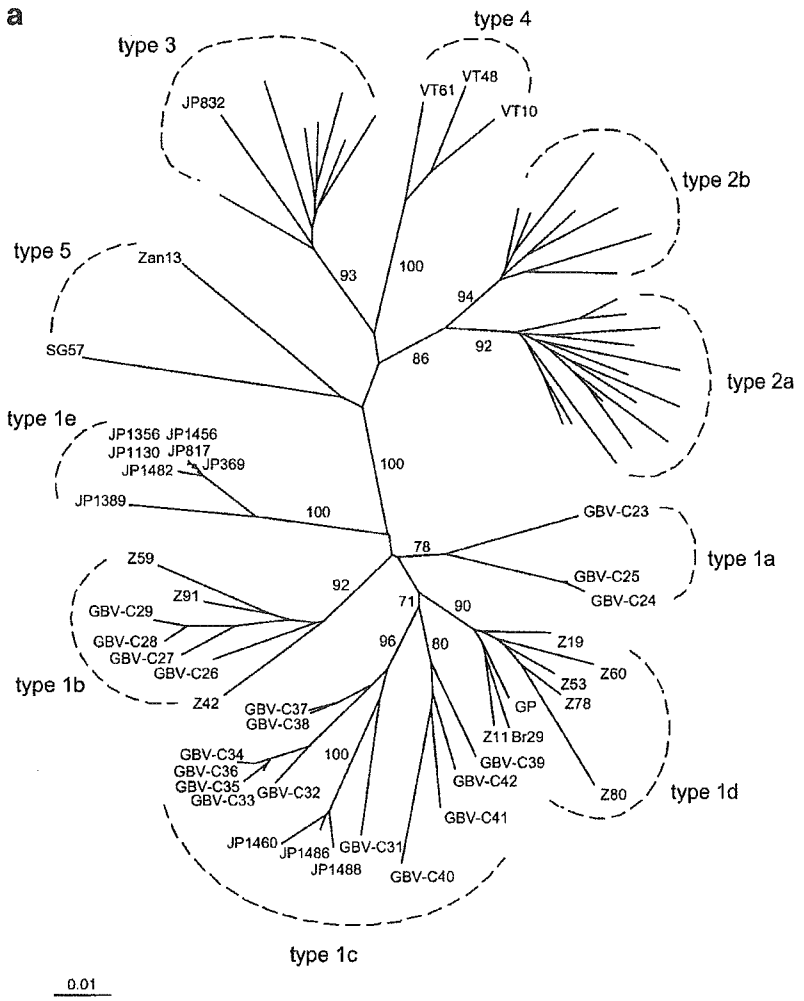

Fig. 1. Unrooted phylogenetic trees of the 11 GB virus C/hepatitis G virus isolates from Japanese patients with hemophilia based on a 592-nucleotide fragment starting from the 5′ non-coding region to part of the E1 gene of the viral genome. The tree was constructed by the (**a**) neighbour-joining method, (**b**) the Fitch and Wagner parsimony method, and (**c**) the maximum likelihood method. The evolutionary distances were calculated using the Felsenstein model with a transition/transversion substitution ratio of 2.5 and empirical base frequencies. The bootstrap values (1,000 bootstrap samples) are indicated beside the branches in percentage. In the maximum likelihood tree, double stars (**) represent a significantly positive branch ($P < 0.01$). The accession numbers of the sequences included for comparison in this study are as the following: type 1 clade a: U59540 to U59542; type 1 clade b: U59543 to U59546, Y16437, Y16439, and Y16444; type 1 clade c: U59547 to U59558; type 1 clade d: Y15261, Y16435, Y16436, Y16438, Y16440, Y16442, Y16443, and AJ539460; type 2a: AJ539458, AJ539461, AJ539462, U59518 to U59520, U59525, U59528, D90600, Y15255, Y15259, Y15260, Y15262, and Y15266; type 2b: U59529, U59531 to U59534, U59537, and AJ539459; type 3: D87262, D90601, AB008342, U59539, D87714, D87715, and D87708; type 4: AB013189, AB018667, and AB013187; type 5: AF131115 and AF078048.

Africa, and it is somewhat strange that GBV-C/HGV detected in most Japanese patients with hemophilia belong to genotype 1. To clarify the real, detailed origin of GBV-C/HGV in Japanese patients with hemophilia, we investigated the phylogeny of 11 GBV-C/HGV isolates from Japanese patients with hemophilia by a more detailed analysis with a longer fragment (around 600 bp) spanning from the 5′NCR to part of the E1 gene, comparing with many type 1 sequences.

Among the eleven sequences of these patients, one was found to be GBV-C/HGV genotype 3, which is mainly an Asian genotype. The patient with GBV-C/HGV genotype 3 had been receiving only blood products, manufactured from Japanese blood donors, and had no history of receiving imported blood products.

All the other isolates (10/11) belonged to GBV-C/HGV genotype 1. All patients with GBV-C/HGV genotype 1 had a history of receiving imported blood products, manufactured in the United States or in Europe. In phylogenetic comparison including many other genotype 1 sequences, 5 main clades were identified and GBV-C/HGV of these patients clustered in two different clades. Among these 10 isolates, three clustered into the subtype 1c clade. Subtype 1c clade mainly consisted of viral sequences from West Africa (mainly from Ghana). This suggests that some blood products used by the

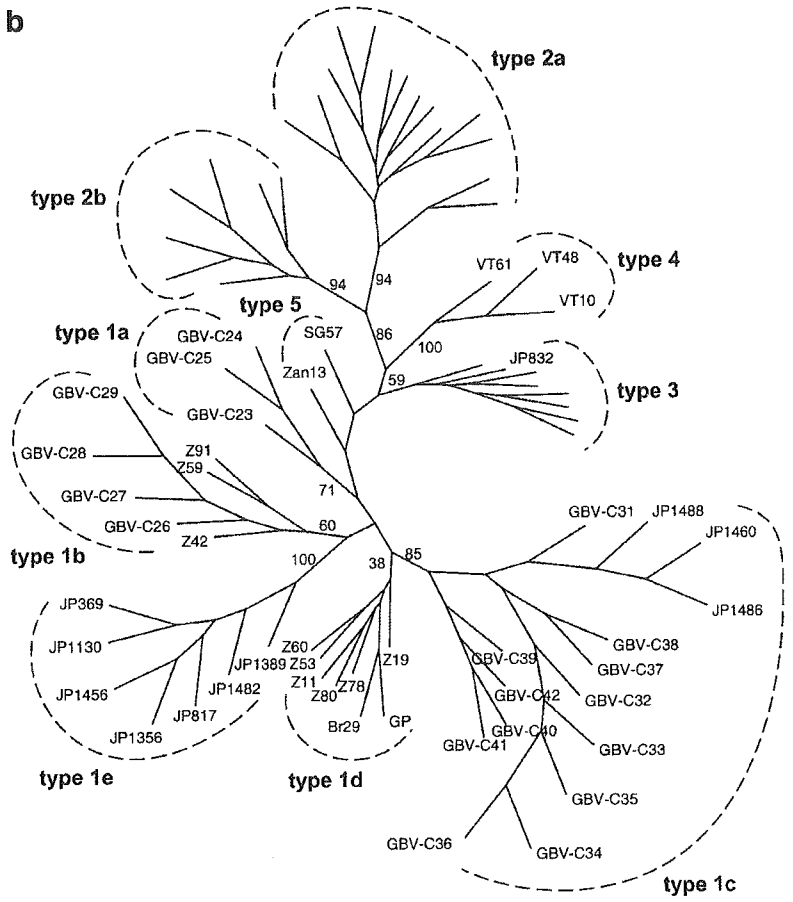

Fig. 1. (*Continued*)

Japanese patients with hemophilia might have originated directly or indirectly from West Africa.

Of interest, the other seven strains formed a new well-supported monophyletic group (clade 1e) with a bootstrap value of 100% in the NJ and pars methods and the branch length is also statistically significant ($P < 0.01$) in the ML tree. In a previous study, four genotype 1 groups were identified [Liu et al., 2000]. However, in the present study, it was found that GBV-C/HGV genotype 1 can be classified further into at least 5 clades, and the strains detected in Japanese patients with hemophilia represented a new clade in the GBV-C/HGV genotype 1 sequences. We could not identify the geographical origin of this subtype for lack of other reported strains in this clade. For clade 1b and 1d, in spite of a bootstrap value of 60 and 38% in the pars tree (Fig. 1b), they obtained 92 and 90% bootstrap support in the NJ tree (Fig. 1a) and their branch length is also significant ($P < 0.01$) in the ML tree (Fig. 1c). Since they have been supported by at least two methods, clade 1b and 1d can therefore be considered as true ones. The phenomenon of weak bootstrap support of some type 1 clusters in the pars tree has been reported before [Liu et al., 2000].

Because it is often the case that patients with hemophilia use blood products consecutively, it is difficult to identify precisely the lot of blood product with which they had exposed and had been infected with GBV-C/HGV. However, there is the possibility that some of our patients had been infected with GBV-C/HGV with the same lot of product, which included the same or closely related GBV-C/HGV strains, because they are followed up at the same hospital, and this could have influenced the clustering pattern of GBV-C/HGV isolates detected in these patients.

In conclusion, Japanese patients with hemophilia are infected mainly with genotype 1 GBV-C/HGV, a genotype reported mainly from the African continent. This raises questions on the origin of coagulation factors imported into Japan in the past. This is important because HCV and perhaps HIV could have also been transmitted by imported coagulation factors. The GBV-C/HGV viruses originated probably from 2 different infected pools, as shown by the 2 different clades (one within 1c and one representing 1e) infecting patients. Further, it was observed that patients infected with GBV-C/HGV subtype 1e are co-infected mainly with HCV subtype 1a and that those infected by HGV-C/HGV

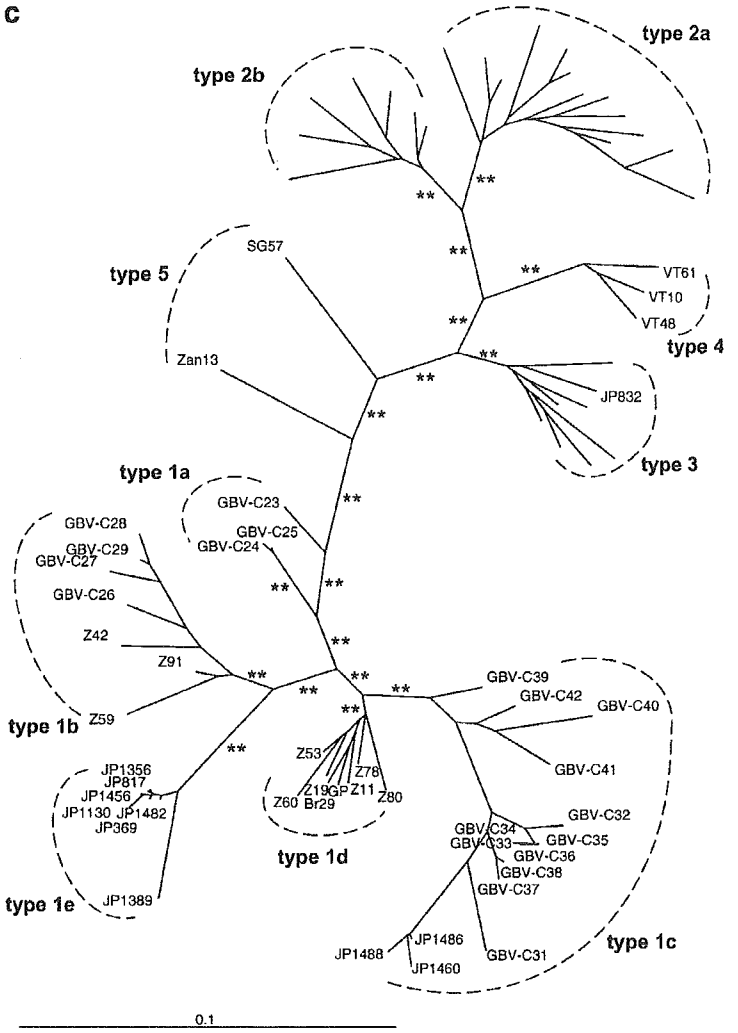

Fig. 1. (*Continued*)

TABLE I. GB Virus C/Hepatitis G Virus of New Subtype (1e) and Other Subtypes (1c or 3) in Japanese Patients With Hemophilia*

Sample no.	Year of birth	Sex	HCV RNA	HCV genotype[a]	Anti-HIV-1
Subtype 1e (new subtype)					
369	1947	M	+	1a+1b	−
817	1978	M	+	1a	+
1130	1957	M	+	Unclassifiable	+
1356	1983	M	+	1a	+
1386	1960	M	+	1a	+
1456	1954	M	+	1a	+
1482	1968	M	+	1a	+
Subtype 1c					
1460	1972	M	+	1b	+
1486	1968	M	+	1a	−
1488	1970	M	+	1b	−
Subtype 3					
832	1973	M	+	1b	−

*HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1.
[a]HCV genotype was measured by the method of Okamoto et al. [1996].

subtype 1c are more often infected with HCV subtype 1b. HIV infection is observed mainly in patients with GBV-C/HGV subtype 1e. Further comparisons with strains of GBV-C/HGV in the general Japanese population and in groups infected by genotype 1 of GBV-C/HGV should clarify these issues.

REFERENCES

Cornu C, Jadoul M, Loute G, Goubau P. 1997. Hepatitis G virus infection in haemodialysed patients: epidemiology and clinical relevance. Nephrol Dial Transplant 12:1326–1329.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Felsenstein J. 1989. PHYLIP-Phylogeny inference package (Version 3.2). Cladistics 5:164–166.

Fukushi S, Kurihara C, Ishiyama N, Okamura H, Hoshino FB, Oya A, Katayama K. 1996. Nucleotide sequence of the 5′ noncoding region of hepatitis G virus isolated from Japanese patients: comparison with reported isolates. Biochem Biophys Res Commun 226: 314–318.

Handajani R, Soetjipto Lusida MI, Suryohudoyo P, Adi P, Setiawan PB, Nidom CA, Soemarto R, Katayama Y, Fujii M, Hotta H. 2000. Prevalence of GB Virus C/hepatitis G virus infection among various populations in Surabaya, Indonesia, and identification of novel groups of sequence variants. J Clin Microbiol 38:662–668.

Linnen J, Wages J, Zhang-Keck ZY, Fry K, Krawczynski KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih JW-K, Young L, Piatak M, Hoover C, Fernandez J, Chen S, Zou J-C, Morris T, Hyams KC, Ismay S, Lifson JD, Hess G, Foung SKH, Thomas H, Bradley D, Margolis H, Kim JP. 1996. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. Science 271:505–508.

Liu H-F, Cornu C, Jadoul M, Dahan K, Loute G, Goubau P. 1998. Molecular analysis of GB virus C isolates in Belgian hemodialysis patients. J Med Virol 55:118–122.

Liu H-F, Muyembe-Tamfum JJ, Dahan K, Desmyter J, Goubau P. 2000. High prevalence of GB virus C/hepatitis G virus in Kinshasa, Democratic Republic of Congo: a phylogenetic analysis. J Med Virol 60:159–165.

Muerhoff AS, Simons JN, Leary TP, Erker JC, Chalmers ML, Pilot-Matias TJ, Dawson GJ, Desai SM, Mushahwar IK. 1996. Sequence heterogeneity within the 5′-terminal region of the hepatitis GB virus C genome and evidence for genotypes. J Hepatol 25:379–384.

Muerhoff AS, Simons JN, Leary TP, Erker JC, Desai SM, Mushahwar IK. 1997. Identification of GB virus C variants by phylogenetic analysis of 5′-untranslated and coding region sequences. J Virol 71: 6501–6508.

Mushahwar IK. 2000. Recently discovered blood-borne viruses: Are they hepatitis viruses or merely endosymbionts. J Med Virol 62: 399–404.

Naito H, Hayashi S, Abe K. 1999. Identification of a novel genotype of hepatitis G virus in southeast Asia. J Clin Microbiol 37:1217–1220.

Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK. 1995. Isolation of novel virus-like sequences associated with human hepatitis. Nat Med 1: 564–569.

Smith DB, Cuceanu N, Davidson F, Jarvis LM, Mokili JLK, Hamid S, Ludlam CA, Simmonds P. 1997. Discrimination of hepatitis G virus/GBV-C geographical variants by analysis of the 5′ non-coding region. J Gen Virol 78:1533–1542.

Smith DB, Basaras M, Frost S, Haydon D, Cuceanu N, Prescott L, Kamenka C, Millband D, Sathar MA, Simmonds P. 2000. Phylogenetic analysis of GBV-C/hepatitis G virus. J Gen Virol 81:769–780.

Strimmer K, von Haeseler A. 1996. Quartet puzzling: A quartet maximum-likelihood method for reconstructing tree topologies. Mol Biol Evol 13:964–969.

Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H, Okamoto H. 1998. GB virus C/hepatitis G virus isolates in Japanese haemophiliacs and their origins. Thromb Haemost 80:242–245.

Tucker TJ, Smuts H. 2000. GBV-C/HGV genotypes: proposed nomenclature for genotypes 1-5. J Med Virol 62:82–83.

Tucker TJ, Smuts H, Eickhaus P, Robson SC, Kirsch RE. 1999. Molecular characterization of the 5′ non-coding region of South African GBV-C/HGV isolates: major deletion and evidence for a fourth genotype. J Med Virol 59:52–59.

Journal of Medical Virology 73:195–199 (2004)

Prevalence and Clinical Implications of Occult Hepatitis B Viral Infection in Hemophilia Patients in Japan

Hidenori Toyoda,[1,3]* Kazuhiko Hayashi,[1] Yoshiki Murakami,[4] Takashi Honda,[1] Yoshiaki Katano,[1] Isao Nakano,[1] Hidemi Goto,[1] Takashi Kumada,[3] and Junki Takamatsu[2]

[1]*Department of Gastroenterology, Nagoya University School of Medicine, Nagoya, Japan*
[2]*Department of Transfusion Medicine, Nagoya University School of Medicine, Nagoya, Japan*
[3]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan*
[4]*Department of Gastroenterology, National Tsuruga Hospital, Tsuruga, Japan*

The prevalence and clinical implications of occult hepatitis B virus (HBV) infection were investigated in the Japanese patients with hemophilia in whom a high prevalence of infection with transfusion-transmissible viruses has been reported. HBV DNA was detected in the sera of 22 of 43 (51.2%) patients with hemophilia who were negative for HBV surface antigen (HBs), indicating that these patients had occult HBV infection. No factor, including age, type or severity of hemophilia, presence of HBs or HBV core (HBc) antibody, or coinfection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) was associated with occult HBV infection, except for high anti-HBc titer and/or coinfection with HCV genotype 1 (1a or 1b). In general, occult HBV infection did not appear to have significant clinical implications. However, in patients in whom HBV was detected by PCR specific for the surface (S)-region, higher alanine aminotransferase levels were observed. The genotype of the occult HBV in the present study was exclusively the domestic type indigenous to Japan (genotype C), suggesting a different route of transmission for HBV in comparison to HCV and HIV in this population. ***J. Med. Virol. 73:195–199, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: hemophilia; hepatitis B virus; occult infection; genotype; route of transmission

INTRODUCTION

Patients with hemophilia are at high risk of infection with parenterally transmissible viruses due to the frequent use of blood products. High prevalence of infection with hepatitis C virus (HCV) [Makris et al., 1990; Troisi et al., 1993], human immunodeficiency virus (HIV) [Tsuchie et al., 1985; Kroner et al., 1994], and GB virus C (GBV-C) [Hanley et al., 1998; Toyoda et al., 1998] has been reported. A few studies have also been carried out on hepatitis B virus (HBV) infection in this population by serological evaluation [Kumar et al., 1993; Goedert et al., 2002].

Recently, several studies have reported occult HBV infection in subjects without HBV surface (HBs) antigen (HBsAg), and its clinical implications are suggested [Cacciola et al., 1999; Brechot et al., 2001; Torbenson and Thomas, 2002]. In addition to a history of frequent use of blood products, a large number of hemophilia patients are infected with HIV, which can result in dynamic changes in the immune status. Reactivation of HBV in association with changes in the immune status can occur and can cause liver damage, which sometimes results in liver failure [Xunrong et al., 2001]. Thus, hemophilia patients are a population in which the assessment of occult HBV infection is important, especially for those patients with HIV.

In the present study, we attempted to clarify the prevalence and clinical importance of occult HBV infection in the Japanese hemophilia patients without HBs antigen. As a result, occult HBV infection was found in around one-half of the patients.

PATIENTS AND METHODS

Patients

Among 44 patients with hemophilia who had been followed-up as outpatients at Nagoya University Hospital and who were admitted regularly to the hospital during 2002, 1 patient had HBs antigen and the other

*Correspondence to: Hidenori Toyoda, MD, PhD, Department of Gastroenterology, Ogaki Municipal Hospital, 4-86 Minaminokawa, Ogaki, Gifu, 503-8502, Japan.
E-mail: tkumada@he.mirai.ne.jp

Accepted 18 February 2004
DOI 10.1002/jmv.20075
Published online in Wiley InterScience (www.interscience.wiley.com)

43 patients were negative for HBs antigen. These 43 patients were enrolled in this study. All were males with a mean age of 34.0 ± 12.1-years-old. Thirty-four patients had hemophilia A, and the remaining 9 had hemophilia B. Thirty-seven patients had severe, 4 patients had moderate, and 2 patients had mild hemophilia. All 24 patients with HIV infection were receiving HAART therapy at the time of sampling serum. Eight of 38 patients with HCV infection had a history of interferon therapy, but no patients were treated with interferon at the time serum was sampled.

Evaluation for Coinfection With HIV and HCV

HIV infection was confirmed by anti-HIV1 antibody detected by particle agglutination (SERODIA-HIV, Fuji Rebio, Tokyo, Japan). Serum HIV RNA concentration was measured by the Amplicor HIV Monitor test (Roche Diagnostics K.K., Tokyo, Japan). The presence of HCV was confirmed by both an HCV antibody assay (2nd generation, Dinabot; Tokyo, Japan), and detection of HCV RNA by nested reverse transcription-polymerase chain reaction (RT-PCR) [Okamoto et al., 1990]. HCV genotypes, according to Simmonds et al. [1994] classification, were determined by RT-PCR with genotype-specific primers [Okamoto et al., 1996]. Serum HCV RNA concentrations were measured by Amplicor Monitor assay (Roche Diagnostics K.K., Tokyo).

Serological Tests for HBV Infection and Detection of HBV DNA

HBV serum markers (HBs antigen, HBs antibody, and HBV core [HBc] antibody) were examined by means of commercial immunoenzyme assays (Abbott Laboratories, North Chicago, IL). For detection of HBV DNA, extracted DNA was amplified by nested touchdown PCR [Don et al., 1991] with three independent primer sets specific for HBV surface (s)–(sense: 5′-CTCTTGTCCTCCAATTTGTCCT-3′ and antisense: 5′-CAGCAAAGCCCAAAAGACCCAC-3′ for the first PCR, and sense: 5′-AGGTA-TGTTGCCCGTTTGTCCT-3′ and antisense: 5′-GGGTTTAAATGTATACCCA-3′ for the second PCR), core (c)–(sense: 5′-ACTGTTCAAGCCTCCAAGCT-3′ for the first and second PCR, antisense: 5′-GGAATACTAACATTGAGATTCCCGAG-3′ for the first PCR, and antisense: 5′-AGTGCGAATCCACACTC-3′ for the second PCR), and X-regions (sense: 5′-TGCCAAGTGTTTGCTGACGC-3′ for the first PCR, sense: 5′-CTGCCGATCCATACTGCGGAAC-3′ for the second PCR, and antisense: 5′-TTCCTGCAGTGGAGACCACCGTGAACG-3′ for the first and second PCR). HBV DNA was amplified from 100 ng of extracted DNA in a total volume of 50 μl, in the presence of 10 pmol of each primer, 125 μM dNTP, and 2.5 U Taq polymerase (Toyobo, Tokyo, Japan). PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR program consisted of 20 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec with a 0.5°C decrease per one cycle (55.5°C at final cycle), and extension at 70°C for 3 min with an initial denaturation at 94°C for 1 min, and a subsequent 20 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 70°C for 3 min with a final extension at 70°C for 10 min. The same PCR program was used for both the first and second PCR amplifications. Amplified PCR products were analyzed by electrophoresis on 1.0% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia, Buckinghamshire, UK). The amplified products were detected by hybridization with a specific probe based on the entire HBV sequences. This probe was generated with a DIG DNA Labeling and Detection kit (Roche Diagnostics, Mannheim, Germany). Results were considered to be valid only if identical results were obtained in at least two separate experiments.

The patients who were positive for both the S- and C-regions were considered to be positive for HBV. The patients who were positive for only one of the two regions examined were then referred to the result of PCR specific for the X-region, and HBV infection was confirmed according to this result.

Genotyping of HBV DNA

Genotyping of occult HBV was performed in seven patients in whom HBV DNA was detected by PCR specific for the S-region. For genotyping of HBV DNA, the PCR product from amplification of the S-region was sequenced directly, and phylogenetic analysis was performed with the neighbor-jointing and bootstrap methods.

The entire protocol was approved by the hospital ethics committee and carried out in compliance with the Helsinki Declaration.

RESULTS

Serological Prevalence of Hepatitis B Viral Markers

Among the 43 patients with hemophilia but without HBs anitgen, HBs antibody was detected in 27 patients (62.8%), and HBc antibody was detected in 37 patients (86.0%). There was no correlation between the type and/or severity of hemophilia and serological prevalence of HBV. There was no association between coinfections with HIV and/or HCV and HBV serological status.

Detection of HBV DNA in Serum

HBV DNA was detected in 22 of the 43 hemophilia patients (51.2%). HBV was detected by PCR specific for the S-region in 7 patients and by PCR specific for the C-region in 19 patients. In 4 patients HBV DNA was detected by both methods. In the 18 patients in whom HBV DNA was detected by only one of the PCR methods, additional PCR targeting the X-region was performed and all patients were positive for this region.

We compared the background, serological markers for HBV, and coinfection status between HBV DNA-positive and -negative patients (Table I). There were no significant differences in the background of patients, including age, and type or severity of hemophilia. Both

TABLE I. Characteristics of Patients With or Without Occult HBV Infection

	HBV DNA (−)	HBV DNA (+)
Number	21	22
Mean age	35.2 ± 10.5	32.8 ± 13.5
Type of hemophilia (A/B)	17/4	17/5
Severity of hemophilia (mild/moderate/severe)	1/1/19	1/3/18
HBs-antibody (+/−)	14/7	13/9
HBc-antibody (+/−)	17/4	20/2
HBc-antibody titer#	129.4 (39.1–897.8)*	291.3 (80.4–914.5)*
HIV (+/−)	13/8	11/11
HIV RNA concentration (copies/μl)##	7.6 (0.2–35)	0.4 (0.2–0.5)
Under limit of quantitation sensitivity	7 (53.8%)	9 (81.8%)
CD4+ cell count##	449.9 ± 300.1	577.7 ± 272.6
HCV (+/−)	20/1	18/4
HCV RNA concentration (copies/μl)###	383.5 (17–810)	498.8 (85–830)
HCV genotype (1a/1b/2a/2b/3a/4a)###	3/3/3/2/6/3**	10/3/1/2/2/0**
Serum ALT levels (IU/L)###	57.1 (12–208)	71.8 (10–209)

HBV, hepatitis B virus; HBs, hepatitis B viral surface; HBc, hepatitis B viral core; HIV, human immunodeficiency virus; HCV, hepatitis C virus; ALT, alanine aminotransferase.
#Only in patients with positive HBc-antibody.
##Only in patients with HIV coinfection.
###Only in patients with HCV coinfection.
*$P = 0.0476$ by Mann–Whitney U test.
**$P = 0.0230$ for 1a or 1b versus other genotypes by Chi-square test.

the rates of positive HBs antibody and HBc antibody were similar between HBV DNA-positive and -negative patients. The rates of both HIV and HCV coinfection were similar regardless of occult HBV infection. In addition, when compared in combination with HBs and HBc antibody, or in combination with HIV and HCV, there was no difference in the rate of patients with HBV DNA (Table II). In patients with positive HBc antibody, however, the antibody titer was higher in HBV DNA-positive patients than in HBV DNA-negative patients ($P = 0.0476$, Mann–Whitney U test). In patients with HCV, HCV genotype 1 (1a or 1b) was significantly more prevalent in patients with HBV DNA than in patients without HBV DNA ($P = 0.0230$, Chi-square test).

In all five patients without HCV coinfection, serum alanine aminotransferase (ALT) levels were continuously normal regardless of occult infection with HBV. In patients with HCV infection, there was no significant difference in serum ALT level which was calculated as the average value of four to six analyses over 1 year, between HBV DNA-positive and -negative patients. When this comparison was restricted to patients with HBV detectable by PCR specific for the S-region only, serum ALT levels in HBV DNA-positive patients were significantly higher than those in HBV DNA-negative patients (patients with HBV, 120.3 ± 66.6 vs. patients without HBV, 57.1 ± 44.0; $P = 0.0162$, Mann–Whitney U test).

Genotype of Occult HBV

HBV genotyping was carried out based on the sequence of the S-region in seven patients in whom HBV DNA was detected by PCR specific for the S-region. Genotype C, which is the major genotype observed in the Japanese patients with chronic hepatitis B without hemophilia, was detected in all seven patients.

DISCUSSION

The clinical significance of occult HBV infection for patients with chronic hepatitis C has been described in recent reports [Cacciola et al., 1999; Sagnelli et al., 2001] and remains controversial [Kao et al., 2002]. These reports consider the influence of HBV occult infection on advanced liver disease [Cacciola et al., 1999; Sagnelli et al., 2001], development of hepatocellular carcinoma [Sheu et al., 1992; Paterlini et al., 1993], and reduced response to interferon [Zignego et al., 1997; Cacciola et al., 1999]. The importance of HBV occult infection has been reported in immunosuppressive patients, even in those without HCV coinfection [Xunrong et al., 2001]. In these patients, reactivation of HBV caused liver damage and sometimes resulted in liver failure.

Patients with hemophilia are at high risk of exposure to transfusion-transmissible virus such as HIV, HBV, HCV, and GBV-C. The high prevalence of infection with HIV [Tsuchie et al., 1985; Kroner et al., 1994], HCV

TABLE II. Rate of Hepatitis B Virus DNA Detection (%)

HBsAb(+) HBcAb(+)#	HBsAb(+) HBcAb(−)	HBsAb(−) HBcAb(+)	HBsAb(−) HBcAb(−)
13/25 (52.0)	0/2 (0)	7/12 (58.3)	2/4 (50.0)
HIV(+) HCV(+)##	HIV(+) HCV(−)	HIV(−) HCV(+)	HIV(−) HCV(−)
11/21 (52.4)	3/3 (100)	10/17 (58.8)	1/2 (50.0)

#HBsAb, hepatitis B viral surface antibody; HBcAb, hepatitis B viral core antibody.
##HIV, human immunodeficiency virus; HCV, hepatitis C virus.

[Makris et al., 1990; Troisi et al., 1993], and GBV-C [Hanley et al., 1998; Toyoda et al., 1998] has been reported in many studies. The status of serological markers on HBV infection has also been reported [Kumar et al., 1993; Goedert et al., 2002]. However, occult HBV infection in this population has not been examined. Because a large number of patients with hemophilia have HIV infection and changes in immune status in these patients can occur partly due to the disease itself and partly to the effect of HAART therapy, clarification of the status of occult HBV infection in these patients is important because of the potential for reactivation of occult HBV in association with changes in immune status.

HBV DNA was detected in serum in around one-half of the patients. The rate of detection was similar to that of HBV DNA detected in serum of the Japanese patients without hemophilia who have chronic HCV infection [Fukuda et al., 1999]. Neither the severity of hemophilia nor coinfection with HIV and HCV indicated the potential for occult HBV infection. In a previous study, Nunez et al. [2002] found no HIV-infected patients (most were intravenous drug users) in whom occult HBV infection could be confirmed. In contrast, we confirmed occult HBV infection in 11 HIV-infected patients. Only a high HBc antibody titer, which has already been reported to be an indicator of occult HBV infection [Nirei et al., 2000], and HCV genotype 1 (1a or 1b) in patients coinfected with HCV may indicate the high risk of occult HBV coinfection.

On the basis of our results, occult HBV infection appears to have no significant clinical impact when the infection is evaluated by the HBV detection for the C-region. On the contrary, occult HBV may increase serum ALT levels, which indicates severe liver damage, in patients with HCV infection when HBV DNA is positive by PCR for the S-region. Further study will be required to clarify the difference in clinical significance of HBV occult infection between PCR positive for the C-region and that positive for the S-region.

The HBV genotype detected in the Japanese patients with hemophilia was exclusively genotype C, which is the most common genotype in Japan. This shows the distinct characteristics of occult HBV infection in hemophilia patients in Japan, which are different from those of other transfusion-transmissible viruses in this population. The genotypes of viruses such as HCV or GBV-C in the Japanese hemophilia patients are foreign and not domestic genotypes [Kinoshita et al., 1993; Toyoda et al., 1998]. This is because, in this population, transmission of these viruses has been by imported blood products, as well as HIV transmission in this population [Tsuchie et al., 1985]. In contrast, only the domestic HBV genotype was found in the Japanese hemophilia patients. This, together with the lack of difference in the prevalence of occult HBV infection between hemophilia patients with HCV and HCV-infected patients who have not undergone repeated transfusions in Japan, suggests a route of transmission of this virus, different from that in cases of HIV, HCV, and GBV-C infection. The lack of correlation in the rate of coinfection between HIV or HCV and HBV also supports this suggestion. Screening for HBV in blood donors using HBs antigen as a marker started in 1973 in Japan. Some patients have a history of blood transfusion, which may have caused the occult HBV infection. Nosocomial infection in relation to the injection of blood products through the repeated use of needles, syringes, or other medical instruments, which could have occurred under medical conditions in Japan prior to the 1970s, might have also played a role.

In summary, among 43 Japanese patients with hemophilia, occult HBV infection was observed in about one-half of patients without detectable HBs antigen, a prevalence similar to that of the Japanese patients with chronic HCV infection. Occult HBV infection did not have significant clinical implications as a whole, although patients in whom HBV was detected with S-region-specific PCR showed higher ALT levels. The HBV genotype was exclusively a domestic type, suggesting a different route of transmission of HBV from that of HCV or HIV in this population. Further studies are required to determine occult HBV infection in the Japanese patients with hemophilia.

REFERENCES

Brechot C, Thiers V, Kremsdorf D, Nalpas B, Pol S, Paterlini-Brechot P. 2001. Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: Clinically significant or purely "occult?" Hepatology 34:194–203.

Cacciola I, Pollicino T, Souadrito G, Cerenzia G, Orlando ME, Raimondo G. 1999. Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. N Engl J Med 341:22–26.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 1991. Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008.

Fukuda R, Ishimura N, Niigaki M, Hamamoto S, Satoh S, Tanaka S, Kushiyama Y, Uchida Y, Ihihara S, Akagi S, Watanabe M, Kinoshita Y. 1999. Serologically silent hepatitis B virus coinfection in patients with hepatitis C virus-associated chronic liver disease: Clinical and virological significance. J Med Virol 58:201–207.

Goedert JJ, Eyster ME, Lederman MM, Mandalaki T, de Moerloose P, White GC II, Angiolillo AL, Luban NLC, Sherman KE, Manco-Johnson M, Preiss L, Leissinger C, Kessler CM, Cohen AR, DiMichele D, Hilgartner MW, Aledort LM, Kroner BL, Rosenberg PS, Hatzakis A—For the Multicenter Hemophilia Cohort Study. 2002. End-stage liver disease in persons with hemophilia and transfusion-associated infections. Blood 100:1584–1589.

Hanley JP, Jarvis LM, Hayes PC, Lee AJ, Simmonds P, Ludlam CA. 1998. Patterns of hepatitis G viraemia and liver disease in haemophiliacs previously exposed to non-virus inactivated coagulation factor concentrates. Thromb Haemost 79:291–295.

Kao J-H, Chen P-J, Lai M-Y, Chen D-S. 2002. Occult hepatitis B virus infection and clinical outcomes of patients with chronic hepatitis C. J Clin Microbiol 40:4068–4071.

Kinoshita T, Miyake K, Okamoto H, Mishiro S. 1993. Imported hepatitis C virus genotypes in Japanese hemophiliacs. J Infect Dis 168:249–250.

Kroner BL, Rosenberg PS, Aledort LM, Alvord WG, Goedert JJ. 1994. HIV-1 infection incidence among persons with hemophilia in the United States and western Europe, 1978–1990. Multicenter Hemophilia Cohort Study. J Acquir Immune Defic Syndr Hum Retrovirol 7:279–286.

Kumar A, Kulkarni R, Murray DL, Gera R, Scott-Emuakpor AB, Bosma K, Penner JA. 1993. Serologic markers of viral hepatitis A, B, C, and D in patients with hemophilia. J Med Virol 41:205–209.

Makris M, Preston FE, Triger DR, Underwood JCE, Choo QL, Kuo G, Houghton M. 1990. Hepatitis C antibody and chronic liver disease in haemophilia. Lancet 335:1117–1119.

Nirei K, Kaneko M, Moriyama M, Arakawa Y. 2000. The clinical features of chronic hepatitis C are not affected by the coexistence of hepatitis B virus DNA in patients negative for hepatitis B surface antigen. Intervirology 43:95–101.

Nunez M, Rios P, Perez-Olmeda M, Soriano V. 2002. Lack of 'occult' hepatitis B virus infection in HIV-infected patients. AIDS 16:2099–2101.

Okamoto H, Okada S, Sugiyama Y, Tanaka T, Sugai Y, Akahane Y, Machida A, Mishiro S, Yoshizawa H, Miyakawa Y, Mayumi M. 1990. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5′-noncoding region. Jpn J Exp Med 60:215–222.

Okamoto H, Kobata S, Tokita H, Inoue T, Woodfield GD, Holland PV, Al-Knawy BA, Uzunalimoglu O, Miyakawa Y, Mayumi M. 1996. A second-generation method of genotyping hepatitis C virus by the polymerase chain reaction with sense and antisense primers deduced from the core gene. J Virol Methods 57:31–45.

Paterlini P, Driss F, Nalpas B, Pisi E, Franco D, Berthelot P, Brechot C. 1993. Persistence of hepatitis B and hepatitis C viral genomes in primary liver cancers from HbsAg-negative patients: A study of a low-endemic area. Hepatology 17:20–29.

Sagnelli E, Coppola N, Scolastico C, Mogavero AR, Filippini P, Piccinino F. 2001. HCV genotype and 'silent' HBV coinfection: Two main risk factors for a more severe liver disease. J Med Virol 64:350–355.

Sheu JC, Huang GT, Shih LN, Lee WC, Chou HC, Wang JT, Lee PH, Lai MY, Wang CY, Yang PM, Lee HS, Chen DS. 1992. Hepatitis C and hepatitis B viruses in hepatitis B surface antigen-negative hepatocellular carcinoma. Gastroenterology 103:1322–1327.

Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan S-W, Chayama K, Chen D-S, Choo Q-L, Colombo M, Cuypers HTM, Date T, Dusheiko GM, Esteban JI, Fay O, Hadziyannis SJ, Han J, Hatzakis A, Holmes EC, Hotta H, Houghton M, Irvine B, Kohara M, Kolberg JA, Kuo G, Lau JYN, Lelie PN, Maertens G, McOmish F, Miyamura T, Mizokami M, Nomoto A, Prince AM, Reesink HW, Rice C, Roggendorf M, Schalm SW, Shikata T, Shimotohno K, Stuyver L, Trepo C, Weiner A, Yap PL, Urdea M. 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 19:1321–1324.

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

Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H, Okamoto H. 1998. GB virus C/hepatitis G virus isolates in Japanese haemophiliacs and their origins. Thromb Haemost 80:242–245.

Troisi CL, Hollinger FB, Hoots WK, Contant C, Gill J, Ragni M, Parmley R, Sexauer C, Gomperts E, Buchanan G. 1993. A multicenter study of viral hepatitis in a United States hemophilic population. Blood 81:412–418.

Tsuchie H, Kawatani T, Nakayama E, Matsui T, Kurimura T, Hinuma Y. 1985. Distribution of the level of antibody to AIDS-associated virus (LAV) in sera from AIDS or AIDS related complex and Japanese hemophiliacs infected with AIDS-associated virus. Microbiol Immunol 29:1083–1087.

Xunrong L, Yan AW, Liang R, Lau GKK. 2001. Hepatitis B virus (HBV) reactivation after cytotoxic or immunosuppressive therapy-pathogenesis and management. Rev Med Virol 11:287–299.

Zignego AL, Fontana R, Puliti S, Barbagli S, Monti M, Careccia G, Giannelli F, Giannini C, Buzzelli G, Brunetto MR, Bonino F, Gentilini P. 1997. Relevance of inapparent co-infection by hepatitis B virus in alpha interferon-treated patients with hepatitis C virus chronic hepatitis. J Med Virol 51:313–318.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Hepatology Research 25 (2003) 409–414
Hepatology Research
www.elsevier.com/locate/ihepcom

Prevalence and characterization of hepatitis C virus genotype 4 in Japanese hepatitis C carriers

Kazuhiko Hayashi [a], Yoshihide Fukuda [a,*], Isao Nakano [a], Yoshiaki Katano [a], Hidenori Toyoda [a], Shouichi Yokozaki [a], Tetsuo Hayakawa [a], Kiyoshi Morita [b], Daisaku Nishimura [b], Katsumoto Kato [b], Fumihiro Urano [c], Junki Takamatsu [d]

[a] *Second Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[b] *Department of Gastroenterology, Kamo Hospital, Toyota, Japan*
[c] *Department of Gastroenterology, Toyohashi Municipal Hospital, Toyohashi, Japan*
[d] *Department of Transfusion Medicine, Nagoya University School of Medicine, Nagoya, Japan*

Received 5 July 2002; received in revised form 6 December 2002; accepted 16 January 2003

Abstract

Hepatitis C virus (HCV) can be classified into six major genotypes, the prevalences of which differ around the world. In Japan, the main genotypes are HCV 1 and HCV 2; others are found only rarely. Little is known about the prevalence in Japan of HCV genotype 4 which, is found frequently in North and Central Africa and the Middle East. Thus, we conducted a study to clarify distribution of HCV genotype 4 and the clinical demographics of patients with HCV genotype 4 in Japan. We examined HCV genotypes in 899 Japanese individuals with HCV viremia living in Aichi Prefecture, including 63 hemophiliacs. Four patients (0.4%) were infected with HCV genotype 4. All four of these patients were male hemophiliacs who had received clotting factors from foreign countries. Three patients were co-infected with human immunodeficiency virus (HIV); none were co-infected with GB virus-C/hepatitis G virus. Phylogenetic analysis of the El region indicated that all four patients were infected with subtype 4a. This subtype is related genetically to a subtype previously reported in Japanese and Italian hemophiliacs. HCV genotype 4 is indeed rare in Japan and may be detected only among hemophiliacs who have received inactivated clotting factor concentrates from foreign countries.

Keywords: Chronic hepatitis C; Hepatitis G virus; Hemophilia

1. Introduction

Hepatitis C virus (HCV) infects about 170 million persons worldwide and has become a big health problem. HCV causes chronic hepatitis that slowly develops into cirrhosis and hepatocellular carcinoma. The progression rate for liver disease

* Corresponding author. Tel.: +81-52-744-2169; fax: +81-52-744-2178.
E-mail address: yfukuda@med.nagoya-u.ac.jp (Y. Fukuda).

1386-6346/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1386-6346(03)00016-0

related to HCV differs between individuals, and the rates are thought to be regulated by various factors such as HCV genotype and co-infection with human immunodeficiency virus (HIV) [1,2]. HCV genotype is one of the most important factors affecting the pathogenesis of HCV infection, and knowing the genotype is useful for the clinical management of chronic hepatitis C. HCV can be classified into six major genotypes [3], of which the prevalences differ around the world [4]. HCV genotypes 1, 2, and 3 make up the majority worldwide. HCV genotype 4 has been thought to be limited to North and Central Africa and the Middle East but some cases of HCV 4 infection have been found among immigrants from areas where HCV 4 is highly endemic, among intravenous drug users, and among hemophiliacs worldwide [5–11]. In Japan, HCV genotypes 1 and 2 account for the majority of cases of HCV infection, and other genotypes are very rare except among hemophiliacs who received inactivated clotting factor concentrates from foreign countries [5,12]. Little is known about the prevalence of HCV genotype 4 in Japan. Thus, the aim of this study was to clarify distribution of the HCV genotype 4 in Japan and characterize the clinical features of patients infected with HCV genotype 4.

2. Materials and methods

2.1. Patients

Among 989 individuals with anti-HCV antibody consulted to Nagoya University Hospital, Kamo Hospital, and Toyohashi Municipal Hospital, 899 patients with serum HCV RNA were enrolled in this study. Our institutions were located in Aichi Prefecture, the center of main island of Japan. Sixty-three of these patients were hemophiliacs who received inactivated clotting factor concentrates from foreign countries. There were 515 male and 384 female patients, and the mean age of patients was 56.2 ± 15.2 years (range, 12–80 years). All patients were positive for serum anti-HCV antibody and HCV RNA. No patient had hepatitis B surface antigen, autoimmune disease, metabolic disease, or a history of chronic alcohol abuse. Informed consent was obtained from each patient participating in the study, and the study was approved by our institution's ethics committee.

2.2. Virologic tests

Serum anti-HCV antibody was measured by commercial enzyme-linked immunosorbent assay (HCV AxSYM, Dainabot, Tokyo, Japan), and qualitative analysis of HCV RNA was performed by RT-PCR (Amplicor-HCV version 2.0; Roche Diagnostic Systems, Tokyo, Japan). Anti-HIV antibody was detected with the use of commercial particle agglutination assay kit (Fuji Rebio, Tokyo, Japan). Detection of GB virus-C/hepatitis G virus (GBV-C/HGV) was performed as previously described [13]. Genotyping of HCV was performed by direct sequencing of the 5′UTR according to a previously described method [14]. Direct sequencing of the E1 region was done among patients infected with HCV 4. Briefly, RNA was extracted from 140 μl of sera with the use of a commercial kit (QIAamp Viral RNA kit; QIAGEN, Valencia, CA) and redissolved in 50 μl diethylpyrocarbonate-treated water. Ten microliters of the RNA solution was used for reverse transcription with anti-sense primer EF1 (5′-CGCCGACCTCATGGGGTA-3′). Amplification of E1 was achieved with primers EF1 and ER1 (5′-CGACCAGTTCATCATCATATCCCA-3′) for the first-round DNA sequencing, and EF2 (5′-TTGCCCGGTTGCTCTTTTTCTATC-3′) and ER1 for the second round. PCR products were subjected to cycle sequencing performed with an ABI PRISM Cycle Sequence Kit (Perkin Elmer, Branchburg, NJ) with EF2 as the sequencing primer and were analyzed with the ABI 310 DNA Sequencer (PE Applied Biosystems, Foster City, MI). Phylogenetic analysis of the E1 region was conducted by the neighbor-joining method [15].

2.3. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). Two-tailed Student's t-test was used to analyze differences in HCV genotypes according to the age of subjects. $P \leq 0.05$ was considered

statistically significant. The statistical software used was STATVIEW 5.0 (SAS Institute Inc., Cary, NC).

2.4. Reference sequences

Reference sequences used for comparison with those obtained in this study were found in GenBank and were as follows: HCV 4, FrSSD1S8 (AJ401099); HCV 4a, FrSSD77 (AJ401096), FrSSD25 (AJ401094), Ve3069 (AJ250204), HEMA51 (D45193) and Ve2666 (AJ250205); HCV 4a (B), FrSSD120 (AJ401097), Z4 (L16652), 1196E1-4 (D43677), GB809.4 (L29628) and FrSSD136 (AJ401098); HCV 4b, Z1 (L16677); HCV 4c, B203 (L39284), GB116 (L29601) and GB358 (L29606); HCV 4d, Ve2560 (AJ250210), Ve 2744 (AJ250209), FrSSD171 (AJ401101), and FrSSD50 (AJ401095); HCV 4e, CAM600 (L29587), GB809 (L29624) and CAM736 (L38323); HCV 4f, G27 (L29597), G22 (L29592) and FrSSD160 (AJ401100); HCV 4g, GB549 (L29620); HCV 4h, GB438 (L29610); HCV 4k, B14 (L39282).

3. Results

HCV genotypes found were 1a ($n = 19$), 1b ($n = 645$), 2a ($n = 143$), 2b ($n = 73$), 3a ($n = 15$), and 4 ($n = 4$). The four of 899 patients (0.4%) infected with HCV genotype 4 were all male hemophiliacs who had received clotting factors from foreign countries. All patients with genotype 4 were not related by birth. The clinical characteristics of these four patients and their HCV, HIV, and GBV-C/HGV statuses are summarized in Table 1.

Hemophiliacs infected with HCV4 tended to be younger than hemophiliacs infected with other genotypes (27.3 ± 4.5 vs. 35.9 ± 12.6 years), but the difference was not significant ($P = 0.17$). Liver function tests revealed no marked abnormalities. We retrospectively reviewed hospital records of four patients, and checked that ALT levels are persistently normal in one patient (Case No. 4). This patient was not performed liver biopsy, so whether he was diagnosed healthy carrier who had normal liver histology is not clear. Three patients

Table 1
Clinical profiles

Number	Age (years)	Hemophilia	Sex	AST (IU/l)	ALT (IU/l)	T. Bil (mg/dl)	HA (ng/ml)	HCV viral loads (K IU/ml)	HIV	HGV
1	30	B	Male	21	32	0.8	48	240	P	N
2	27	A	Male	88	119	0.9	200	440	P	N
3	31	A	Male	47	39	0.2	16	17	P	N
4	21	A	Male	18	17	0.4	50	130	N	N

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; T. Bil, total bilirubin; HA, hyaluronan; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HGV, hepatitis G virus; N, negative; P, positive.

were co-infected with HIV and none were co-infected with GBV-C/HGV. The results of phylogenetic analysis of the E1 region are shown in Fig. 1. All HCV 4 genotypes found in this study were classified into subtype 4a, a subtype also detected among Japanese and Italian hemophiliacs previously reported (HEMA51, Ve2666, and Ve3069) [5,10].

4. Discussion

Outside the regions where HCV 4 is the major genotype, investigators have found a small number of individuals infected with HCV 4. These individuals are usually immigrants, intravenous drug users, or hemophiliacs [5–11]. Of these three populations, only hemophiliacs are present in fairly large numbers in Japan. Thus, HCV 4 is a rare genotype among Japanese patients with chronic hepatitis C, and it was detected in only four hemophiliacs in our study. These four patients with genotype 4 received inactivated clotting factor concentrates from foreign countries; they had not traveled to countries where HCV genotype 4 is highly endemic. Clotting factor concentrates are made from thousands of donors; thus, contamination by HCV genotype 4 occurs fairly easily even though prevalence is of the genotype low. HCV genotype 4 has been detected

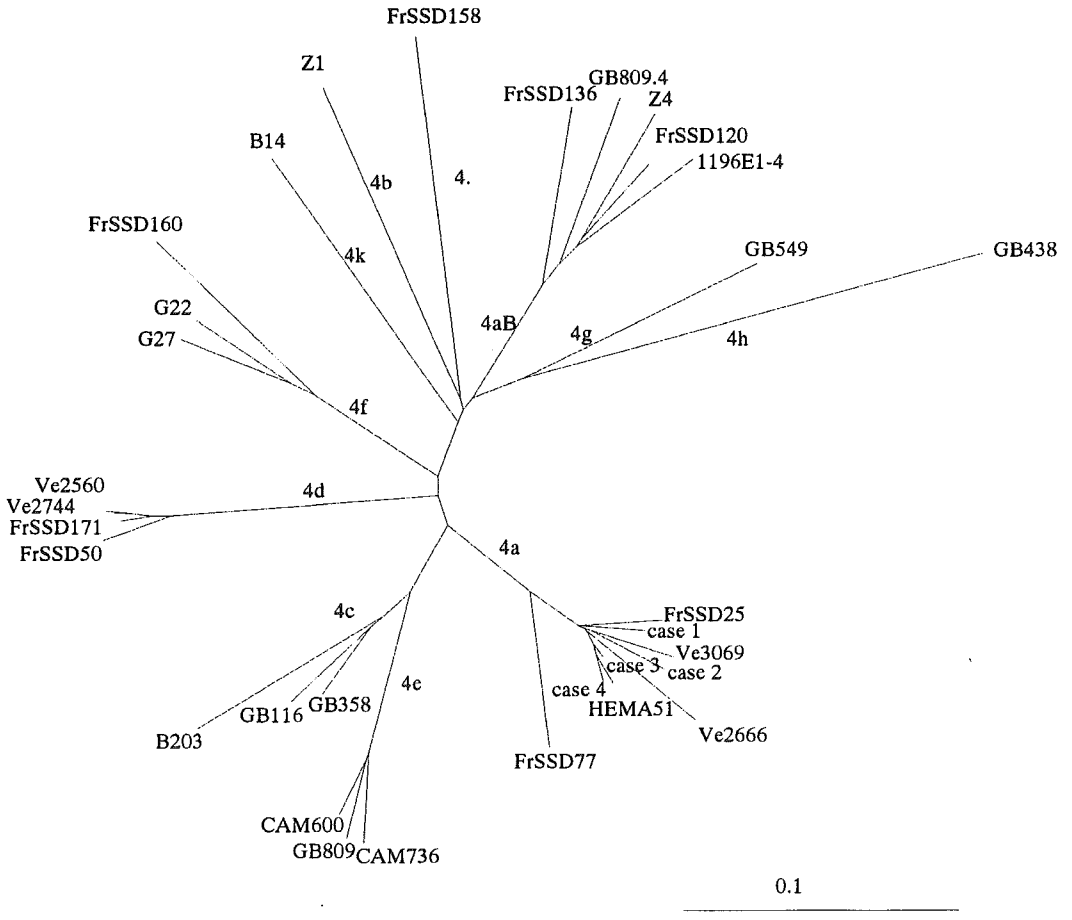

Fig. 1. Phylogenetic results from 32 HCV genotype 4 E1 region sequences including four cases found in this study. Our four cases were classified into subtype 4a.

among hemophiliacs in many countries where HCV genotype 4 is not endemic area [5,6,9,10]. So, clotting factor concentrates are likely the source of HCV genotype 4 infection in Japan.

Egypt and Saudi Arabia are countries in which the prevalence of genotype 4 is high [16,17], but the predominant subtypes differ. HCV genotype 4 have high sequence heterogeneity and the subtype most often found in Egypt is 4a, and in Saudi Arabia it is 4c/d. Not many HCV4 subtypes were detected in the present study, according to phylogenetic analysis of HCV 4. These results indicated that there were not many sources for the HCV genotype 4 infections. The HCV genotype 4 found in our subjects and among the Italian and Japanese hemophiliacs reported previously are genetically related, and a common source for the infection is suspected.

The hemophiliacs infected with HCV genotype 4 were younger than hemophiliacs infected with the HCV of other genotypes. Eyster reported that HCV genotype 4 was detected in hemophiliacs in USA after 1988 [9]. This suggests that the genotype was introduced into inactivated clotting factor concentrates in the 1980s and then spread among hemophiliacs in many countries.

The transaminase levels in our patients infected with HCV genotype 4 were nearly normal. Differences in age and ethnicity seemed to explain the differences in this reaction. There are no similar data indicative of low HCV genotype 4 activity in patients from endemic areas [16–19]. There were too small in number for proper assessment of the relation between HCV genotype 4 and minimal liver damage in Japanese patients. We previously found that there were no relationships between HCV genotype and co-infection with HIV, but co-infection with GBV-C/HGV were associated with HCV genotype 1a [20]. All patients with HCV 4 were young and had only received non-inactivated clotting factor concentrates a few times or who were switched from non-inactivated to inactivated clotting factor concentrates early in life. HCV genotype and age would be the factors of co-infection with GBV-C/HGV. The reason for the coinfection with HIV in our three patients are still unclear. Further studies are needed to clarify whether HCV genotype 4 induces minimal liver damage and favors co-infection with HIV. Patients who were consulted to our hospitals in Aichi Prefecture were enrolled in this study. Therefore, the prevalence of HCV genotype 4 in patients living in another regions of Japan and in individuals who did not come to hospital were not closely observed. Our results need to confirm in larger numbers of patients.

In conclusion, We found HCV genotype 4 to be rare in Aichi Prefecture, Japan, infecting only hemophiliacs. All patients infected with genotype 4 were classified into subtype 4a with phylogenetic analysis and appeared to be transmitted through inactivated clotting factor concentrates.

References

[1] Bruno S, Silini E, Crosignani A, et al. Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. Hepatology 1997;25:754–8.
[2] Benhamou Y, Di Martino V, Bochet M, et al. Factors affecting liver fibrosis in human immunodeficiency virus- and hepatitis C virus-coinfected patients: impact of protease inhibitor therapy. Hepatology 2001;34:283–7.
[3] Robertson B, Myers G, Howard C, et al. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch Virol 1998;143:2493–503.
[4] McOmish F, Yap PL, Dow BC, et al. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J Clin Microbiol 1994;32:884–92.
[5] Fujimura Y, Ishimoto S, Shimoyama T, et al. Genotypes and multiple infections with hepatitis C virus in patients with haemophilia A in Japan. J Viral Hepat 1996;3:79–84.
[6] Araujo F, Koch MC, Henriques I, Araujo AR, Cunha-Ribeiro L. Hepatitis C virus type 4 in Portuguese haemophiliacs, Thromb Haemost 1997;777:805.
[7] Sanehez-Quijano A, Abad MA, Torronteras R, et al. Unexpected high prevalence of hepatitis C virus genotype 4 in Southern Spain. J Hepatol 1997;27:25–9.
[8] Kaba S, Dutta U, Byth K, et al. Molecular epidemiology of hepatitis C in Australia. J Gastroenterol Hepatol 1998;13:914–20.
[9] Eyster ME, Sherman KE, Goedert JJ, Katsoulidou A, Hatzakis A. Prevalence and changes in hepatitis C virus genotypes among multitransfused persons with hemophilia. The Multicenter Hemophilia Cohort Study. J Infect Dis 1999;179:1062–99.
[10] Argentini C, Dettori S, Villano U, et al. Molecular characterisation of HCV genotype 4 isolates circulating in Italy. J Med Virol 2000;62:84–90.

[11] Phylogenetic analyses confirm the high prevalence of hepatitis C virus (HCV) type 4 in the Seine-Saint-Denis district (France) and indicate seven different HCV-4 subtypes linked to two different epidemiological patterns, J Gen Virol 2001;82:1001–012.
[12] Takada N, Takase S, Takada A, Date T. Differences in the hepatitis C virus genotypes in different countries. J Hepatol 1993;3:277–83.
[13] Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H, Okamoto H. GB virus C/hepatitis G virus isolates in Japanese haemophiliacs and their origins. Thromb Haemost 1998;80:242–5.
[14] Otagiri H, Fukuda Y, Nakano I, et al. Evaluation of a new assay for hepatitis C virus genotyping and viral load determination in patients with chronic hepatitis C. J Virol Methods 2002;103:137–43.
[15] Saitou N, Nei M. The neighbor-joining method: a new phylogenetic tree. Mol Biol Evol 1987;4:406–25.
[16] Shobokshi OA, Serebour FE, Skakni L, Al-Saffy YH, Ahdal MN. Hepatitis C genotypes and subtypes in Saudi Arabia. J Med Virol 1999;58:44–8.
[17] Ray SC, Arthur RR, Carella A, Bukh J, Thomas DL. Genetic epidemiology of hepatitis C virus throughout Egypt. J Infect Dis 2000;182:698–707.
[18] Koshy A, Marcellin P, Martinot M, Madda JP. Improved response to ribavirin interferon combination compared with interferon alone in patients with type 4 chronic hepatitis C without cirrhosis. Liver 2000;20:335–9.
[19] Al-Knawy B, Okamoto H, Ahmed El-Mekki A, et al. Distribution of hepatitis C genotype and co-infection rate with hepatitis G in Saudi Arabia. Hepatol Res 2002;24:95–8.
[20] Toyoda H, Takahashi I, Fukuda Y, Hayakawa T, Takamatsu J. Comparison of characteristics between patients with GB virus C/hepatitis G virus (GBV-C/HGV) RNA and those with GBV-C/HGV E2-antibody in patients with hemophilia. J Med Virol 2000;60:34–8.

Journal of Medical Virology 73:195–199 (2004)

Prevalence and Clinical Implications of Occult Hepatitis B Viral Infection in Hemophilia Patients in Japan

Hidenori Toyoda,[1,3]* Kazuhiko Hayashi,[1] Yoshiki Murakami,[4] Takashi Honda,[1] Yoshiaki Katano,[1] Isao Nakano,[1] Hidemi Goto,[1] Takashi Kumada,[3] and Junki Takamatsu[2]

[1]*Department of Gastroenterology, Nagoya University School of Medicine, Nagoya, Japan*
[2]*Department of Transfusion Medicine, Nagoya University School of Medicine, Nagoya, Japan*
[3]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan*
[4]*Department of Gastroenterology, National Tsuruga Hospital, Tsuruga, Japan*

The prevalence and clinical implications of occult hepatitis B virus (HBV) infection were investigated in the Japanese patients with hemophilia in whom a high prevalence of infection with transfusion-transmissible viruses has been reported. HBV DNA was detected in the sera of 22 of 43 (51.2%) patients with hemophilia who were negative for HBV surface antigen (HBs), indicating that these patients had occult HBV infection. No factor, including age, type or severity of hemophilia, presence of HBs or HBV core (HBc) antibody, or coinfection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) was associated with occult HBV infection, except for high anti-HBc titer and/or coinfection with HCV genotype 1 (1a or 1b). In general, occult HBV infection did not appear to have significant clinical implications. However, in patients in whom HBV was detected by PCR specific for the surface (S)-region, higher alanine aminotransferase levels were observed. The genotype of the occult HBV in the present study was exclusively the domestic type indigenous to Japan (genotype C), suggesting a different route of transmission for HBV in comparison to HCV and HIV in this population. ***J. Med. Virol. 73:195–199, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: hemophilia; hepatitis B virus; occult infection; genotype; route of transmission

INTRODUCTION

Patients with hemophilia are at high risk of infection with parenterally transmissible viruses due to the frequent use of blood products. High prevalence of infection with hepatitis C virus (HCV) [Makris et al., 1990; Troisi et al., 1993], human immunodeficiency virus (HIV) [Tsuchie et al., 1985; Kroner et al., 1994], and GB virus C (GBV-C) [Hanley et al., 1998; Toyoda et al., 1998] has been reported. A few studies have also been carried out on hepatitis B virus (HBV) infection in this population by serological evaluation [Kumar et al., 1993; Goedert et al., 2002].

Recently, several studies have reported occult HBV infection in subjects without HBV surface (HBs) antigen (HBsAg), and its clinical implications are suggested [Cacciola et al., 1999; Brechot et al., 2001; Torbenson and Thomas, 2002]. In addition to a history of frequent use of blood products, a large number of hemophilia patients are infected with HIV, which can result in dynamic changes in the immune status. Reactivation of HBV in association with changes in the immune status can occur and can cause liver damage, which sometimes results in liver failure [Xunrong et al., 2001]. Thus, hemophilia patients are a population in which the assessment of occult HBV infection is important, especially for those patients with HIV.

In the present study, we attempted to clarify the prevalence and clinical importance of occult HBV infection in the Japanese hemophilia patients without HBs antigen. As a result, occult HBV infection was found in around one-half of the patients.

PATIENTS AND METHODS

Patients

Among 44 patients with hemophilia who had been followed-up as outpatients at Nagoya University Hospital and who were admitted regularly to the hospital during 2002, 1 patient had HBs antigen and the other

*Correspondence to: Hidenori Toyoda, MD, PhD, Department of Gastroenterology, Ogaki Municipal Hospital, 4-86 Minaminokawa, Ogaki, Gifu, 503-8502, Japan.
E-mail: tkumada@he.mirai.ne.jp

Accepted 18 February 2004
DOI 10.1002/jmv.20075
Published online in Wiley InterScience (www.interscience.wiley.com)

43 patients were negative for HBs antigen. These 43 patients were enrolled in this study. All were males with a mean age of 34.0 ± 12.1-years-old. Thirty-four patients had hemophilia A, and the remaining 9 had hemophilia B. Thirty-seven patients had severe, 4 patients had moderate, and 2 patients had mild hemophilia. All 24 patients with HIV infection were receiving HAART therapy at the time of sampling serum. Eight of 38 patients with HCV infection had a history of interferon therapy, but no patients were treated with interferon at the time serum was sampled.

Evaluation for Coinfection With HIV and HCV

HIV infection was confirmed by anti-HIV1 antibody detected by particle agglutination (SERODIA-HIV, Fuji Rebio, Tokyo, Japan). Serum HIV RNA concentration was measured by the Amplicor HIV Monitor test (Roche Diagnostics K.K., Tokyo, Japan). The presence of HCV was confirmed by both an HCV antibody assay (2nd generation, Dinabot; Tokyo, Japan), and detection of HCV RNA by nested reverse transcription-polymerase chain reaction (RT-PCR) [Okamoto et al., 1990]. HCV genotypes, according to Simmonds et al. [1994] classification, were determined by RT-PCR with genotype-specific primers [Okamoto et al., 1996]. Serum HCV RNA concentrations were measured by Amplicor Monitor assay (Roche Diagnostics K.K., Tokyo).

Serological Tests for HBV Infection and Detection of HBV DNA

HBV serum markers (HBs antigen, HBs antibody, and HBV core [HBc] antibody) were examined by means of commercial immunoenzyme assays (Abbott Laboratories, North Chicago, IL). For detection of HBV DNA, extracted DNA was amplified by nested touchdown PCR [Don et al., 1991] with three independent primer sets specific for HBV surface (s)–(sense: 5′-CTCTTGTCCTCCAATTTGTCCT-3′ and antisense: 5′-CAGCAAAGCCCAAAAGACCCAC-3′ for the first PCR, and sense: 5′-AGGTA-TGTTGCCCGTTTGTCCT-3′ and antisense: 5′-GGGTTTAAATGTATACCCA-3′ for the second PCR), core (c)–(sense: 5′-ACTGTTCAAGCCTCCAAGCT-3′ for the first and second PCR, antisense: 5′-GGAATACTAACATTGAGATTCCCGAG-3′ for the first PCR, and antisense: 5′-AGTGCGAATCCACACTC-3′ for the second PCR), and X-regions (sense: 5′-TGCCAAGTGTTTGCTGACGC-3′ for the first PCR, sense: 5′-CTGCCGATCCATACTGCGGAAC-3′ for the second PCR, and antisense: 5′-TTCCTGCAGTGGAGACCACCGTGAACG-3′ for the first and second PCR). HBV DNA was amplified from 100 ng of extracted DNA in a total volume of 50 μl, in the presence of 10 pmol of each primer, 125 μM dNTP, and 2.5 U Taq polymerase (Toyobo, Tokyo, Japan). PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR program consisted of 20 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec with a 0.5°C decrease per one cycle (55.5°C at final cycle), and extension at 70°C for 3 min with an initial denaturation at 94°C for 1 min, and a subsequent 20 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 70°C for 3 min with a final extension at 70°C for 10 min. The same PCR program was used for both the first and second PCR amplifications. Amplified PCR products were analyzed by electrophoresis on 1.0% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia, Buckinghamshire, UK). The amplified products were detected by hybridization with a specific probe based on the entire HBV sequences. This probe was generated with a DIG DNA Labeling and Detection kit (Roche Diagnostics, Mannheim, Germany). Results were considered to be valid only if identical results were obtained in at least two separate experiments.

The patients who were positive for both the S- and C-regions were considered to be positive for HBV. The patients who were positive for only one of the two regions examined were then referred to the result of PCR specific for the X-region, and HBV infection was confirmed according to this result.

Genotyping of HBV DNA

Genotyping of occult HBV was performed in seven patients in whom HBV DNA was detected by PCR specific for the S-region. For genotyping of HBV DNA, the PCR product from amplification of the S-region was sequenced directly, and phylogenetic analysis was performed with the neighbor-jointing and bootstrap methods.

The entire protocol was approved by the hospital ethics committee and carried out in compliance with the Helsinki Declaration.

RESULTS

Serological Prevalence of Hepatitis B Viral Markers

Among the 43 patients with hemophilia but without HBs anitgen, HBs antibody was detected in 27 patients (62.8%), and HBc antibody was detected in 37 patients (86.0%). There was no correlation between the type and/or severity of hemophilia and serological prevalence of HBV. There was no association between coinfections with HIV and/or HCV and HBV serological status.

Detection of HBV DNA in Serum

HBV DNA was detected in 22 of the 43 hemophilia patients (51.2%). HBV was detected by PCR specific for the S-region in 7 patients and by PCR specific for the C-region in 19 patients. In 4 patients HBV DNA was detected by both methods. In the 18 patients in whom HBV DNA was detected by only one of the PCR methods, additional PCR targeting the X-region was performed and all patients were positive for this region.

We compared the background, serological markers for HBV, and coinfection status between HBV DNA-positive and -negative patients (Table I). There were no significant differences in the background of patients, including age, and type or severity of hemophilia. Both

TABLE I. Characteristics of Patients With or Without Occult HBV Infection

	HBV DNA (−)	HBV DNA (+)
Number	21	22
Mean age	35.2 ± 10.5	32.8 ± 13.5
Type of hemophilia (A/B)	17/4	17/5
Severity of hemophilia (mild/moderate/severe)	1/1/19	1/3/18
HBs-antibody (+/−)	14/7	13/9
HBc-antibody (+/−)	17/4	20/2
HBc-antibody titer#	129.4 (39.1–897.8)*	291.3 (80.4–914.5)*
HIV (+/−)	13/8	11/11
HIV RNA concentration (copies/μl)##	7.6 (0.2–35)	0.4 (0.2–0.5)
Under limit of quantitation sensitivity	7 (53.8%)	9 (81.8%)
CD4+ cell count##	449.9 ± 300.1	577.7 ± 272.6
HCV (+/−)	20/1	18/4
HCV RNA concentration (copies/μl)###	383.5 (17–810)	498.8 (85–830)
HCV genotype (1a/1b/2a/2b/3a/4a)###	3/3/3/2/6/3**	10/3/1/2/2/0**
Serum ALT levels (IU/L)###	57.1 (12–208)	71.8 (10–209)

HBV, hepatitis B virus; HBs, hepatitis B viral surface; HBc, hepatitis B viral core; HIV, human immunodeficiency virus; HCV, hepatitis C virus; ALT, alanine aminotransferase.
#Only in patients with positive HBc-antibody.
##Only in patients with HIV coinfection.
###Only in patients with HCV coinfection.
*$P = 0.0476$ by Mann–Whitney U test.
**$P = 0.0230$ for 1a or 1b versus other genotypes by Chi-square test.

the rates of positive HBs antibody and HBc antibody were similar between HBV DNA-positive and -negative patients. The rates of both HIV and HCV coinfection were similar regardless of occult HBV infection. In addition, when compared in combination with HBs and HBc antibody, or in combination with HIV and HCV, there was no difference in the rate of patients with HBV DNA (Table II). In patients with positive HBc antibody, however, the antibody titer was higher in HBV DNA-positive patients than in HBV DNA-negative patients ($P = 0.0476$, Mann–Whitney U test). In patients with HCV, HCV genotype 1 (1a or 1b) was significantly more prevalent in patients with HBV DNA than in patients without HBV DNA ($P = 0.0230$, Chi-square test).

In all five patients without HCV coinfection, serum alanine aminotransferase (ALT) levels were continuously normal regardless of occult infection with HBV. In patients with HCV infection, there was no significant difference in serum ALT level which was calculated as the average value of four to six analyses over 1 year, between HBV DNA-positive and -negative patients. When this comparison was restricted to patients with HBV detectable by PCR specific for the S-region only, serum ALT levels in HBV DNA-positive patients were significantly higher than those in HBV DNA-negative patients (patients with HBV, 120.3 ± 66.6 vs. patients without HBV, 57.1 ± 44.0; $P = 0.0162$, Mann–Whitney U test).

Genotype of Occult HBV

HBV genotyping was carried out based on the sequence of the S-region in seven patients in whom HBV DNA was detected by PCR specific for the S-region. Genotype C, which is the major genotype observed in the Japanese patients with chronic hepatitis B without hemophilia, was detected in all seven patients.

DISCUSSION

The clinical significance of occult HBV infection for patients with chronic hepatitis C has been described in recent reports [Cacciola et al., 1999; Sagnelli et al., 2001] and remains controversial [Kao et al., 2002]. These reports consider the influence of HBV occult infection on advanced liver disease [Cacciola et al., 1999; Sagnelli et al., 2001], development of hepatocellular carcinoma [Sheu et al., 1992; Paterlini et al., 1993], and reduced response to interferon [Zignego et al., 1997; Cacciola et al., 1999]. The importance of HBV occult infection has been reported in immunosuppressive patients, even in those without HCV coinfection [Xunrong et al., 2001]. In these patients, reactivation of HBV caused liver damage and sometimes resulted in liver failure.

Patients with hemophilia are at high risk of exposure to transfusion-transmissible virus such as HIV, HBV, HCV, and GBV-C. The high prevalence of infection with HIV [Tsuchie et al., 1985; Kroner et al., 1994], HCV

TABLE II. Rate of Hepatitis B Virus DNA Detection (%)

HBsAb(+) HBcAb(+)#	HBsAb(+) HBcAb(−)	HBsAb(−) HBcAb(+)	HBsAb(−) HBcAb(−)
13/25 (52.0)	0/2 (0)	7/12 (58.3)	2/4 (50.0)
HIV(+) HCV(+)##	HIV(+) HCV(−)	HIV(−) HCV(+)	HIV(−) HCV(−)
11/21 (52.4)	3/3 (100)	10/17 (58.8)	1/2 (50.0)

#HBsAb, hepatitis B viral surface antibody; HBcAb, hepatitis B viral core antibody.
##HIV, human immunodeficiency virus; HCV, hepatitis C virus.

[Makris et al., 1990; Troisi et al., 1993], and GBV-C [Hanley et al., 1998; Toyoda et al., 1998] has been reported in many studies. The status of serological markers on HBV infection has also been reported [Kumar et al., 1993; Goedert et al., 2002]. However, occult HBV infection in this population has not been examined. Because a large number of patients with hemophilia have HIV infection and changes in immune status in these patients can occur partly due to the disease itself and partly to the effect of HAART therapy, clarification of the status of occult HBV infection in these patients is important because of the potential for reactivation of occult HBV in association with changes in immune status.

HBV DNA was detected in serum in around one-half of the patients. The rate of detection was similar to that of HBV DNA detected in serum of the Japanese patients without hemophilia who have chronic HCV infection [Fukuda et al., 1999]. Neither the severity of hemophilia nor coinfection with HIV and HCV indicated the potential for occult HBV infection. In a previous study, Nunez et al. [2002] found no HIV-infected patients (most were intravenous drug users) in whom occult HBV infection could be confirmed. In contrast, we confirmed occult HBV infection in 11 HIV-infected patients. Only a high HBc antibody titer, which has already been reported to be an indicator of occult HBV infection [Nirei et al., 2000], and HCV genotype 1 (1a or 1b) in patients coinfected with HCV may indicate the high risk of occult HBV coinfection.

On the basis of our results, occult HBV infection appears to have no significant clinical impact when the infection is evaluated by the HBV detection for the C-region. On the contrary, occult HBV may increase serum ALT levels, which indicates severe liver damage, in patients with HCV infection when HBV DNA is positive by PCR for the S-region. Further study will be required to clarify the difference in clinical significance of HBV occult infection between PCR positive for the C-region and that positive for the S-region.

The HBV genotype detected in the Japanese patients with hemophilia was exclusively genotype C, which is the most common genotype in Japan. This shows the distinct characteristics of occult HBV infection in hemophilia patients in Japan, which are different from those of other transfusion-transmissible viruses in this population. The genotypes of viruses such as HCV or GBV-C in the Japanese hemophilia patients are foreign and not domestic genotypes [Kinoshita et al., 1993; Toyoda et al., 1998]. This is because, in this population, transmission of these viruses has been by imported blood products, as well as HIV transmission in this population [Tsuchie et al., 1985]. In contrast, only the domestic HBV genotype was found in the Japanese hemophilia patients. This, together with the lack of difference in the prevalence of occult HBV infection between hemophilia patients with HCV and HCV-infected patients who have not undergone repeated transfusions in Japan, suggests a route of transmission of this virus, different from that in cases of HIV, HCV, and GBV-C infection. The lack of correlation in the rate of coinfection between HIV or HCV and HBV also supports this suggestion. Screening for HBV in blood donors using HBs antigen as a marker started in 1973 in Japan. Some patients have a history of blood transfusion, which may have caused the occult HBV infection. Nosocomial infection in relation to the injection of blood products through the repeated use of needles, syringes, or other medical instruments, which could have occurred under medical conditions in Japan prior to the 1970s, might have also played a role.

In summary, among 43 Japanese patients with hemophilia, occult HBV infection was observed in about one-half of patients without detectable HBs antigen, a prevalence similar to that of the Japanese patients with chronic HCV infection. Occult HBV infection did not have significant clinical implications as a whole, although patients in whom HBV was detected with S-region-specific PCR showed higher ALT levels. The HBV genotype was exclusively a domestic type, suggesting a different route of transmission of HBV from that of HCV or HIV in this population. Further studies are required to determine occult HBV infection in the Japanese patients with hemophilia.

REFERENCES

Brechot C, Thiers V, Kremsdorf D, Nalpas B, Pol S, Paterlini-Brechot P. 2001. Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: Clinically significant or purely "occult?" Hepatology 34:194–203.

Cacciola I, Pollicino T, Souadrito G, Cerenzia G, Orlando ME, Raimondo G. 1999. Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. N Engl J Med 341:22–26.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 1991. Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008.

Fukuda R, Ishimura N, Niigaki M, Hamamoto S, Satoh S, Tanaka S, Kushiyama Y, Uchida Y, Ihihara S, Akagi S, Watanabe M, Kinoshita Y. 1999. Serologically silent hepatitis B virus coinfection in patients with hepatitis C virus-associated chronic liver disease: Clinical and virological significance. J Med Virol 58:201–207.

Goedert JJ, Eyster ME, Lederman MM, Mandalaki T, de Moerloose P, White GC II, Angiolillo AL, Luban NLC, Sherman KE, Manco-Johnson M, Preiss L, Leissinger C, Kessler CM, Cohen AR, DiMichele D, Hilgartner MW, Aledort LM, Kroner BL, Rosenberg PS, Hatzakis A—For the Multicenter Hemophilia Cohort Study. 2002. End-stage liver disease in persons with hemophilia and transfusion-associated infections. Blood 100:1584–1589.

Hanley JP, Jarvis LM, Hayes PC, Lee AJ, Simmonds P, Ludlam CA. 1998. Patterns of hepatitis G viraemia and liver disease in haemophiliacs previously exposed to non-virus inactivated coagulation factor concentrates. Thromb Haemost 79:291–295.

Kao J-H, Chen P-J, Lai M-Y, Chen D-S. 2002. Occult hepatitis B virus infection and clinical outcomes of patients with chronic hepatitis C. J Clin Microbiol 40:4068–4071.

Kinoshita T, Miyake K, Okamoto H, Mishiro S. 1993. Imported hepatitis C virus genotypes in Japanese hemophiliacs. J Infect Dis 168:249–250.

Kroner BL, Rosenberg PS, Aledort LM, Alvord WG, Goedert JJ. 1994. HIV-1 infection incidence among persons with hemophilia in the United States and western Europe, 1978–1990. Multicenter Hemophilia Cohort Study. J Acquir Immune Defic Syndr Hum Retrovirol 7:279–286.

Kumar A, Kulkarni R, Murray DL, Gera R, Scott-Emuakpor AB, Bosma K, Penner JA. 1993. Serologic markers of viral hepatitis A, B, C, and D in patients with hemophilia. J Med Virol 41:205–209.

Makris M, Preston FE, Triger DR, Underwood JCE, Choo QL, Kuo G, Houghton M. 1990. Hepatitis C antibody and chronic liver disease in haemophilia. Lancet 335:1117–1119.

Nirei K, Kaneko M, Moriyama M, Arakawa Y. 2000. The clinical features of chronic hepatitis C are not affected by the coexistence of hepatitis B virus DNA in patients negative for hepatitis B surface antigen. Intervirology 43:95–101.

Nunez M, Rios P, Perez-Olmeda M, Soriano V. 2002. Lack of 'occult' hepatitis B virus infection in HIV-infected patients. AIDS 16:2099–2101.

Okamoto H, Okada S, Sugiyama Y, Tanaka T, Sugai Y, Akahane Y, Machida A, Mishiro S, Yoshizawa H, Miyakawa Y, Mayumi M. 1990. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5′-noncoding region. Jpn J Exp Med 60:215–222.

Okamoto H, Kobata S, Tokita H, Inoue T, Woodfield GD, Holland PV, Al-Knawy BA, Uzunalimoglu O, Miyakawa Y, Mayumi M. 1996. A second-generation method of genotyping hepatitis C virus by the polymerase chain reaction with sense and antisense primers deduced from the core gene. J Virol Methods 57:31–45.

Paterlini P, Driss F, Nalpas B, Pisi E, Franco D, Berthelot P, Brechot C. 1993. Persistence of hepatitis B and hepatitis C viral genomes in primary liver cancers from HbsAg-negative patients: A study of a low-endemic area. Hepatology 17:20–29.

Sagnelli E, Coppola N, Scolastico C, Mogavero AR, Filippini P, Piccinino F. 2001. HCV genotype and 'silent' HBV coinfection: Two main risk factors for a more severe liver disease. J Med Virol 64:350–355.

Sheu JC, Huang GT, Shih LN, Lee WC, Chou HC, Wang JT, Lee PH, Lai MY, Wang CY, Yang PM, Lee HS, Chen DS. 1992. Hepatitis C and hepatitis B viruses in hepatitis B surface antigen-negative hepatocellular carcinoma. Gastroenterology 103:1322–1327.

Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan S-W, Chayama K, Chen D-S, Choo Q-L, Colombo M, Cuypers HTM, Date T, Dusheiko GM, Esteban JI, Fay O, Hadziyannis SJ, Han J, Hatzakis A, Holmes EC, Hotta H, Houghton M, Irvine B, Kohara M, Kolberg JA, Kuo G, Lau JYN, Lelie PN, Maertens G, McOmish F, Miyamura T, Mizokami M, Nomoto A, Prince AM, Reesink HW, Rice C, Roggendorf M, Schalm SW, Shikata T, Shimotohno K, Stuyver L, Trepo C, Weiner A, Yap PL, Urdea M. 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 19:1321–1324.

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

Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H, Okamoto H. 1998. GB virus C/hepatitis G virus isolates in Japanese haemophiliacs and their origins. Thromb Haemost 80:242–245.

Troisi CL, Hollinger FB, Hoots WK, Contant C, Gill J, Ragni M, Parmley R, Sexauer C, Gomperts E, Buchanan G. 1993. A multicenter study of viral hepatitis in a United States hemophilic population. Blood 81:412–418.

Tsuchie H, Kawatani T, Nakayama E, Matsui T, Kurimura T, Hinuma Y. 1985. Distribution of the level of antibody to AIDS-associated virus (LAV) in sera from AIDS or AIDS related complex and Japanese hemophiliacs infected with AIDS-associated virus. Microbiol Immunol 29:1083–1087.

Xunrong L, Yan AW, Liang R, Lau GKK. 2001. Hepatitis B virus (HBV) reactivation after cytotoxic or immunosuppressive therapy-pathogenesis and management. Rev Med Virol 11:287–299.

Zignego AL, Fontana R, Puliti S, Barbagli S, Monti M, Careccia G, Giannelli F, Giannini C, Buzzelli G, Brunetto MR, Bonino F, Gentilini P. 1997. Relevance of inapparent co-infection by hepatitis B virus in alpha interferon-treated patients with hepatitis C virus chronic hepatitis. J Med Virol 51:313–318.