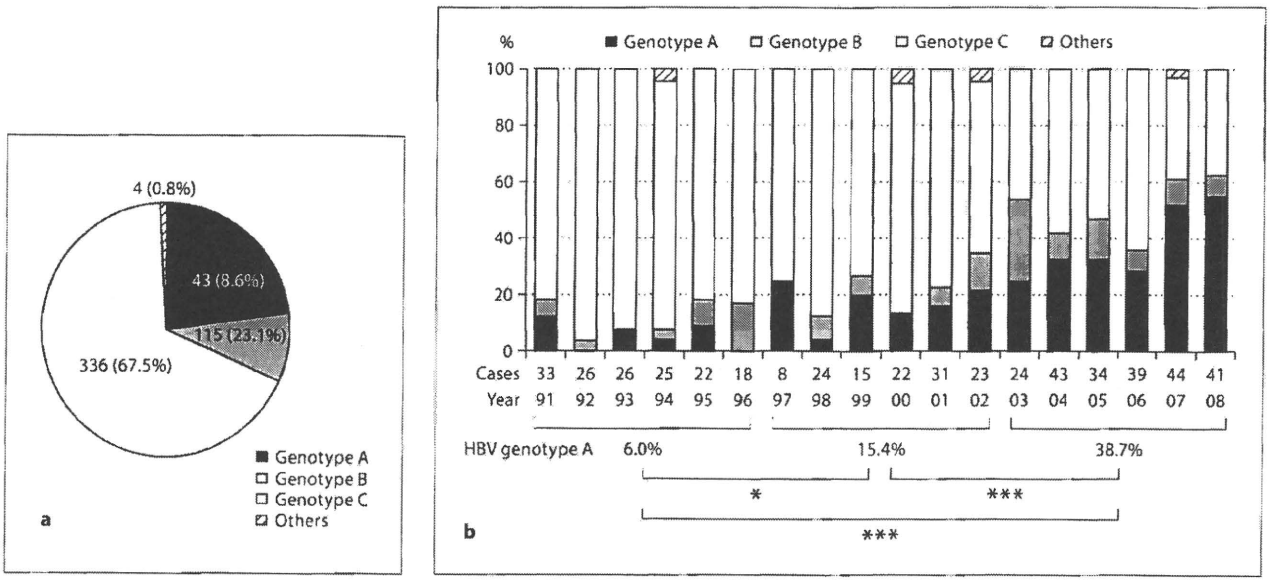

Fig. 2. a Genotype change in AVB (1991–2008, n = 498). **b** Trends of HBV genotypes (1991–2008). Note that HBV genotype A has increased in the course of the observation period. * $p < 0.05$; *** $p < 0.001$.

the period, whereas 1 case (0.2%) each of genotypes D, E, G (co-infection with genotype A) and H was found. The trend of genotypes of AHB is shown in figure 2b. HBV genotype A was confirmed in 9 of 150 cases (6.0%) in phase 1 (1991–1996), 19 of 123 (15.4%) in phase 2 (1997–2002) and 87 of 225 (38.7%) in phase 3 (2002–2008). An apparent increment of AHB by HBV genotype A proportion is observed ($p < 0.05$, 1st vs. 2nd phase; $p < 0.0001$, 1st vs. 3rd and 2nd vs. 3rd phases). In contrast, HBV genotype B was observed in 9 of 150 cases (6.0%) in phase 1, 9 of 123 (15.4%) in phase 2 and 26 of 225 (11.6%) in phase 3. There was no significant difference among the frequency of AHB by HBV genotype B in the 3 phases. Characteristics of AHB by HBV genotype A include male dominance (94.8% for genotype A, 76.7% for B and 56.0% for C; $p < 0.001$), higher total bilirubin (10.1 ± 7.9 mg/dl for genotype A, 8.1 ± 7.6 mg/dl for B and 6.9 ± 5.9 mg/dl for C; $p < 0.001$), lower frequency of severe cases (3.5% for A, 11.0% for B and 8.3% for C; $p < 0.05$), and higher rate of chronicity (3% for A and 0% for both B and C; $p < 0.05$).

Domestic or Imported Hepatitis E in Japan

In the 1,163 cases of non-ABC hepatitis, 896 (77.0%) samples were available for anti-hepatitis E (HEV) analysis. Of the 896 cases, 8 were positive for anti-HEV IgM alone, 78 were positive for anti-HEV IgG alone, and 44 cases tested positive for both anti-HEV IgM and IgG. Based upon studies on specificity tests (data not shown), the 44 cases (4.9%) which were positive for both anti-HEV IgM and IgG were given a final diagnosis of AHE. Among the 44 samples, RT-PCR for HEV was performed for 30 samples which all showed positive. Eleven cases had a history of international travel, whereas 20 did not. Travel history was not confirmed in the remaining 13 patients, who probably acquired HEV infection locally. Phylogenetic analysis revealed that all but 1 (genotype 4) domestic infections were caused by HEV genotype 3, whereas imported infections were caused by either genotype 1, 3 or 4.

Figure 3 shows the trend of non-ABC and hepatitis E that had been buried in non-ABC from 1980–2008 in this study. In Japan, which has been believed to be a non-endemic country for HEV, AHE occurred sporadically as early as the 1980s. Although the number of hepatitis E (1–6 cases per year) and the ratio to non-A, B, C and E hepatitis (0–14.5%) are not very high and insignificant, the occurrence of hepatitis E became constant after 2002. Hepatitis E constituted 11.0% (25/228) of non-ABC hepatitis after 2002. All patients with AHE recovered, and none of the cases showed prothrombin time less than 40%.

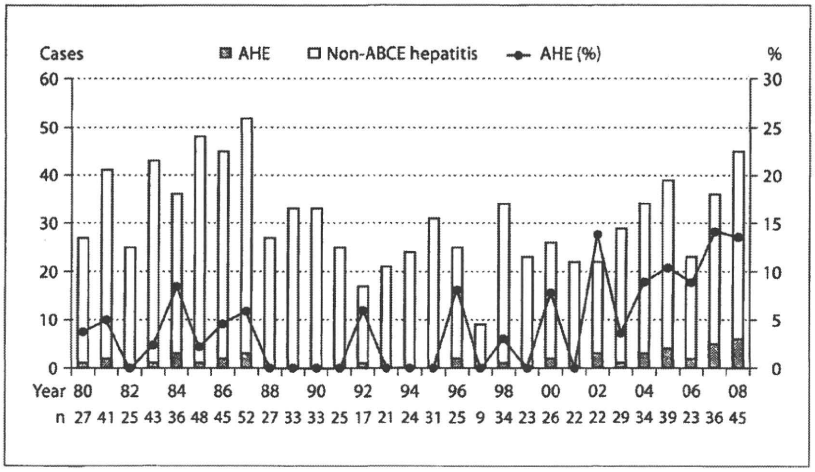

Fig. 3. Trends of non-ABC hepatitis (1980~2008, n = 895). Bars indicate numbers of non-ABC hepatitis in each year. Closed bars within, indicate absolute numbers of AHE, and the line plot indicates the ratio of AHE to non-ABC hepatitis.

Discussion

Despite being notifiable infectious diseases, very limited information has been available on the epidemiology of AVH in Japan. Under-reporting of cases has been linked to lack of awareness of the reporting system by medical practitioners and a rather complex process of notification. This study, which is a nationwide longitudinal survey carried out over a long period, provides reliable data on trends of AVH in Japan.

In this study, AHV is classified into 4 categories, namely, AHA, AHB, AHC and non-ABC hepatitis. Other etiologies of AVH, such as Epstein-Barr virus, cytomegalovirus, and herpes simplex virus-associated hepatitis, were excluded to focus on the epidemiology of 'hepatitis virus'. Accordingly, the category non-ABC hepatitis may consist of undiscovered or submerged types of hepatitis, such as AHE which had been paid little attention as a cause of domestic AVH in most industrialized countries, including Japan, until recently.

The overview of trends among all 4,302 cases in chronological fashion reveals that the cause of hepatitis in Japan has been drastically changed during the past 3 decades. This change is largely due to a major decrease of AHA, probably because of improvements in hygienic conditions in this country.

In connection with this decrease of AHA incidence, a marked decrease of anti-hepatitis A prevalence among the healthy population has been observed. More than 99% of individuals who were under 50 years old were sensitive to hepatitis A in 2006 (unpubl. observation). This may paradoxically rouse a debate over the need for vaccination in this low-endemic country. In fact, many outbreaks have been observed in low-endemic countries [3, 4], which can be linked to imported foods.

There was a remarkable increase in AHB by HBV genotype A from the 1st phase (1991–1996) to the 2nd (1997–2002) and 3rd (2003–2008) phases. This observation is consistent with previous reports [5–11]. Like other investigators, we observed an increase of HBV genotype A which had not been indigenous to Japan, probably because of a marked increase of immigration from countries where HBV genotype A is common. Taking into consideration that all AHB by HBV genotype A patients reported in our study were Japanese and most of them had neither travel histories nor contact with foreign people, it seems to be natural to speculate that original transmission had happened from foreign individuals to Japanese, and secondary spread from Japanese to other native individuals were carried out.

Clinical characteristics of AHB by genotype A is of particular importance. Our data indicate a milder, but prolonged course of AHB genotype A compared to genotype B or genotype C. Although chronicity of HBV genotype A has been a matter of concern, the reported chronicity rate varies [5, 7, 8]. If HBV genotype A is prone to cause chronic infection in immunocompetent adults, the increase of such strains in acute hepatitis may change the picture of chronic infection in this country. Indeed, Matsuura et al. [12] reported that HBV genotype A in chronic hepatitis B between 2005 and 2006 was twice as frequent (3.5 vs. 1.7%; p = 0.02) as it was in their previous cohort between 2000 and 2001 [13]. A further study, especially a prospective one, is necessary to confirm the scenario.

HEV is a major cause of acute hepatitis in many developing countries where AHE is an important public health concern. However, cases of sporadic AHE in people with no history of recent travel have been reported in developed regions such as North America, Europe, Japan and Australia [14–21]. The reporting of such infections together with the availability of more comprehensive molecular and serological data has led to the re-evaluation of HEV epidemiology and to the acceptance that autochthonous AHE is a clinical problem in developed countries [14]. Information on AHE in the non-ABC hepatitis population in Japan is limited, although there are many reports of sporadic or epidemic occurrence of AHE [21–28]. The current study also showed the trend of AHE in Japan. AHE constituted 4.9% (44/896) of non-ABC hepatitis. Although the number of AHE cases (1–6 cases per year) and its ratio to non-ABC hepatitis (0–14.5%) are not very high and insignificant, the occurrence of AHE became constant after 2002. Surprisingly, AHE constituted as high as 11,0% (25/228) of non-ABC hepatitis after 2002. The clinical course was generally modest, and none of the patients showed a severe type of hepatitis, probably because most domestic cases were caused by HEV genotype 3 which has been implicated with milder clinical outcome compared with HEV genotype 4 [29–31]. This phenomenon may reflect the fact that our sentinels involve only a few institutes in Hokkaido where HEV genotype 4 is endemic. Nevertheless, the trend of AHE requires particular attention, because mode of transmission is still often unknown, even after taking very careful history of eating particular foods such as raw meat of deer, pigs and boars [29].

Acknowledgment

This study was partially supported by a grant-in-aid from the National Hospital Organization and from the Ministry of Health, Labor and Welfare of Japan.

Disclosure Statement

All authors do not have conflicts of interest to declare.

Appendix

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今月の症例

輸血の6カ月後に発症したB型急性肝炎の1例

井上　淳[1)]　上野　義之[1)]　福島　耕治[1)]　近藤　泰輝[1)]
嘉数　英二[1)]　小原　範之[1)]　木村　修[1)]　涌井　祐太[1)]
下瀬川　徹[1)]　内田　茂治[2)]

要旨　症例は31歳，女性．6年前より特発性血小板減少症にてプレドニゾロン（PSL）を内服中．出産に伴い血小板輸血を行い，PSLを60 mgに増量．その後PSL漸減中にB型急性肝炎を発症した．この時には輸血から6カ月が経過していたが，調査を行ったところ，献血者のうちの一人がその後に再度献血し，HBV DNAが陽転化していたことが判明した．HBVの塩基配列の解析により感染の原因となっていたことが確認された．

〔日内会誌　99：1910～1912，2010〕

Key words：B型急性肝炎，輸血後肝炎，プレドニゾロン

症　例

患者：31歳，女性．**主訴**：肝機能障害．**既往歴**：2002年より特発性血小板減少性紫斑病（ITP）にてプレドニゾロン（PSL）内服．2004年，2006年，2008年に出産し，いずれも血小板輸血を受けた（それぞれ120単位，225単位，120単位）．**家族歴**：特記事項なし．**現病歴**：2008年4月の出産時に当院血液免疫科にてITPに対するPSLを60 mgに増量，その後漸減していたが，2008年10月になりAST 95 IU/*l*，ALT 296 IU/*l*の肝機能障害を認め，6月は陰性であったHBs抗原が11月の検査で陽性であることが判明し，12月に当科紹介となった．**初診時現症**：身長153 cm，体重53 kg，眼瞼結膜に貧血なし，眼球結膜に黄疸なし，表在リンパ節触知せず，心音・呼吸音異常なし，腹部異常所見なし，下腿浮腫なし．**初診時検査所見**：WBC 9,600/μl，RBC 462×10⁴/μl，Hb 13.9 g/dl，PLT 7.8×10⁴/μl，T-Bil 0.7 mg/dl，D-Bil 0.1 mg/dl，AST 77 IU/*l*，ALT 223 IU/*l*，ALP 404 IU/*l*，γ-GTP 101 IU/*l*，LDH 206 IU/*l*，BUN 13 mg/dl，Cr 0.5 mg/dl，TP 6.6 g/dl，Alb 3.6 g/dl，Na 143 mEq/*l*，K 3.7 mEq/*l*，Cl 108 mEq/*l*，CRP 0.2 mg/dl，PT 100.5%，HBs抗原≧250 IU/ml，HBe抗原 1,184.1 index，HBe抗体 陰性，HBc抗体 15.79 index，IgM-HBc抗体 1.8 index，HBV DNA 7.4 log copies/ml．腹部超音波検査では特に異常所見なし．

臨床経過

これまでの経過とIgM-HBc抗体が陽性であることからB型急性肝炎と診断．PSL内服中（15 mgと12.5 mgを隔日投与）であり，HBV DNA量も多く，B型肝炎の重症化・慢性化が懸念された．授乳中であったため，断乳が可能となった2009年3月よりエンテカビル0.5 mgの内服を開

〔第189回東北地方会（2009/09/12）推薦〕〔受稿2010/03/02，採用2010/03/03〕

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A case of acute hepatitis B who had an onset 6 months after blood transfusion.

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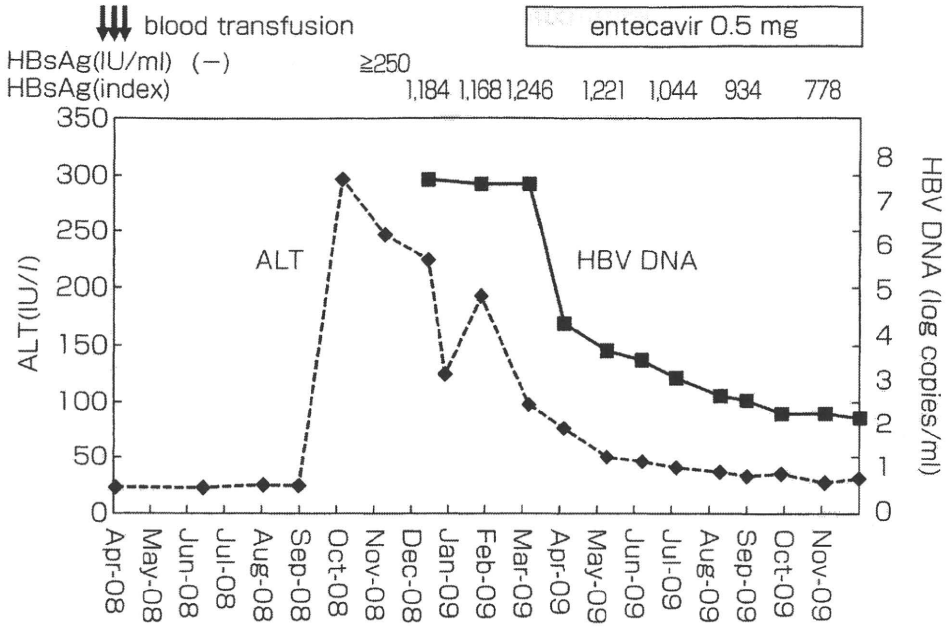

図 1. 臨床経過

始した. その後はHBV DNA, ALTともに低下し経過は良好である（図 1）.

HBVの感染源についてであるが, 本患者に輸血した 12 本の血小板の献血者のうちの一人が当該献血から 45 日後に再度献血し, この時にHBV DNAが陽転化していたことが判明した. 宮城県赤十字血液センターから, 遡及調査の結果,「当該血液は個別の核酸増幅検査（NAT）は陰性であるが, window periodに採血されており, 検出限界以下のウイルスが存在する可能性がある」との連絡が当院に入った. この時点（輸血の 2 カ月後）での患者のHBs抗原は陰性であったが, 輸血から 6 カ月後の肝炎発症時にはHBs抗原が陽転化していたため血液センターに調査を依頼したところ, ①輸血に使用した 12 本の血液はいずれも血液は個別NAT陰性であること, ②患者および陽転時の献血者のHBVの塩基配列に高い相同性があること, などが明らかとなった.

HBVの遺伝子解析はPre-S/S遺伝子を含むP遺伝子前半部 1,550 塩基（塩基 2,333-3,215/1-667）をダイレクトシークエンス法にて解析した. その結果, 1 塩基のみに相違があり, それ以外に献血者のHBVにおいて 30 カ所に 2 種類の塩基の混在が認められたが, 患者のHBVはいずれもその一方の塩基と一致していた（一部を図 2 に示す）. このことから, 献血者には 2 株のHBVが共存しており, そのうちの 1 株が本患者に感染したものと考えられた. いずれのHBV株もgenotypeは日本に最も多いCであった.

考　察

近年, 急性B型肝炎の状況に変化が見られている. 献血された血液に対する核酸増幅検査（NAT）の進歩によりwindow periodが短縮され, 輸血による感染例は激減している[1]. 一方, sexual transmitted disease（STD）としての性格が鮮明となっており, 欧米型のgenotype AのHBVの感染が都市部を中心に増加している[2,3]. さらに, 共通した感染経路をもつHIV（human immunodeficiency virus）の重複感染例の増加も危惧されている[3]. しかし, 本症例は輸血以外の感染経路が考えにくかったため, 肝炎発症の 6 カ月前の輸血が原因であることを疑い, 詳細な調査にてその関連を証明することができた.

一般にHBVの感染から発症までの潜伏期間は 1〜6 カ月とされており[4], 本症例の潜伏期間は比較的長いものであった. 長い潜伏期間を経て

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Patient 272 AATTTTCTAGGGGGAGCACCCACGTGTCCTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCTTGTCC
Donor   272 ...........................................................................................

Patient 352 TCCAATTTGTCCTGGCTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCT
Donor   352 ...........................................................................................

Patient 432 TCTTGTTGGTTCTTCTGGACTACCAAGGTATGTTGCCCGTTTGTCCTCTACTTCCAGGAACATCAACTACCAGCACGGGA
Donor   432 ....R......................................................................................

Patient 512 CCATGCAAGACCTGCACGATTCCTGCTCAAGGAACCTCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGACGGAAA
Donor   512 ........R...................................................................................

Patient 592 CTGCACTTGTATTCCCATCCCATCATCCTGGGCTTTCGCAAGATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTCC
Donor   592 ..........................................R................................................
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(R=A or G)

図2. HBVのS遺伝子内の部分塩基配列（396塩基）の比較．上列が本症例，下列が献血者．

感染が成立した原因としては，輸血された血液中のHBVが個別NATでも検出できないほど微量であったが，PSL内服中のためにHBVが徐々に増殖したことが推測された．PSLによるHBV増殖の機序としては，宿主の免疫が抑制されることに加え，HBVに存在するglucocorticoid-responsive elementの作用が考えられている[5]．

最近，HBV既往感染者に免疫抑制薬や抗腫瘍薬を投与した場合にB型肝炎が再活性化する*de novo* B型肝炎と呼ばれる病態があり，死亡率が高いことが注目されている[6]．この病態は，治療前にHBs抗体あるいはHBc抗体が陽性の既往感染状態であることを確認できていないと通常のB型急性肝炎との鑑別が難しい場合がある．免疫抑制薬や抗腫瘍薬などの治療を行っている患者は輸血を受けている場合も多く，治療中にB型肝炎が生じた場合には現在では頻度は低いと思われるが輸血からの感染の可能性も考慮しなければいけないことが本症例から示唆された．今回のケースは個別NATでも陰性のwindow periodの血液によるHBV感染であり，献血者がHBV DNA陽性の時期に再度献血したこと，献血者の陽転時のHBVの遺伝子解析により感染源を特定できたこと，など貴重な症例と考えられ報告した．

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Transfusion Medicine

Official Journal of the British Blood Transfusion Society

Transfusion Medicine, 2011, **21**, 107–115 doi: 10.1111/j.1365-3148.2010.01057.x

ORIGINAL ARTICLE

Infectivity of HBV DNA positive donations identified in look-back studies in Hyogo-Prefecture, Japan

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Received 30 July 2010; accepted for publication 3 November 2010

SUMMARY

Aims/Objectives: To clarify transfusion incidence of hepatitis B virus (HBV) infected blood negative for mini pool-nucleic acid amplification testing (MP-NAT).

Background: Japanese Red Cross (JRC) blood centres screen donated blood to avoid contamination with HBV. However, a low copy number of HBV may be overlooked.

Methods/Materials: In Hyogo-Prefecture, JRC blood centres screened 787 695 donations for HBV from April 2005 to March 2009. Of these, 685 844 were donations from the repeat donors. To detect the donors with HBV, serological tests, MP-NAT and/or individual donation (ID)-NAT were performed. To detect the recipients with transfusion-transmitted HBV infection (TTHBI), serological analysis and/or ID-NAT were performed.

Results: In this study, 265 of the 685 844 repeat donations were serologically and/or MP-NAT positive for HBV. Their repository samples from the previous donation were examined in a look-back study: 13 of the 265 repository samples proved ID-NAT positive. Twelve recipients were transfused with HBV-infected blood components derived from 10 of the 13 HBV-infected donors. Only 1 of the 12 recipients was identified as TTHBI case. Seven of the 12 recipients escaped from our follow-up study and 4 recipients were negative for HBV during the observation period.

Conclusion: On the basis of the look-back study among the repeat donors in Hyogo-Prefecture, Japan, donations with HBV-infected blood negative for MP-NAT occurred with a frequency of 13 in 685 844 donations (~1/53 000 donations). However, more than half of the recipients transfused with HBV-infected blood negative for MP-NAT could not be followed up. It is necessary to establish a more cautious follow-up system.

Key words: HBV, ID-NAT, look-back study, MP-NAT, TTHBI.

For the purpose of the prevention of transfusion-transmitted hepatitis B virus infection (TTHBI), Japanese Red Cross (JRC) blood centres have been screening blood with serological tests for hepatitis B surface antigen (HBsAg) since 1972 and for hepatitis B core antibody (HBcAb) since 1989 (Japanese Red Cross NAT Screening Research Group, 2000). Currently, nucleic acid amplification testing (NAT) methods are widely used in Japan as useful diagnostic tools for virus detection.

In 1999, mini pool (MP)-NAT for hepatitis B virus (HBV) was implemented: MP-NAT with 50-member mini pool samples (50-MP-NAT) had been included in the JRC donor screening system since 2000, but that was replaced by 20-MP-NAT in 2004 (Yugi *et al.*, 2006). The screening system including 20-MP-NAT or individual donation-NAT (ID-NAT) has been the standard method in Japan since 2004. Table 1 shows the HBV tests for donor selection that are now conducted in JRC.

However, it may be impossible to exterminate HBV infections, despite the introduction of sensitive NAT. The current NAT system cannot completely eliminate the risk

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Table 1. HBV tests for donor selection

Period	Items	Test	Methods	Kit (Company)	Normal range
~July 2008	HBsAg	Serological test for screening	Reverse passive haemagglutination	JRC HBsAg (JRC)	$<2^1$
	HBsAg	Serological test for confirmation	Enzyme immunoassay	AxSYM® HBsAg Dynapack (Abbott)	<2·0 S/N
	HBcAb	Serological test for screening	Haemagglutination inhibition	JRC HBcAb (JRC)	$<2^5$
	HBcAb	Serological test for confirmation	Enzyme immunoassay	AxSYM® HBcAb Dynapack (Abbott)	<50·0%INH
	HBsAb	Serological test for screening	Passive haemagglutination	JRC HBsAb (JRC)	$<2^4$
	HBsAb	Serological test for confirmation	Enzyme immunoassay	AxSYM® HBsAb Dynapack (Abbott)	<200 mIU/mL
	HBV DNA	Mini pool NAT for screening	TaqMan PCR	AMPLINAT MPX (Roche Diagnostics)	<100 copies/mL[1]
	HBV DNA	Individual NAT for confirmation	TaqMan PCR	Single NAT HBV (Roche Diagnostics)	<100 copies/mL[1]
August 2008~	HBsAg	Serological test for screening and confirmation	Chemiluminescence enzyme immunoassay	Lumipulse® HBsAg (Fujirebio)	<1·0 C.O.I.
	HBcAb	Serological test for screening and confirmation	Chemiluminescence enzyme immunoassay	Lumipulse® HBcAb (Fujirebio)	<12·0 C.O.I.
	HBsAb	Serological test for screening and confirmation	Chemiluminescence enzyme immunoassay	Lumipulse® HBsAb (Fujirebio)	<200 mIU/mL
	HBV DNA	Mini pool NAT for screening	TaqMan PCR	Cobas TaqScreen MPX (Roche Diagnostics)	<3·2 IU/mL[1]
	HBV DNA	Individual NAT for confirmation	TaqMan PCR	Cobas TaqScreen HBV (Roche Diagnostics)	<2·4 IU/mL[1]

[1]HBV DNA detection limit.
C.O.I, cut off index; S/N, sample rate/index calibrator mean rate.

of TTHBI, because the limited sensitivity of NAT may overlook blood contaminated with extremely low levels of HBV donated by individuals with an early stage HBV infection (window period) or with HBV carrier status (Tadokoro, 2007).

When a new donation of a repeat donor turned out to be positive for HBsAg, HBcAb or NAT for HBV, repository samples of the preceding donation are subjected to a look-back study. In the look-back study, repository tubes from previous donations are re-investigated by ID-NAT. JRC has stored repository tubes of every blood donation for 11 years since 1996 (Satake *et al.*, 2007). A look-back system of JRC may detect donors with acute HBV infection in the window period and occult carrier donors with low HBcAb titers (Satake & Tadokoro, 2008).

To investigate transfusion incidents with HBV-infected blood negative for MP-NAT in the current JRC screening system, we calculated their incidence based on the cumulative data of the look-back study during the period between 1 April 2005 and 31 March 2009. In addition, to investigate the outcome of the recipients who were transfused with HBV-infected blood, we searched for the transfusion incident victims.

MATERIALS AND METHODS

Subjects studied

In Japan, a total of 20 314 745 donated blood samples were screened for HBV infection during the period 1 April 2005 to 31 March 2009. Among 17 875 065 donations from the repeat donors, 10 380 were serologically positive or MP-NAT positive for HBV.

In Hyogo-Prefecture, a total of 787 695 donated blood samples were screened for HBV infection during the

period between 1 April 2005 and 31 March 2009. Among 685 844 donations from the repeat donors, 265 were serologically positive or MP-NAT positive for HBV.

In addition to the donors identified in the look-back study of the repeat donations, a donor with HBV-infected blood negative for MP-NAT, D1, in Hyogo-Prefecture was identified in the look-back study based on a hospital report.

Serological tests of donation samples for HBV

For the detection of HBV-infected blood, a battery of HBsAg, HBcAb, HBsAb and 20-MP-NAT (or ID-NAT) was used to judge the presence or absence of HBV in the donated blood samples. Donated blood samples had been screened for HBsAg by reverse passive haemagglutination (RPHA), for HBcAb by haemagglutination inhibition (HI) and for antibody to HBsAg (HBsAb) by passive haemagglutination (PHA) in JRC blood centres. Since August 2008, to improve the sensitivity of screening for HBsAg, HBcAb and HBsAb, all RPHA, HI and PHA methods had been replaced by chemiluminescence enzyme immunoassay (CLEIA) method (Table 1). Donated blood samples which were positive for HBV tests or which showed elevated alanine aminotransferase (ALT) (>60 IU/L) were excluded (Yoshikawa *et al.*, 2005; Yugi *et al.*, 2005).

MP-NAT and ID-NAT of HBV DNA

The outlines of MP-NAT and ID-NAT in the JRC screening system were reported by Mine *et al.* (2003) and Minegishi *et al.* (2003), respectively. In this study, MP-NAT for screening of HBV DNA was performed with AMPLINAT MPX (Roche Diagnostics, Mannheim, Germany) or Cobas TaqScreen MPX (Roche Diagnostics) according to the manufacturer's instruction. ID-NAT for confirmation was performed with Single NAT HBV (Roche Diagnostics) or Cobas TaqScreen HBV (Roche Diagnostics) according to the manufacturer's instruction. The detection limits using these reagents are shown in Table 1. The genotypes of HBV were determined by the sequencing analysis of Okamoto *et al.* (1990).

Detection of recipients with TTHBI

For the detection of recipients with TTHBI, serological analysis, ID-NAT for HBV and biochemical analysis including ALT were performed.

RESULTS

Look-back studies of the repeat donations

In Japan, a total of 20 314 745 donated blood samples were screened for HBV infection during the period 1 April 2005 to 31 March 2009. Of the total 20 314 745 donations, 17 875 065 were from the repeat donors. Of the 17 875 065 repeat donors, 10 380 were serologically positive or MP-NAT positive for HBV and subjected to look-back study (Ministry of Health, Labour and Welfare of Japan, 2009). When a new donation of a repeat donor turned out to be positive for serological tests and/or MP-NAT, repository samples of that donor's preceding donation were subjected to look-back study using ID-NAT. The look-back study of 10 380 repeat donors revealed that the previously donated blood samples of 247 repeat donors were ID-NAT positive (Table 2). On the basis of the nationwide look-back study in Japan, donations with HBV-infected blood negative for MP-NAT occurred with frequency of 247 in 17 875 065 donations (~1/72 000 donations).

Table 2. Donors[1] in Japan-wide and Hyogo-Prefecture surveys during the period 1 April 2005 to 31 March 2009

	Japan-wide survey[2]	Hyogo-Prefecture survey
Total donors	20 314 745	787 695
First donors	2 439 680	101 851
Repeat donors[3]	17 875 065	685 844
Donors who were subjected to the look-back study	10 380	265
Donors who were ID-NAT positive at the preceding donation	247	13

[1]The donors in this study were the people who came to the JRC blood centres to donate their blood.
[2]Ministry of Health, Labour and Welfare of Japan (2009).
[3]Double counting took place if the same people came to the JRC blood centres to donate their blood more than one time. We call these people "repeat donors".

In Hyogo-Prefecture, a total of 787 695 donated blood samples were screened for HBV infection during the period between 1 April 2005 and 31 March 2009. Of the 787 695 donations, 685 844 were from the repeat donors and 265 were serologically positive or MP-NAT positive for HBV and subjected to the look-back study. The look-back study of 265 repeat donors revealed that the previously donated blood samples of 13 repeat donors were ID-NAT positive (Table 2). On the basis of the look-back study in Hyogo-Prefecture, donations with HBV-infected blood negative for MP-NAT occurred with a frequency of 13 in 685 844 donations (~1/53 000 donations).

Transfusion incidents in Hyogo-Prefecture

Table 3 shows the numbers of recipients transfused with HBV-infected blood negative for MP-NAT (or victims of

Table 3. Recipients[1] transfused with HBV-infected blood negative for MP-NAT in Japan-wide and Hyogo-Prefecture surveys during the period 1 April 2005 to 31 March 2009

	Japan-wide survey[2]	Hyogo-Prefecture survey
Recipients transfused with HBV-infected blood negative for MP-NAT	204	12
Recipients who converted to HBV positive	13	1
Recipients who did not convert to HBV positive	58	4
Recipients who escaped from the follow-up study	133	7

[1]The recipients in this study were the people who were transfused with JRC blood products.
[2]Ministry of Health, Labour and Welfare of Japan (2009).

the transfusion incidents) in the Japan-wide and Hyogo-Prefecture surveys during the study period. According to the nationwide survey in Japan, 13 of the 204 recipients (6·3%) who had been transfused with HBV-infected blood negative for 20-MP-NAT were proved to be TTHBI. According to Hyogo-Prefecture survey, only 1 of the 12 recipients (8·3%) who had been transfused with HBV-infected blood negative for 20-MP-NAT was proved to be TTHBI.

HBV markers of the 'look-back study' donors in Hyogo-Prefecture

Table 4 shows the HBV markers at the final donation of 265 repeat donors in Hyogo-Prefecture who were subjected to look-back study. A total of 204 of the 265 'look-back study' donors presented HBsAg negative, NAT negative and HBcAb positive.

Analysis of individual cases of transfusion incidents in Hyogo-Prefecture

Table 5 shows the information of the 14 donors with HBV-infected blood negative for MP-NAT in Hyogo-Prefecture. All except D1 proved to be ID-NAT positive in the repository sample of the donation prior to the final donation. D1 was accidentally found to be ID-NAT positive in the look-back study started from the hospital report of a patient with TTHBI, R1. The case of D1 was not identified in the look-back study in the repeat donations, because D1 did not donate any more after the index donation. Thus, there was a possibility of higher incidence of TTHBI due to the transfusion with HBV-infected blood negative for MP-NAT than ~1/53 000 donations.

Table 4. HBV markers at the final donation of the 'look-back study' donors

Type	HBV markers	Donor numbers
A	[HBsAg (+)]	48
B	[HBsAg (−), NAT (+)]	13
C	[HBsAg (−), NAT (−), HBcAb (+)]	204
	Total	265

Table 6 shows the clinical data of the 13 recipients transfused with HBV-infected blood negative for MP-NAT in Hyogo-Prefecture. We followed 13 recipients in Hyogo-Prefecture who were transfused with HBV-infected blood negative for MP-NAT. Only one recipient was identified to be suffering from TTHBI. Four of the 12 recipients were negative for HBV during the follow-up period. However, 7 of the 12 recipients did not undergo the post-transfusion HBV examination, because they died soon after the transfusion or did not return for a follow-up.

Case 1: Look-back study based on the hospital report

D1 donated a red blood cell (RBC) component causing TTHBI in a 63-year-old male with myelodysplastic syndrome (MDS), R1. R1 was transfused 28 times and got infected with HBV during chemotherapy. The index RBC component from D1 was ID-NAT positive with low HBV DNA levels, but was not detected by the regular 50-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D1 was in the window period of HBV infection at the index donation.

R1 was negative for HBsAg on 18 February 2002, according to the hospital records. However, he was positive for HBV DNA on 13 November 2002, 175 days after the date of the HBV-positive RBC component transfusion (22 May 2002). The recipient showed to be positive for HBsAg 254 days after the date of the transfusion and positive for HBcAb 315 days after the date of the transfusion (Table 6). R1 showed a transient elevation of ALT just after the negative-to-positive conversion of HBsAg and HBcAb, although HBsAb was still under detection level during his disease course (Fig. 1). R1 died of intracerebral haemorrhage, gastrointestinal haemorrhage and sepsis on 4 January 2004.

To confirm that HBV was transmitted through the blood from D1, HBV strains of the donor and the recipient were determined by the PCR/direct-sequencing method. The sequences in both samples showed genotype

Table 5. Donors involved in HBV-infected blood transfusion incidents

Case	Donor	Recipient	Donation number	Donation date	Index and final donations	HBV markers			
						MP-NAT	ID-NAT	HBsAg	HBcAb
1	D1[1]	R1	3rd	15 Apr 1997		N.D.	N.D.	—	—
			4th	08 May 2002	Index donation	—	+[2]	—	—
2	D2	R2	7th	02 Jun 2003		—	—	—	—
			8th	11 Oct 2004	Index donation	—	+[2]	—	—
			9th	04 May 2005	Final donation	N.D.	—	—	+
3,4	D3	R3, R4	5th	11 Jun 2007		—	—	—	—
			6th	09 Oct 2007	Index donation	—	+[2]	—	—
			7th	20 Jul 2008	Final donation	N.D.	—	—	+
5	D4	R5	7th	18 Jan 2005		—	—	—	—
			8th	16 Mar 2005	Index donation	—	+[2]	—	—
			9th	19 Dec 2008	Final donation	N.D.	—	—	+
6	D5	R6	11th	15 Jun 2008		—	—	—	—
			12th	14 Sep 2008	Index donation	—	+[2]	—	—
			13th	15 Feb 2009	Final donation	+	+	—	—
7	D6	R7	24th	27 Apr 2005		—	—	—	—
			25th	25 Jan 2006	Index donation	—	+[2]	—	—
			26th	04 Mar 2006	Final donation	N.D.	N.D	+	—
8,9	D7	R8, R9	1st	04 Oct 1995		N.D.	N.D.	—	—
			2nd	08 Dec 2004	Index donation	—	+[2]	—	—
			3rd	07 Mar 2006	Final donation	N.D.	N.D	+	+
10	D8	R10	33rd	21 Jun 2006		—	—	—	—
			34th	06 Jul 2006	Index donation	—	+[2]	—	—
			35th	20 Jul 2006	Final donation	+	+	—	—
11	D9	R11	9th	06 Sep 2005		—	—	—	—
			10th	06 Sep 2006	Index donation	—	+[2]	—	—
			11th	27 Feb 2007	Final donation	N.D.	—	—	+
12	D10	R12	5th	04 Dec 2006		—	—	—	—
			6th	01 Feb 2007	Index donation	—	+[2]	—	—
			7th	02 Apr 2007	Final donation	N.D.	N.D	+	+
13	D11	R13	3rd	03 May 2007		—	—	—	—
			4th	04 May 2008	Index donation	—	+[2]	—	+
			5th	24 Feb 2009	Final donation	N.D.	N.D	+	+
	D12[3]	—	18th	14 Jan 2003		—	—	—	—
			19th	05 Oct 2004	Index donation	—	+[2]	—	—
			20th	31 Mar 2005	Final donation	N.D.	—	—	+
	D13[3]	—	8th	16 Mar 2004		—	—	—	—
			9th	25 Mar 2005	Index donation	—	+[2]	—	—
			10th	12 Jan 2006	Final donation	N.D.	—	—	+
	D14[3]	—	19th	10 Jun 2007		—	—	—	—
			20th	21 Oct 2007	Index donation	—	+[2]	—	—
			21st	24 Jul 2008	Final donation	N.D.	—	—	+

N.D., not done.

[1]D1 was identified as HBV carrier based on the hospital reports. No laboratory data of the fifth donation were available, because the fifth donation was not done.

[2]HBV DNA analysis using repository samples.

[3]The components from these donations were not transfused.

C and they were identical except for one nucleotide at nt 533, which was C for the donor and A for the recipient. Thus, R1 was infected by HBV in the RBC component offered by D1.

Case 2: Look-back study of the repeat donations

D2 donated blood nine times. The blood at the final donation (the ninth donation on 4 May 2005) was HBcAb positive, although it was ID-NAT negative. The

Table 6. Recipients involved in HBV-infected blood transfusion incidents

Case	Recipient (age, sex)[1]	Donor	Component	Transfusion date	Examination date (days after transfusion)	Examination result	Diagnosis of TTHBI	Present state
1	R1 (63y, M)[2]	D1	RBC	22 May 2002	13 Nov 2002 (175)	Positive for HBV DNA	Definite	Dead
					31 Jan 2003 (254)	Positive for HBsAg		
					02 Apr 2003 (315)	Positive for HBcAb		
					~29 Oct 2003 (525)	Negative for HBsAb		
2	R2 (63y, M)	D2	RBC	15 Oct 2004	20 May 2005 (217)	Positive for HBsAg, HBcAb and HBV DNA Negative for HBsAb	Definite	Alive
3	R3 (51y, M)	D3	FFP	03 Jun 2008	08 Oct 2008 (127)	Negative for HBsAg, HBcAb, HBsAb and HBV DNA	Uncertain	Alive
4	R4 (87y, F)	D3	RBC	16 Oct 2007	13 Aug 2008 (302)	Negative for HBV DNA	Uncertain	Alive
5	R5 (58y, F)	D4	RBC	22 Mar 2005	20 Jan 2006 (304)	Negative for HBsAg	Uncertain	Dead
					31 Oct 2007 (953)	Negative for HBsAg		
					09 Mar 2008 (1083)	Negative for HBsAg		
6	R6 (68y, M)	D5	RBC	29 Sep 2008	07 Jan 2009 (100)	Negative for HBsAg and HBsAb	Uncertain	Alive
7	R7 (61y, M)	D6	PC	27 Jan 2006	N.D.	N.D.	Uncertain	Dead
8	R8 (67y, F)	D7	FFP	09 Aug 2005	N.D.	N.D.	Uncertain	Dead
9	R9 (82y, F)	D7	RBC	12 Dec 2004	N.D.	N.D.	Uncertain	No available information
10	R10 (75y, M)	D8	PC	08 Jul 2006	N.D.	N.D.	Uncertain	Dead
11	R11 (65y, M)	D9	RBC	14 Sep 2006	N.D.	N.D.	Uncertain	Dead
12	R12 (65y, F)	D10	PC	03 Feb 2007	N.D.	N.D.	Uncertain	Dead
13	R13 (75y, M)	D11	RBC	12 May 2008	N.D.	N.D.	Uncertain	Dead

RBC, red blood cells; FFP, fresh frozen plasma; PC, platelet concentrate; N.D., not done.
[1]Age; age at transfusion of HBV-infected blood.
[2]The index case was found in the hospital reports. Other cases were identified in the look-back study of the repeat donors.

look-back study proved that the repository tube of the index donation (the eighth donation on 11 October 2004) was ID-NAT positive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D2 was in the window period of HBV infection at the index donation.

R2 was a 63-year-old male with severe anaemia who had been transfused with the index RBC component derived from D2 on 15 October 2004. According to the hospital record, his HBsAg was negative on 13 October 2004 and his HBV DNA was positive 217 days after the date of transfusion (Table 6).

To confirm that HBV was transmitted through the blood from D2, HBV strains of the donor and the recipient were determined by the PCR/direct-sequencing method. The sequences in both samples showed genotype C and they were identical except for one nucleotide at nt 519, which was R for the donor and A for the recipient. Thus, R2 was infected by HBV in the RBC component offered by D2.

Case 3: Look-back study of the repeat donations

D3 donated blood seven times. The blood at the final donation (the seventh donation on 20 July 2008) was HBcAb positive, although it showed ID-NAT negative. The look-back study proved that the repository tube of the index donation (the sixth donation on 9 October 2007) was ID-NAT positive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D3 was in the window period of HBV infection at the index donation.

R3 was a 51-year-old male with infectious endocarditis and he had been transfused with the index fresh frozen plasma (FFP) component derived from D3 on 3 June 2008. According to the record in the hospital, his

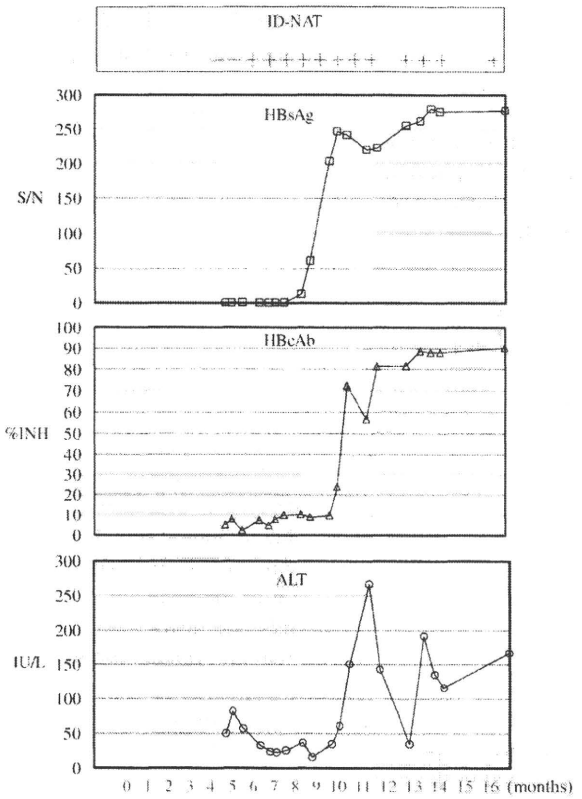

Fig. 1. HBV markers and ALT of R1. R1 was negative for HBsAb during the observation period.

HBsAg, HBsAb, HBcAb and HBV DNA were negative after 127 days from the date of transfusion (Table 6).

Case 4: Look-back study of the repeat donations

R4 was an 87-year-old female with bone fracture and she had been transfused with the index RBC component donated from D3 on 16 October 2007. According to the record in the hospital, her HBV DNA was negative after 302 days from the date of transfusion (Table 6).

Case 5: Look-back study of the repeat donations

D4 donated blood nine times. The blood at the final donation (the ninth donation on 19 December 2008) was HBcAb positive, although it showed ID-NAT negative. The look-back study proved that the repository tube of the index donation (the eighth donation on 16 March 2005) was ID-NAT positive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D4 was in the window period of HBV infection at the index donation.

R5 was a 58-year-old female with aplastic anaemia and she had been transfused with the index RBC component derived from D4 on 22 March 2005. According to the record in the hospital, her HBsAg was negative after 4 months from the date of transfusion, after 304, 953 and 1083 days (Table 6).

Case 6: Look-back study of the repeat donations

D5 donated blood 13 times. The blood at the final donation (the 13 donation on 15 February 2009) was 20-MP-NAT positive and it also showed ID-NAT positive. The look-back study proved that the repository tube of the index donation (the 12th donation on 14 September 2008) was ID-NATpositive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D5 was in the window period of HBV infection at the index donation.

R6 was a 68-year-old male with chronic renal failure and he had been transfused with the index RBC component derived from D5 on 29 September 2008. According to the record in the hospital, his HBsAg and HBsAb were negative after 100 days from the date of transfusion (Table 6).

Cases 7–13: Look-back study of the repeat donations

The index donations of D6 in Case 7, D7 in Cases 8 and 9, D8 in Case 10, D9 in Case 11 and D10 in Case 12 were ID-NAT positive, but their HBV infection was not detected by the regular 20-MP-NAT. HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that they were in the window period of HBV infection at the index donation.

The examination data of the recipients, R7 in Case 7, R8 in Case 8, R9 in Case 9, R10 in Case 10, R11 in Case 11, R12 in Case 12 and R13 in Case 13, were not available in this study.

The index blood components derived from D12, D13 and D14 were not transfused.

DISCUSSION

New HBV infection in the repeat donors

We identified the repeat donors who 'seroconverted' to either HBV DNA in MP-NAT, HBsAg or HBcAb in the look-back study during the period 1 April 2005 to 31 March 2009. If these were all true seroconversions, the incidence of new HBV infection among the repeat donors in Japan and Hyogo-Prefecture would be 1 : 1700 and 1 : 2600 in 4 years, i.e. 1 : 425 and 1 : 650 in a year. The value may be helpful to estimate the incidence

of new HBV infection in Japan and Hyogo-Prefecture, although it may not be the true incidence of new HBV infection.

Frequency of transfusion incidents with HBV-infected blood negative for MP-NAT

We calculated the transfusion incident frequency of HBV-infected blood negative for MP-NAT as 1/53 000–72 000 donations in the current JRC system, based on the look-back study data of the repeat donations. However, it should be noted that the frequency is only a minimum estimate, because donations from first time donors were not be evaluated in the look-back study among the repeat donors.

In addition, even ID-NAT does not always detect a donor with a low copy number of HBV. Some donors with intermittent ID-NAT detectable viremia may not be detected in any HBV screening system. Inaba *et al.* (2006) reported a case of an occult HBV carrier who did not constantly show ID-NAT positive results. The case of Inaba *et al.* suggested that it is necessary to repeat ID-NAT even after the repository tube is once found to be ID-NAT negative.

Incidence of TTHBI due to transfusion with HBV-infected blood negative for MP-NAT

The exact TTHBI incidence in the current JRC system could not be estimated. Two factors hampered estimation of the exact TTHBI incidence: (i) more than half of the recipients who were transfused with HBV-infected blood escaped from the follow-up study and (ii) recipients may become HBV positive after the observation period is over.

In Japan, only 'ID-NAT at 3 months after transfusion' is recommended by the Guideline of Ministry of Health, Labour and Welfare of Japan, because it is thought that recipients will turn out ID-NAT positive within 3 months after HBV-infected blood is transfused (Schreiber *et al.*, 1996; Comanor & Holland, 2006). However, in a recipient, R1, in our study, the period length of the ID-NAT negative window period was more than 161 days. Only 'ID-NAT investigation at 3 months after transfusion' may not be enough to prove the absence of HBV transmission in some recipients.

Our ideas are supported by the report of Satake *et al.* (2007). They reported the difficulty of following the recipients who had been transfused with HBV-infected blood. In those days they screened for HBV with 50-MP-NAT. According to the nationwide data during the period between 2000 and 2004, 12 of the 181 recipients who had been transfused with HBV-infected blood (6·6%) were cases of TTHBI (Satake *et al.*, 2007). Fifty-one of the 181 recipients showed no evidence of HBV infection after index transfusion during their observation period. However, there is no information on whether these 51 recipients stayed in HBV-free status after the observation period. In addition, a total of 104 patients died without leaving any test results regarding HBV infection. This result also suggests that it is difficult to know the exact TTHBI incidence.

Prolonged window period due to long incubation before ID-NAT positive

In this study, we reported a recipient with MDS, R1, who showed no liver dysfunction, no elevation of serologic markers of HBV infection and no detection of HBV DNA until 175 days after transfusion of ID-NAT positive blood. The delayed appearance of HBV may be explained by two factors: (i) a very low copy number of HBV in the transfused blood and (ii) the immunosuppressive condition of the recipient.

Regarding the longer window period in the immune suppressed patients, it is difficult to explain. Immunosuppressive effect may shorten the window period and facilitate viral replication. However, Wendel *et al.* (2008) suggested a possibility that some free virus may have persisted in the liver, escaping the immune system until the level of immunodeficiency was such that viral replication could take place. They also reported two cases of immunodeficient recipients who were transfused with blood components from a single unit containing a very low level of HBV. One of these recipients had acute lymphoblastic leukaemia (ALL) and developed an acute HBV infection 13 months after transfusion despite carrying vaccine-induced HBsAb. The other recipient, who had MDS and received platelets from the same donation while receiving major chemotherapy, remained uninfected.

Unusual circumstances, such as chemotherapy or immunosuppression, can considerably modify the variables classically defining the early stages of a viral infection. When transfused with HBV-infected blood, immunosuppressive recipients need long-term examination, even if HBV-negative data are obtained at 3 months after transfusion (Hollinger & Dodd, 2009).

Then, a question arises how long it is necessary to observe the recipients after transfusion. It is not easy to answer this question. According to the report of Yuki *et al.* (2003), 14 patients who were recalled at a median of 4·2 years (range: 1·8–9·5 years) after the onset of acute hepatitis B all showed clinical and serologic recovery with circulating HBsAg clearance. However, PCR analysis showed that 3 of these 14 patients had low levels of circulating HBV DNA for up to 4·6–8·9 years after the onset. Although Yuki *et al.* did not show the exact

immunological state of their patients, a much longer observation period may be required than had ever been considered. Regarding the observation period after blood transfusion, we can say that it should be longer than 6 months, based on our experience with R1.

Conclusion

In the current Japanese look-back study, we can find the transfusion cases due to HBV-infected blood that could not be detected by serological tests or MP-NAT. On the basis of the look-back study among the repeat donors in Hyogo-Prefecture, Japan, donations with HBV-infected blood negative for MP-NAT occurred with a frequency of 13 in 685 844 donations (~1/53 000 donations). However, more than half of the recipients transfused with HBV-infected blood negative for MP-NAT could not be followed up. It is therefore necessary to establish a more cautious follow-up system.

ACKNOWLEDGMENTS

We are grateful to the patients, doctors and coworkers of the hospitals in Hyogo-Prefecture for their participation in this study. We thank T. Taniguchi (Himeji Dokkyo University) for his helpful comments on the manuscript. We also thank the following members of Hyogo Blood Centre for their kind cooperation in this study: K. Namura, S. Kojima, K. Minamino, Y. Noguchi, M. Doi, M. Yamashita, N. Araki, S. Akita, H. Yabui, K. Izumi and T. Ootake.

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Clinica Chimica Acta 411 (2010) 605–606

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Letter to the Editor

Single amino acid substitution in the hepatitis B virus surface antigen (HBsAg) "a" determinant affects the detection sensitivity of an HBsAg diagnostic kit

Dear editor:

Hepatitis B virus surface antigen (HBsAg) is one of the most important serological markers used in the diagnosis of HBV infection. HBV has been classified into 8 genotypes, designated A to H [1–4]. In our previous report [5], we evaluated the sensitivity of 10 commercially available diagnostic kits to recombinant HBsAgs encoded by HBV of genotypes A to H. None of the diagnostic kits examined failed to detect HBsAg of all the genotypes (A to H) at a concentration of 1.0 IU/ml. When the HBsAg samples were tested at a lower concentration (0.2 IU/ml), 9 out of 10 kits gave positive results, i.e. cut-off index (COI) ≥ 1.0, but 1 kit, Lumipulse II HBsAg, failed to give positive results for genotypes E and F. In this study, we compared the amino acid sequences of the HBsAg "a" determinant among HBV genotypes.

Amino acid-substituted HBsAgs, i.e., S140T, for genotypes E and F were synthesized as follows: the S genes of genotypes E and F were cloned into the eukaryotic expression vector pEF6/V5-His (Invitrogen Co., San Diego, CA), as previously described [5]. We constructed the amino acid-substituted HBsAg mutants, S140T, by inverse PCR of the whole plasmids with KOD-plus polymerase (Toyobo Co., Ltd., Osaka, Japan) following the thermal cycler protocol; denaturation for 2 min at 94 °C and 30 cycles of denaturation at 94 °C for 1 s, annealing at 55 °C for 30 s, and extension at 68 °C for 6 min 30 s. Primers for PCR of the plasmid of genotype E were as follows: forward eS140T-Fw409–430, 5′-ATGTTGCTGTACAAAACCTTCGG-3′; reverse eS140T-Rv408-389, 5′-GAGGGAAACATAGAGGTTCC-3′. Primers for genotype F plasmid were as follows: forward fS140T-Fw409–430, 5′-CTGTTGCTGTACCAAACCCTCGG-3′; reverse fS140T-Rv408-389, 5′-GAGGGAAACATAGAGGTTCC-3′. Underlined characters in these DNA sequences refer to codon S140T in the S protein and boldface characters indicate the substituted nucleotide that was T in the wild-type sequence. PCR products were separated by agarose gel electrophoresis and isolated with the MinElute Gel kit (Qiagen GmbH, Germany), circularized using the Blunting Kination Ligation kit (Takara Bio Inc., Ohtsu, Japan), and used to transform TOP 10 Chemically Competent *Escherchia coli* cells (Invitrogen). The entire S gene regions of the resulting plasmids were sequenced to confirm that they had the desired substitution and no other mutation (data not shown). The above kits were used according to the respective manufacturer's instructions.

Huh-7 cells grown in two 6-well culture plates (Invitrogen) were transiently transfected with each HBsAg plasmid as previously described [5]. After 3 days of culture, approximately 40 ml of culture supernatant was harvested and filtered through a membrane filter with pore size of 0.45 mm (Millex® Filter Unit; Millipore Co., Billerica, MA). The supernatants were concentrated with Amicon Ultra-15 K10 (Millipore Co.) to one-fifth of the volume. The concentration of each recombinant HBsAg sample was tentatively determined using (expressed in IU/ml) Architect HBsAg QT (Abbott Japan Co., Ltd., Chiba, Japan), which is the only quantitative assay kit approved in Japan. The samples were diluted to make 2-fold serial dilutions at concentrations of 1.6 IU/ml to 0.1 IU/ml with a multi-marker negative matrix (Accurum 810; BBI Co. Ltd., Boston, MA). The test samples were measured with 2 diagnostic kits – Lumipulse II HBsAg and Lumipulse Presto HBsAg (Fujirebio Co. Ltd., Tokyo, Japan) – according to the manufacturer's instructions and the results were expressed as the COI.

Comparison of the amino acid sequences of the HBsAg "a" determinant among HBV genotypes revealed that the amino acid at position 140 was T (threonine) in all genotypes except E and F but was S (serine) in genotypes E and F (Fig. 1A). We therefore synthesized amino acid-substituted HBsAgs, i.e., S140T, for genotypes E and F. As shown in Fig. 1B, S140T of both genotypes, E and F, tested positive, i.e., COI ≥ 1.0, by the Lumipulse II HBsAg kit even at a low concentration (0.1 IU/ml) of HBsAg. These results indicated that a single amino acid substitution in the HBsAg "a" determinant affects the sensitivity of the Lumipulse II HBsAg kit. In response to these results, Fujirebio Inc., the manufacturer of the Lumipulse II HBsAg kit, improved the sensitivity of the kit by optimizing it for the amounts of the two types of monoclonal antibody used. As shown in Fig. 1C, the improved kit, named Lumipulse Presto HBsAg, was able to detect a concentration of HBsAg as low as 0.1 IU/ml regardless of the substitution at amino acid 140.

In this report, we demonstrate that a single amino acid substitution affects the sensitivity of an in vitro diagnostic kit for HBsAg detection. This issue has been of wide concern because it was well documented that some diagnostic kits failed to detect particular mutant HBsAgs that have an amino acid substitution in the "a" determinant, such as a Gly/Arg mutation at amino acid 145, i.e., G145R [6–8]. Our study clearly verified the influence of amino acid substitution in the HBsAg "a" determinant on the detection capacity of a diagnostic kit by using the amino acid substitution technique.

The effect of an amino acid substitution on the sensitivity of diagnostic kits may not be restricted to detection kits for HBsAg. The phenomenon reported here should be borne in mind when using in vitro diagnostic kits not only for HBsAg but also for other analytes, such as HCV core Ag and HIV Ag. In fact, our recent study demonstrated that a single amino acid substitution within the HCV core antigen sequence reduced the sensitivity of a commonly used immunoassay [9].

In conclusion, we have verified that a particular amino acid residue in the HBsAg "a" determinant of a particular HBV genotype is critical for HBsAg detection sensitivity. Furthermore, it was demonstrated that optimization for the amounts of monoclonal antibodies improved the sensitivity of an HBsAg detection kit. These results indicate that caution should be exercised when detecting HBsAgs of various genotypes as well as mutant HBsAgs.

Acknowledgments

We thank Fujirebio Inc. who supplied the HBsAg kits and in performing the assays. This study was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare, Japan.

0009-8981/$ – see front matter
doi:10.1016/j.cca.2009.12.020

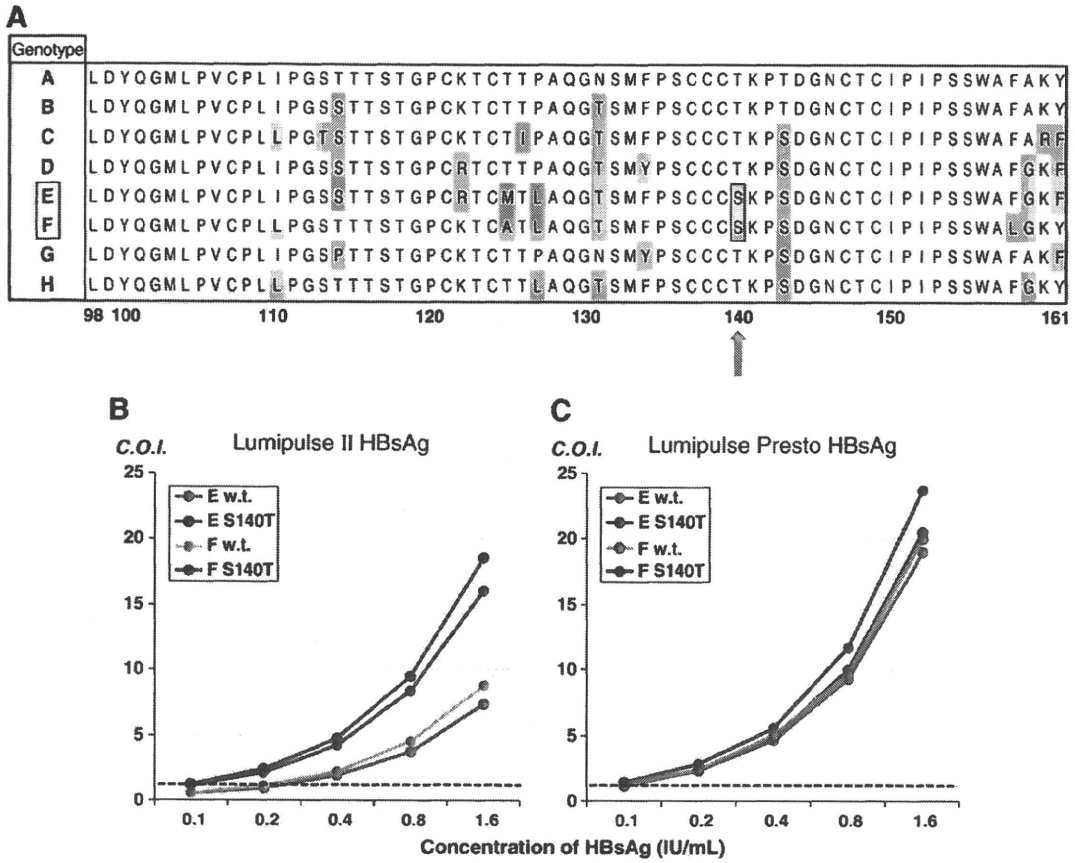

Fig. 1. A Comparison of amino acid sequences of HBsAg "a" determinants of various HBV genotypes. Accession numbers for amino acid sequences of genotypes A to H shown in the figure are listed below. Genotype A (AY902775, http://www.ncbi.nlm.nih.gov/nuccore/59802797); genotype B (AY293309, http://www.ncbi.nlm.nih.gov/nuccore/38147024); genotype C (AY205125, http://www.ncbi.nlm.nih.gov/nuccore/60279615); genotype D (AY796032, http://www.ncbi.nlm.nih.gov/nuccore/56090033); genotype E (DQ060829, http://www.ncbi.nlm.nih.gov/nuccore/70794948); genotype F (AY090459, http://www.ncbi.nlm.nih.gov/nuccore/22135721); genotype G (AB056516, http://www.ncbi.nlm.nih.gov/nuccore/15425700); genotype H (AB179747, http://www.ncbi.nlm.nih.gov/nuccore/60115422). B: Detection of recombinant HBsAg derived from genotypes E (E w.t.) and F (F w.t.) and their amino acid-substituted counterparts (E S140T and F S140T) by Lumipulse II HBsAg and C: Lumipulse Presto HBsAg kits. Each point indicates the mean of triplicate results. Error bars are too short to be indicated. Dashed lines indicate COI = 1.0.

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13 October 2009