Fig. 2. Virus production of JFH-1/S2 chimeric constructs in HuH-7 cells. One million cells were transfected with 10 μg in vitro-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean ± SD.

infected HCV RNA titer among these inoculated mice. Sequence analyses of the complete open reading frames revealed that infecting JFH-1/wt virus and variant strains had no nonsynonymous mutations at the time of development of infection. From these data, we concluded that not only JFH-1/wt virus but also JFH-1/S1, JFH-1/S2, and JFH-1/C viruses were able to establish productive infection in human hepatocyte-transplanted mice.

Apoptosis Induction Assay. To investigate the survival strategy against the host defense system, we examined the susceptibility of JFH-1/wt and variant strains to TNF-α–mediated apoptosis induction. After transfection with *in vitro*–transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, Huh-7.5.1 cells were exposed to TNF-α plus actinomycin D. Without exposure, apoptosis was observed in a limited number of HCV-positive cells (Supporting Fig. 2A). Forty-eight hours later, cells were harvested, fixed, and
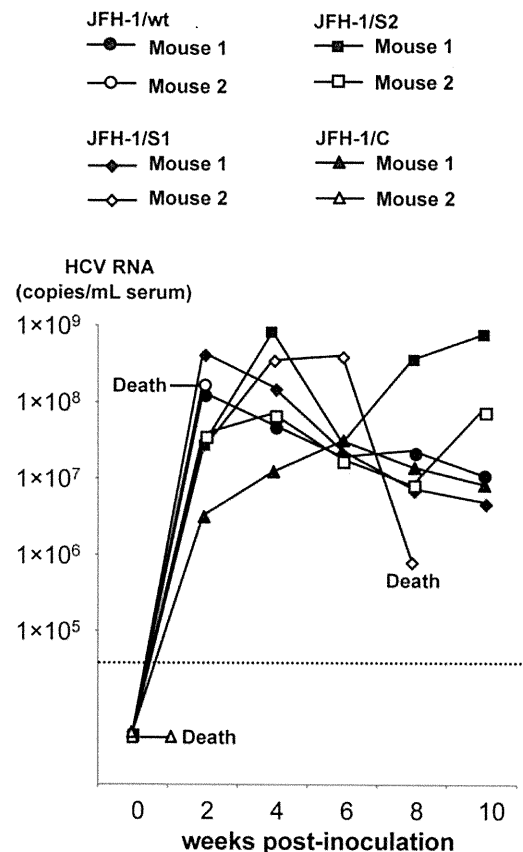

Fig. 3. *In vivo* infection study of JFH-1/wt and its variants in human hepatocyte-transplanted mice. Cell culture medium containing 1 × 10⁷ HCV RNA copies of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C were inoculated into human hepatocyte-transplanted mice, and HCV RNA levels in mice serum were monitored.

hepatocyte-transplanted mice. Two mice were used for each virus. Two weeks after intravascular inoculation, all mice but one became HCV RNA–positive (Fig. 3). Two mice died 3 weeks after inoculation; one was inoculated with JFH-1/wt and had developed infection, and the other was inoculated with JFH-1/C and died without developing infection. HCV RNA levels in infected mice fluctuated, ranging from 10^6 to 10^9 copies/mL. We could not observe much difference of

subjected to terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and anti-HCV NS5A staining. The effects of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C transfection on apoptosis induction were determined by calculating the ratio of apoptosis between HCV-positive and HCV-negative populations and expressed as an apoptosis induction index. After treatment of JFH-1/wt–transfected cells with TNF-α, apoptosis was observed in 36.8% of the HCV-positive population and in 19.3% of the HCV-negative population, and the apoptosis induction index was 1.85 ± 0.06 (Fig. 4). The apoptosis induction indexes of JFH-1/S1–transfected and JFH-1/C– transfected cells were 1.23 ± 0.06 and 1.16 ± 0.10, respectively, suggesting lower susceptibility to apoptosis induction compared with JFH-1/wt. On the other hand, the apoptosis induction index of JFH-1/S2 was 0.74 ± 0.17, which was substantially lower than that of JFH-1/wt, demonstrating the more reduced apoptosis in the cells harboring this strain. Similar results were obtained by treatment with FasL plus actinomycin D (Supporting Fig. 2B). To confirm the lower susceptibility of JFH-1/S2–transfected cells, apoptosis was also detected by staining with anticleaved poly(adenosine diphosphate ribose) polymerase (PARP) antibody. The apoptosis induction indexes of JFH-1/wt and JFH-1/S2–transfected cells were 2.28 ± 0.24 and 1.15 ± 0.14, respectively, and were consistent with TUNEL assay (Fig. 5). Although the HCV NS5A-positive rate in JFH-1/S2–transfected cells was higher than that in JFH-1/wt, the mean fluorescence intensity of the NS5A-positive population in JFH-1/S2–transfected cells was significantly lower (185.0 ± 8.7) than that in JFH-1/wt–transfected cells (395.0 ± 98.0), corresponding to the observed phenotype of the JFH-1/S2 strain in the single cycle virus production assay (i.e., lower replication efficiency and rapid spread to surrounding cells).

To clarify the genomic region responsible for lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, we examined the effect of TNF-α on the cells carrying subgenomic reporter replicons. The apoptosis induction index of SGR-JFH1/Luc/S2–transfected cells was lower than that of SGR-JFH1/Luc/wt–transfected cells (Supporting Fig. 2C); however, the difference was not as pronounced as with full-genome constructs, indicating that mutations in the NS3-NS5B region contribute to lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, but they are not sufficient to explain the difference between JFH-1/wt and JFH-1/S2. We confirmed these results by use of the chimeric
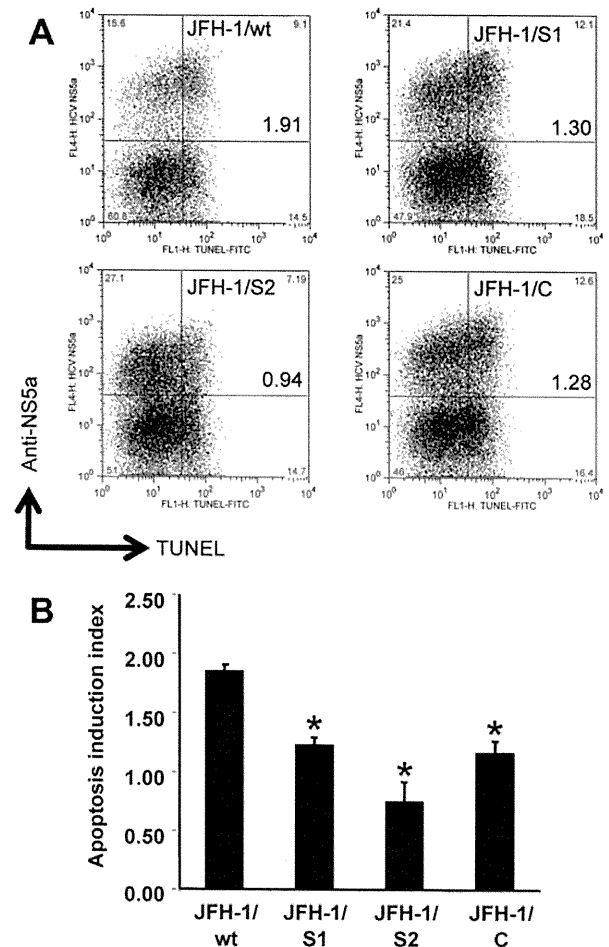

Fig. 4. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt and its variants. (A) Three million cells were transfected with 3 μg in vitro-transcribed full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. Forty-eight hours later, apoptosis was induced by exposing cells to 20 ng/mL TNF-α plus 50 ng/mL actinomycin D. Cells were harvested after 48 hours of treatment and subjected to TUNEL and anti-HCV NS5A staining. Dot plots show HCV replication and apoptosis at the single cell level. Quadrant gates were determined using unstained and a terminal deoxynucleotidyltrasferase-untreated control in each culture condition. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S1-, JFH-1/S2-, and JFH-1/C- transfected cells. The mean ± SD of three independent experiments is shown. *P < 0.005 versus JFH-1/wt.

constructs JFH-1/S2-wt and JFH-1/wt-S2. The apoptosis induction indexes of JFH-1/S2-wt–transfected and JFH-1/wt-S2–transfected cells were 1.42 ± 0.13 and 1.71 ± 0.08, respectively (Fig. 5). These data indicate that both structural and nonstructural regions of JFH-1/S2 were associated with lower susceptibility to cytokine-induced apoptosis, although mutations in core-NS2 seemed to have higher contribution toward this phenotype. Together, these results indicate that the JFH-1/S2 strain, which was selected after passage in
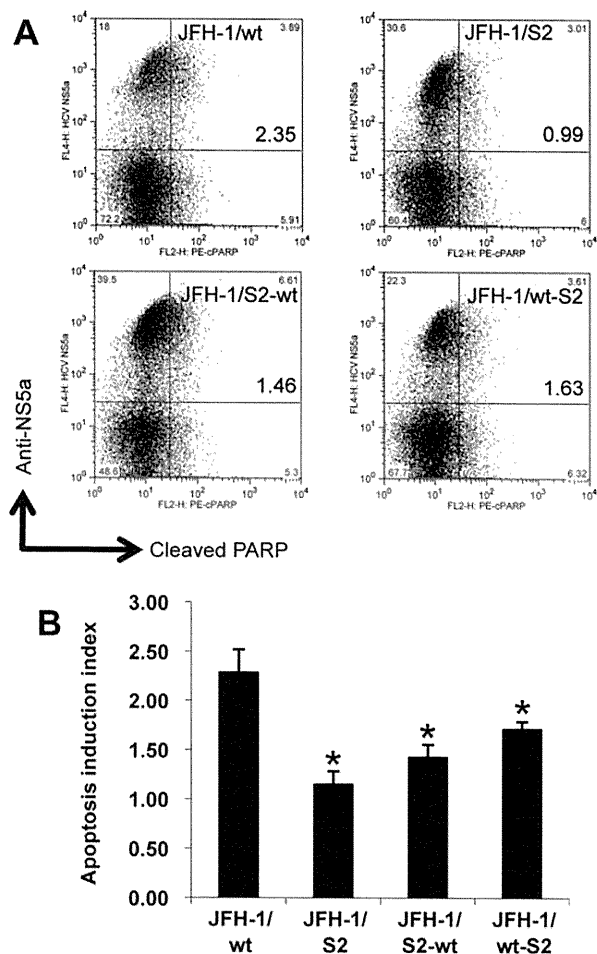
Fig. 5. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt, JFH-1/S2, and their chimeric constructs. (A) Three million cells were transfected with 3 μg in vitro-transcribed full-genome RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. Apoptosis was induced by exposing cells to 20 ng/mL TNF-α plus 50 ng/mL actinomycin D and detected by anticleaved PARP staining. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S2-, JFH-1/S2-wt-, and JFH-1/wt-S2-transfected cells. The mean ± SD of three independent experiments is shown. *$P < 0.05$ versus JFH-1/wt.

the patient serum–infected chimpanzee, acquired less susceptibility to the cytokine-induced apoptosis.

Discussion

HCV develops chronic infection in the vast majority of infected patients[1]; however, the mechanisms of its persistence are still under investigation. Many viruses have evolved different strategies to cope with host immune systems, thus causing the development of persistent infection. For example, some viruses interfere with the major histocompatibility complex class I presentation of viral antigens, whereas others modulate

lymphocyte and macrophage functions, including cytokine production.[12-16] In our previous study, we detected an increasing number of mutations in the HCV genome isolated from JFH-1 patient serum–infected chimpanzees. Thus, we reasoned that these detected mutations might have imparted some advantage to this virus for long-time survival. To examine this hypothesis, we compared the phenotypes of JFH-1 variant strains emerged at early and late stages of infection in JFH-1 patient serum–infected and JFH-1cc–infected chimpanzees and found that the JFH-1/S2 strain isolated from the patient serum–infected chimpanzee at a later time point of infection replicated slowly, produced more infectious viruses, and displayed reduced susceptibility to cytokine-induced apoptosis.

The JFH-1 variant strain JFH-1/C, which contains seven nonsynonymous mutations identified in the JFH-1cc–infected chimpanzee at week 7, showed comparatively slower replication kinetics and slightly enhanced infectious virus production in cell culture. The intracellular specific infectivity of this strain in Huh7-25 cells was 3.9 times higher than that of JFH-1/wt (Table 1). These characteristics might have imparted some advantage to this strain for establishing productive infection in the chimpanzee. The other JFH-1 variant strains, JFH-1/S1 and JFH-1/S2, contain 6 and 17 nonsynonymous mutations identified in the JFH-1 patient serum–infected chimpanzee at weeks 2 and 23 postinfection, respectively. Replication kinetics and infectious virus production of the JFH-1/S1 strain were comparable to that of JFH-1/wt in cultured cells (Fig. 1, Table 1). In contrast, the JFH-1/S2 strain showed lower replication efficiency. Although the intracellular HCV RNA level of this strain in Huh7-25 cells was lower than that of JFH-1/wt and JFH-1/S1, and almost the same as that of JFH-1/C (Table 1), intracellular specific infectivity was 18.0 and 12.9 times higher than that of JFH-1/wt and JFH-1/S1, respectively, suggesting a significant increase in the assembly of infectious virus particles ($P < 0.005$, Table 1). The enhanced capacity of this strain to assemble infectious virus particles resulted in a higher extracellular infectivity titer that contributed to the rapid spread of virus to surrounding cells. Flow cytometry analyses of cells transfected with JFH-1/wt and variant strains revealed that the percentage of the HCV NS5A-positive population in JFH-1/S2–transfected cells was higher, but the mean fluorescence intensity of the anti-NS5A signal was lower than that in JFH-1/wt–transfected cells, thus confirming higher spread and lower replication of this strain. Taken together, both JFH-1/C and JFH-1/S2 exhibited a tendency toward

decreased replication and increased infectious virus production. However, the extent of enhanced virus production was substantially lower in JFH-1/C than in JFH-1/S2, which might have led to the earlier elimination of infection in the JFH-1cc–infected chimpanzee. In other words, the potency of infectious virus production and spread seems to correspond to the duration of infection in infected animals.

The association between a lower replication efficiency and persistent infection is still unclear. It has been reported that an escape mutant with an amino acid substitution at the cytotoxic T lymphocyte (CTL) epitope in the NS3 region exhibits lower NS3/ 4 protease activity and replication capacity *in vitro*.[17,18] The JFH-1/S2 strain contains the T1077A mutation in the NS3 region (Supporting Table 1), and this mutation is located close to mutations reported to be associated with immune evasion and lower replication.[17] Thus, the lower replication efficiency of the JFH-1/S2 strain may be a result of an immune escape mutation at the expense of viral fitness. Meanwhile, we cannot deny the advantage of lower replication in establishing persistent infection. Lower replication may contribute to the avoidance of major histocompatibility class I–mediated antigen presentation and to escape from the host immune system. Either way, by acquiring the ability to produce more viral particles, the JFH-1/S2 strain could rapidly spread to surrounding cells, irrespective of its lower replication efficiency. Importantly, these emerged mutations did not attenuate *in vivo* infectivity, unlike cell culture adaptive mutations reported to cause attenuated infection *in vivo*.[19] Upon inoculation into human hepatocyte–transplanted mice, JFH-1/S1, JFH-1/S2, and JFH-1/C strains could establish infection without any mutations, produced levels of viremia similar to JFH-1/wt, and persisted for a similar observed period of infection (Fig. 2). This observation is different from that in chimpanzees, where JFH-1/ wt and JFH-1/C strains were eliminated earlier than JFH-1/S2. In contrast to chimpanzees, human hepatocyte–transplanted mice lack a CTL and natural killer (NK) cell–mediated immune system, which could be responsible for this difference.[6] Taken together, our results suggest that along with efficient infectious virus production, the JFH-1/S2 strain might have acquired an advantage that helps it evade the CTL and NK cell–mediated immune system.

Apoptosis of virus-infected cells by the immune system is crucial as a general mechanism of clearing infections.[20,21] The J6/JFH-1 chimeric virus has been reported to exhibit proapoptotic characteristics in cell culture.[22] However, because HCV needs to escape the host immune system in order to establish chronic infection, immune cell–mediated apoptosis may be inhibited in infected hepatocytes. In the liver, HCV-infected hepatocytes are eliminated by targeted apoptosis induced by NK cells, macrophages, and CTLs with ligand-mediated and receptor-mediated signals such as TNF-α, FasL, and TNF-related apoptosis–inducing ligand.[23-26] Thus, we used TNF-α to mimic natural immunomediated apoptosis and found that the JFH-1/S2–replicating cells have lower susceptibility to the apoptosis induced by these cytokines. In JFH-1/S2– transfected cells, TNF-α–induced apoptosis detected by TUNEL assay was substantially lower than that of JFH-1/wt–transfected cells (Fig. 4). We confirmed it by staining with anticleaved PARP. In complete agreement with the results produced by way of TUNEL assay, the number of anticleaved PARP stained cells among JFH-1/S2–infected cells was significantly lower than that among JFH-1/wt–infected cells (Fig. 5). In our previous study, we reported that HCV-specific immune responses with T cell proliferation and interferon-γ production were maintained until the disappearance of viremia in the patient serum–infected chimpanzee.[11] This finding indicates that continuous selection pressure in the infected chimpanzee might have contributed to the emergence of a clone with an ability to escape the cytokine-induced apoptosis. We are not sure whether this phenotype of JFH-1/S2 is due to its lower replication efficiency and thus lower production of HCV proteins. The accumulation of viral proteins might predispose cells to the apoptosis induced by TNF-α. To answer this question, it will be necessary to investigate the genomic regions of JFH-1/ S2 and cellular host factors responsible for the ability of this strain to escape the apoptosis.

By way of mapping analysis for JFH-1/S2, we could determine responsible regions; NS5B was for lower replication efficiency (Supporting Fig. 1B), and P7 and NS2 were for enhanced viral particle assembly (Supporting Table 2). For the evasion of apoptosis, we could not specify the responsible region, because both chimeric constructs, JFH-1/S2-wt and JFH-1/wt-S2, showed less susceptibility to cytokine-induced apoptosis to a certain extent. These data indicate that both structural and nonstructural regions might have contributed to the acquisition of this phenotype. Previously, a potent antiapoptotic effect of the HCV NS5A protein was described.[27] NS5A interacts with Bin1, which is a nucleocytoplasmic c-Myc–interacting protein with tumor suppressor and apoptotic properties, thus inhibiting Bin1-

associated apoptosis. Because JFH-1/S2 contains several mutations in the NS5A region (Supporting Table 1), one or more mutations in this protein may be associated with antiapoptotic effects.

In conclusion, we demonstrated that the JHF-1/S2 strain acquired phenotypes of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis. These phenotypes were associated with mutations that emerged 23 weeks after infection in a chimpanzee, and might have contributed to long-term infection *in vivo*. Such control of viral functions by specific mutations may be a key viral strategy to establish persistent infection.

References

1. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000;132:296-305.
2. Feld JJ, Liang TJ. Hepatitis C—identifying patients with progressive liver injury. HEPATOLOGY 2006;43:S194-S206.
3. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 2001;194:1395-1406.
4. Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, et al. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc Natl Acad Sci U S A 2002;99: 15661-15668.
5. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927-933.
6. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901-912.
7. Kato T, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, et al. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J Med Virol 2001;64:334-339.
8. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
9. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
10. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
11. Kato T, Choi Y, Elmowalid G, Sapp RK, Barth H, Furusaka A, et al. Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation. HEPATOLOGY 2008;48:732-740.
12. Johannessen I, Crawford DH. In vivo models for Epstein-Barr virus (EBV)-associated B cell lymphoproliferative disease (BLPD). Rev Med Virol 1999;9:263-277.
13. Oglesbee MJ, Pratt M, Carsillo T. Role for heat shock proteins in the immune response to measles virus infection. Viral Immunol 2002;15: 399-416.
14. Stevenson PG, Boname JM, de Lima B, Efstathiou S. A battle for survival: immune control and immune evasion in murine gamma-herpesvirus-68 infection. Microbes Infect 2002;4:1177-1182.
15. Alcami A. Viral mimicry of cytokines, chemokines and their receptors. Nat Rev Immunol 2003;3:36-50.
16. Wilkinson GW, Tomasec P, Stanton RJ, Armstrong M, Prod'homme V, Aicheler R, et al. Modulation of natural killer cells by human cytomegalovirus. J Clin Virol 2008;41:206-212.
17. Soderholm J, Ahlen G, Kaul A, Frelin L, Alheim M, Barnfield C, et al. Relation between viral fitness and immune escape within the hepatitis C virus protease. Gut 2006;55:266-274.
18. Uebelhoer L, Han JH, Callendret B, Mateu G, Shoukry NH, Hanson HL, et al. Stable cytotoxic T cell escape mutation in hepatitis C virus is linked to maintenance of viral fitness. PLoS Pathog 2008;4: e1000143.
19. Bukh J, Pietschmann T, Lohmann V, Krieger N, Faulk K, Engle RE, et al. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc Natl Acad Sci U S A 2002;99:14416-14421.
20. Kagi D, Seiler P, Pavlovic J, Ledermann B, Burki K, Zinkernagel RM, et al. The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. Eur J Immunol 1995;25:3256-3262.
21. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, et al. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science 1994;265:528-530.
22. Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, et al. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 2008;82:10375-10385.
23. Kafrouni MI, Brown GR, Thiele DL. Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. J Immunol 2001;167:1566-1574.
24. Guicciardi ME, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. Gut 2005;54:1024-1033.
25. Fischer R, Baumert T, Blum HE. Hepatitis C virus infection and apoptosis. World J Gastroenterol 2007;13:4865-4872.
26. Stegmann KA, Bjorkstrom NK, Veber H, Ciesek S, Riese P, Wiegand J, et al. Interferon-alpha-induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection. Gastroenterology 2010; 138:1885-1897.
27. Nanda SK, Herion D, Liang TJ. The SH3 binding motif of HCV NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. Gastroenterology 2006;130:794-809.

Viral hepatitis

ORIGINAL ARTICLE

Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

Yoko Tamada,[1,2] Hiroshi Yatsuhashi,[1,2] Naohiko Masaki,[3] Makoto Nakamuta,[4] Eiji Mita,[5] Tatsuji Komatsu,[6] Yukio Watanabe,[7] Toyokichi Muro,[8] Masaaki Shimada,[9] Taizo Hijioka,[10] Takeaki Satoh,[11] Yutaka Mano,[12] Toshiki Komeda,[13] Masahiko Takahashi,[14] Hiroshi Kohno,[15] Hajime Ota,[16] Shigeki Hayashi,[17] Yuzo Miyakawa,[18] Seigo Abiru,[1,2] Hiromi Ishibashi[1,2]

For numbered affiliations see end of article.

Correspondence to
Professor Hiroshi Yatsuhashi, Clinical Research Center, NHO National Nagasaki Medical Center and Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Address: 2-1001-1 Kubara, Omura, Nagasaki 856-8562, Japan; yatsuhashi@nmc.hosp.go.jp

ABSTRACT

Objective To examine recent trends of acute infection with hepatitis B virus (HBV) in Japan by nationwide surveillance and phylogenetic analyses.
Methods During 1991 through 2009, a sentinel surveillance was conducted in 28 national hospitals in a prospective cohort study. Genotypes of HBV were determined in 547 patients with acute hepatitis B. Nucleotide sequences in the preS1/S2/S gene of genotype A and B isolates were determined for phylogenetic analyses.
Results HBV genotype A was detected in 137 (25% (accompanied by genotype G in one)) patients, B in 48 (9%), C in 359 (66%), and other genotypes in the remaining three (0.5%). HBV persisted in five with genotype A including the one accompanied by genotype G; another was co-infected with HIV type 1. The genotype was A in 4.8% of patients during 1991—1996, 29.3% during 1997—2002, and 50.0% during 2003—2008 in the capital region, as against 6.5%, 8.5% and 33.1%, respectively, in other regions. Of the 114 genotype A isolates, 13 (11.4%) were subgenotype A1, and 101 (88.6%) were A2, whereas of the 43 genotype B isolates, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. Sequences of 65 (64%) isolates of A2 were identical, as were three (23%) of A1, and five (18%) of B2, but none of the B1, B3 and B4 isolates shared a sequence.
Conclusions Acute infection with HBV of genotype A, subgenotype A2 in particular, appear to be increasing, mainly through sexual contact, and spreading from the capital region to other regions in Japan nationwide. Infection persisted in 4% of the patients with genotype A, and HBV strains with an identical sequence prevailed in subgenotype A2 infections. This study indicates the need for universal vaccination of young people to prevent increases in HBV infection in Japan.

Significance of this study

What is already known about this subject?
▶ In Japan, a national prevention programme was started in 1986 with selective vaccination of babies born to mothers who carry hepatitis B virus (HBV). Since then, the prevalence of hepatitis B surface antigen among younger generations has decreased sharply.
▶ However, retrospective studies indicate that the frequency of HBV genotype A is increasing among patients with acute hepatitis B (AHB) within the capital region of Japan.
▶ Infection with genotype A more often persists than infection with other genotypes.
▶ Because there is no reliable and comprehensive surveillance system for AHB in Japan, the incidence of AHB and factors responsible for changes over many years are not known.

What are the new findings?
▶ This is a prospective cohort study for surveillance of AHB throughout Japan in a national research programme.
▶ The incidence of AHB in Japan has not decreased, because genotype A infections have increased over time.
▶ Genotype A infections started to increase in the capital region of Japan, and then spread to other regions 5—6 years later.
▶ About 90% of genotype A found in AHB patients in Japan is subgenotype A2.
▶ Subgenotype A2 isolates from patients with AHB tend to preserve sequence identity over time, indicating that particular subgenotype A2 strains have been transmitted without undergoing mutations.

Hepatitis B virus (HBV) has been classified into 10 genotypes, designated A—J, based on a >8% divergence in the full-genome sequence.[1–7] Different genotypes are associated with distinct clinical manifestations, such as severity and progression of liver disease, as well as response to antiviral treatments.[8–10] Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)[11–13];

218

Significance of this study

How might it impact on clinical practice in the foreseeable future?

► It needs to be noted that subgenotype A2 infections are spreading among sexually active generations in Japan.
► Although selective vaccination has prevented mother-to-baby transmission of HBV since 1986, it does not contain sporadic infections in Japan.
► Herd vaccination of younger generations needs to be considered in Japan.

B into B1 (Japanese type) and B2 (Asian type)[14][15]; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).[16] Subgenotypes also influence the replication of HBV and clinical manifestation.[15][17][18]

According to a report from Japan in 2001,[19] genotype C was the most prevalent (84.7%), followed by genotype B (12.2%) and A (1.7%), among patients with chronic hepatitis B. In 2002, genotype A became the most prevalent in patients with acute hepatitis B (AHB) around Tokyo, the capital region of Japan.[20][21] Several reports have shown that infection with HBV genotype A is associated with particular sexual behaviours, such as homosexual activity and promiscuous sexual contacts, and tends to persist longer than that with HBV genotype C.[22][23] These reports have raised concerns about the horizontal HBV infection in adults, which, in general, is considered to resolve spontaneously. However, adult-acquired HBV infection may result in chronic HBV infection in some instances.

Information on changes in genotype distribution over time, as well as genotype-specific clinical manifestations, may help in planning preventive measures and antiviral therapy strategies. Therefore it is important to examine how genotype A infection has spread in Japan, and what clinical and virological characteristics it possesses.

We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions of HBV genotypes/subgenotypes were surveyed, and their influence on clinical outcome was evaluated.

PATIENTS AND METHODS
Patients

A total of 681 patients with sporadic AHB were enrolled consecutively in a survey carried out by the Japan National Hospital Acute Hepatitis Study Group (JNHAHSG). They were admitted to 28 national hospitals from January 1991 to the end of December 2009. They were grouped geographically into two areas: the capital region (Gunma, Saitama, Tokyo and Kanagawa) and other regions (figure 1). Patients were also longitudinally categorised into three periods: 1st (1991—1996), 2nd (1997—2002) and 3rd (2003—2008). In addition, the year 2009 provided the most recent data. Of the 681 patients, 547 (80.3%) entered the study, for whom serum samples were available on admission and had been stored at −20°C.

The diagnosis of AHB was based on the following criteria: (1) acute onset of liver injury without a history of liver dysfunction; (2) detection of hepatitis B surface antigen (HBsAg) in the
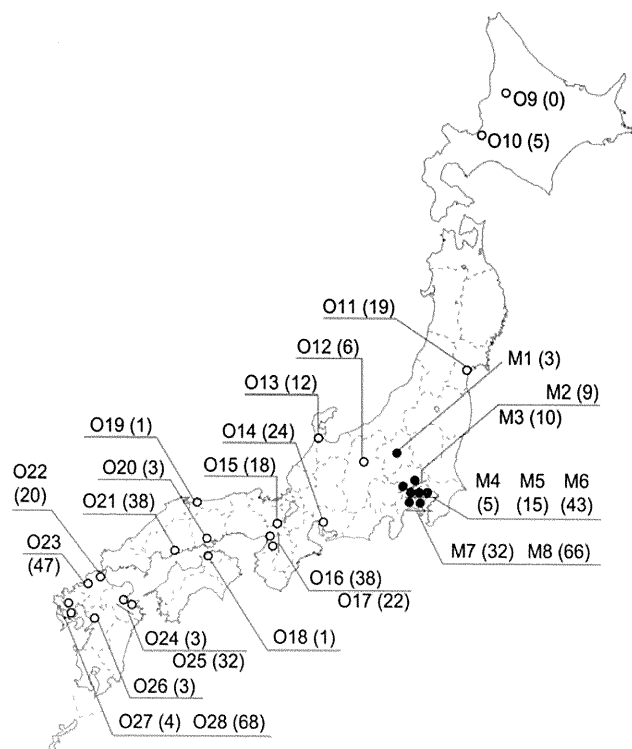

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1—M8) are indicated by eight closed circles, and those in other regions (O9—O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma Hospital, Gunma; M2, Nishisaitama-Chuo Hospital, Saitama; M3, National Disaster Medical Center, Tokyo; M4, Tokyo Hospital, Tokyo; M5, Tokyo Medical Center, Tokyo; M6, National Center for Global Health and Medicine, Tokyo; M7, Sagamihara Hospital, Kanagawa; M8, Yokohama Medical Center, Kanagawa; O9, Asahikawa Medical Center, Hokkaido; O10, Hokkaido Medical Center, Hokkaido; O11, Sendai Medical Center, Miyagi; O12, Matsumoto Medical Center, Nagano; O13, Kanazawa Medical Center, Ishikawa; O14, Nagoya Medical Center, Aichi; O15, Kyoto Medical Center, Kyoto; O16, Osaka National Hospital, Osaka; O17, Osaka-Minami Medical Center, Osaka; O18, Zentsuji Hospital, Kagawa; O19, Yonago Medical Center, Tottori; O20, Okayama Medical Center, Okayama; O21, Kure Medical Center and Chugoku Cancer Center, Hiroshima; O22, Kokura Medical Center, Fukuoka; O23, Kyushu Medical Center, Fukuoka; O24, Beppu Medical Center, Oita; O25, Oita Medical Center, Oita; O26, Kumamoto Medical Center, Kumamoto; O27, Ureshino Medical Center, Saga; and O28, Nagasaki Medical Center, Nagasaki.

serum; (3) positivity for IgM antibody to HBV-core antigen (IgM anti-HBc) in high titres (detectable in sera diluted 10-fold); and (4) absence of past or family history of chronic HBV infection. Severe acute hepatitis (SAH) was defined as prothrombin time (PT) $\leq$40% and hepatic encephalopathy of grade $\leq$I. Fulminant hepatitis (FH) was diagnosed from PT $\leq$40% and hepatic encephalopathy of grade $\geq$II. Patients in whom HBsAg remained in the serum for >6 months after onset were considered to have acquired chronic HBV infection. The following information was collected from each patient: year and age at onset, gender, residential area, HBsAg, IgM anti-HBc, alanine aminotransferase, total bilirubin, PT, severity of liver disease, mortality, routes of transmission, sexual behaviours, travelling abroad in recent past, HBV genotype, mutations in precore (PreC) and core promoter (CP) regions, and RNA of hepatitis D virus. Antibody to HIV type 1 (anti-HIV) was

determined in patients who were at high risk and gave consent to testing.

Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the Ministry of Education, Culture, Sports Science and Technology of Japan, and was approved by the ethics committee of each institution.

Extraction of HBV DNA

HBV DNA was extracted from serum (100 μl) by the SMITEST EX-R&D Nucleic Acid Extraction Kit (MBL Co, Nagoya, Japan) and used for genotyping/subgenotyping and detecting mutations in PreC and CP regions.

HBV genotypes

Genotypes were determined in Nagasaki Medical Center with the SMITEST HBV Genotyping Kit (MBL) by hybridisation with type-specific probes immobilised on a solid-phase support.[24]

Determination of HBV subgenotypes

For subgenotyping, HBV DNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Shiga, Japan). PCR was performed with appropriate nested primers to amplify a ~1.2 kb sequence in the preS1/S2/S gene (nucleotides 2854–835 in the reference isolate (AB116077)). PCR products were purified, subjected to cycle sequencing reaction with the BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan), and applied to the DNA sequencer (3100-Avant; Applied Biosystems).

Mutations in the PreC and CP regions

The A1896 mutation in the PreC region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA; Roche Diagnostics, Tokyo, Japan), and mutations in the CP region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit; Roche Diagnostics). The results were recorded as 'wild-type' and 'mutant types' dominantly expressed by HBV isolates.[25]

Phylogenetic analyses

Nucleotide sequences were aligned, and phylogenetic trees were constructed by the CLUSTAL W program v1.83 (DDBJ homepage: http://clustalw.ddbj.nig.ac.jp/top-j.html). The statistical validity was assessed by bootstrap resampling with 1000 replicates. Reference HBV strains were retrieved from the GenBank database.

Statistical analysis

Results were expressed as percentage or mean±SD. Statistical differences were evaluated by χ^2 and Fisher exact tests for categorical variables, and analysis of variance and Scheffe's test for quantitative variables, using the SPSS software. The 95% CI, for the difference in means, was calculated in analyses for quantitative variables. $p<0.05$ was considered significant.

RESULTS

Distribution of HV genotypes

HBV genotypes were determined in the 547 patients with AHB. The genotype was A in 137 (25.0%) patients (accompanied by G in one (0.2%)), B in 48 (8.8%), C in 359 (65.6%), D in one (0.2%), E in one (0.2%), and H in one (0.2%). Because HBV genotype G is a defective virus and cannot replicate by itself,[26 27] the single patient with mixed genotypes A and G was included in the 137 patients with genotype A in further analyses. RNA of hepatitis

D virus was detected in three of the 453 (0.7%) patients. Anti-HIV was examined in patients at high risk of infection and detected in 14 of the 53 (26.4%) who gave consent to testing.

Demographic and clinical differences among patients infected with HBV of distinct genotypes

Demographic and clinical characteristics of patients with different genotypes are compared in table 1. There was no difference in mean age among patients with genotypes A, B and C. The proportion of men was higher in patients with genotype A than B or C (94.2% vs 79.2%, $p<0.05$; or 56.0%, $p<0.0001$), and in those with genotype B than C (79.2% vs 56.0%, $p<0.05$).

Maximum levels of total bilirubin were higher in patients with genotype A than C (9.6±7.6 vs 7.1±6.2 mg/dl, $p<0.05$), with a difference of 2.5 mg/dl (95% CI 0.93 to 4.08), whereas the highest alanine aminotransferase activity and lowest PT values did not differ among patients with distinct genotypes.

SAH developed in four (2.9%) patients with genotype A, four (8.3%) with genotype B, and 26 (7.2%) with genotype C. FH developed in one (2.1%) patient with genotype B and eight (2.2%) with genotype C; no patients with genotype A developed FH. Eight (1.5%) patients died, including one with genotype B and seven with genotype C. There were no significant differences among patients with different genotypes in the frequency of SAH or FH or mortality.

The outcome of AHB was traceable in 514 of the 547 (94.0%) patients. Chronic infection with persistence of HBsAg for >6 months developed in five of the 123 (4.1%) patients with genotype A (including the one accompanied by genotype G), none of the 46 (0%) with genotype B, and none of the 342 (0%) with genotype C; it was more common in patients with genotype A than C ($p<0.05$). HBV infection persisted exclusively in the patients with genotype A, either alone (four patients) or together with genotype G (one).

Among the five patients who acquired chronic HBV infection, four (three with genotype A and one with mixed genotypes A and G) were examined for anti-HIV, and one with genotype A was found to be positive. HBV infection persisted in three (including the one with anti-HIV) of the five patients for >1 year after the onset, and the remaining two (both without anti-HIV) cleared HBsAg from the serum after retaining it for >6 months.

Mutations in the PreC and/or CP region were detected in 3.7% (4/109) of patients with genotype A, 15.4% (6/39) of those with genotype B, and 25.5% (79/310) of those with genotype C. They were significantly less common in patients with genotype A than B or C (A vs B, $p<0.05$; A vs C, $p<0.0001$). The only patient with genotype A who had the PreC mutation was simultaneously infected with genotype G.

Routes of transmission were identifiable in 275 of the 547 (50%) patients, and the main route was heterosexual contacts; those in the remaining patients could not be disclosed. The frequency of heterosexual activity did not differ among patients with distinct genotypes. However, homosexual activity was more common in patients with genotype A than B or C (21.2%, 0% and 0.8%, respectively (A vs B, $p<0.001$; A vs C, $p<0.0001$)). Among the 32 homosexual men, HBV genotype A was detected in 29 (91%). Consent to anti-HIV testing was given by 10 of the 29 patients, and four of these (40%) were positive.

Longitudinal changes in the distribution of genotypes

Figure 2 illustrates changes in the distribution of HBV genotypes through three 6-year periods over 18 years (1991–2008). In addition, data from 2009 are shown. HBV genotype A accounted

Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with HBV of different genotypes (1991–2009)

Feature	Total (n=547)	HBV genotypes			
		A (n=137)† (25.0%)	B (n=48) (8.8%)	C (n=359) (65.6%)	Others (n=3)‡ (0.5%)
Age (years)	35.6±14.8	35.2±12.2	39.6±15.6	35.1±15.5	49.7±13.6
Male	367 (67.1%)	129 (94.2%)¶ * †† ***	38 (79.2%)†† *	201 (56.0%)	3 (100%)
ALT (IU/l)§	2553±1563	2289±1069	2557±1412	2342±1728	3333±2406
T-Bil (mg/dl)§	7.8±6.7	9.6±7.6††*	7.7±7.4	7.1±6.2	9.0±2.5
PT (%)§	74.6±22.6	75.2±15.9	73.8±24.5	74.7±24.5	15.8‡‡
Severe hepatitis	34 (6.2%)	4 (2.9%)	4 (8.3%)	26 (7.2%)	0 (0.0%)
Fulminant hepatitis	10 (1.8%)	0 (0.0%)	1 (2.1%)	8 (2.2%)	1 (33.3%)
Mortality	8 (1.5%)	0 (0.0%)	1 (2.1%)	7 (1.9%)	0 (0.0%)
HBsAg persisting >6 months	5/514 (1.0%)	5/123 (4.1%)†† *	0/46 (0.0%)	0/342 (0%)	0/3 (0.0%)
PreC/CP mutations					
PreC	43/461 (9.3%)	1/109 (0.9%)¶ * †† *	6/39 (15.4%)	34/310 (11.0%)	2/3 (66.7%)
CP	69/461 (15.0%)	3/109 (2.8%)†† ***	0/39 (0.0%)†† *	63/310 (20.3%)	3/3 (100%)
PreC and/or CP	92/461 (20.0%)	4/109 (3.7%)¶ * †† ***	6/39 (15.4%)	79/310 (25.5%)	3/3 (100%)
Transmission route					
Homosexual	32 (5.9%)	29 (21.2%)¶ ** †† ***	0 (0.0%)	3 (0.8%)	0 (0.0%)
Heterosexual	217 (39.5%)	52 (38.0%)	25 (52.1%)	139 (39.6%)	1 (33.3%)
Medical procedure	16 (2.9%)	2 (1.5%)	2 (4.2%)	12 (3.3%)	0 (0.0%)
Other	10 (1.8%)	1 (0.7%)	1 (2.1%)	7 (1.9%)	1 (33.3%)
Undetermined	272 (49.7%)	53 (38.7%)†† *	20 (41.7%)	198 (55.2%)	1 (33.3%)
Anti-HIV	14/53 (26.4%)	11/35 (31.4%)	0/3 (0.0%)	3/15 (20.0%)	0/0

Values are mean±SD or number (%).
†One patient with genotype A was simultaneously infected with genotype G.
‡Each patient was infected with genotype D, E or H.
§Highest values during the clinical course are shown for ALT and T-Bil, and lowest values for PT.
Statistical analysis was performed to compare genotypes A, B and C.
¶Significantly different compared with genotype B.
††Significantly different compared with genotype C.
*p<0.05, **p<0.001, ***p<0.0001.
‡‡Data from the patient with genotype E only.
ALT, alanine aminotransferase; CP, core promoter; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PreC, precore; PT, prothrombin time; T-Bil, total bilirubin.

for 6% (9/150) in the 1st period, 15.4% (19/123) in the 2nd, and 39.4% (89/226) in the 3rd, with significant differences between 1st and 2nd (p<0.05), 2nd and 3rd (p<0.0001), and 1st and 3rd (p<0.0001). Conversely, AHB associated with genotype C decreased through three periods with significant differences, while AHB associated with genotype B did not change appreciably.

On the basis of these results, the yearly incidence in each of the three 6-year periods is calculated to be: 25.0 cases including 1.5 with genotype A in the 1st period; 20.5 cases including 3.2 with genotype A in the 2nd; and 37.7 cases including 14.8 with genotype A in the 3rd. Hence, the incidence of AHB had not changed markedly over the 12 years from 1991 to 2002, but increased thereafter until 2008. Of the increment in the 3rd period of 17.2 (37.7 minus 20.5) cases, there were 11.6 (14.8 minus 3.2) with genotype A; they accounted for 67% (11.6/17.2) of the recent increase in AHB.

Regional distributions and longitudinal changes in genotype A

Among the 183 patients from the capital region, the genotype was A in 65 (35.5%), B in 22 (12.0%), C in 94 (51.4%), E in one (0.5%), and H in one (0.5%) (table 2). Of the remaining 364 (66.5%) patients from other regions, by contrast, the genotype was A in 72 (19.8%), B in 26 (7.1%), C in 265 (72.8%), and D in one (0.3%). Genotype A was significantly more common in the capital than in other regions (35.5% vs 19.8%, p<0.0001). In the capital region, genotype A accounted for 4.8% (2/42) in the 1st period, 29.3% (12/41) in the 2nd, and 50.0% (42/84) in the 3rd. There were significant differences between the 1st and 2nd periods (p<0.05), 2nd and 3rd (p<0.05), and 1st and 3rd (p<0.0001). In other regions, by contrast, genotype A accounted for 6.5% (7/108) in the 1st period, 8.5% (7/182) in the 2nd, and

33.1% (47/142) in the 3rd. For the first time in other regions, genotype A increased in the 3rd period, in comparison with the 1st and 2nd (1st vs 3rd, p<0.0001; 2nd vs 3rd, p<0.0001).
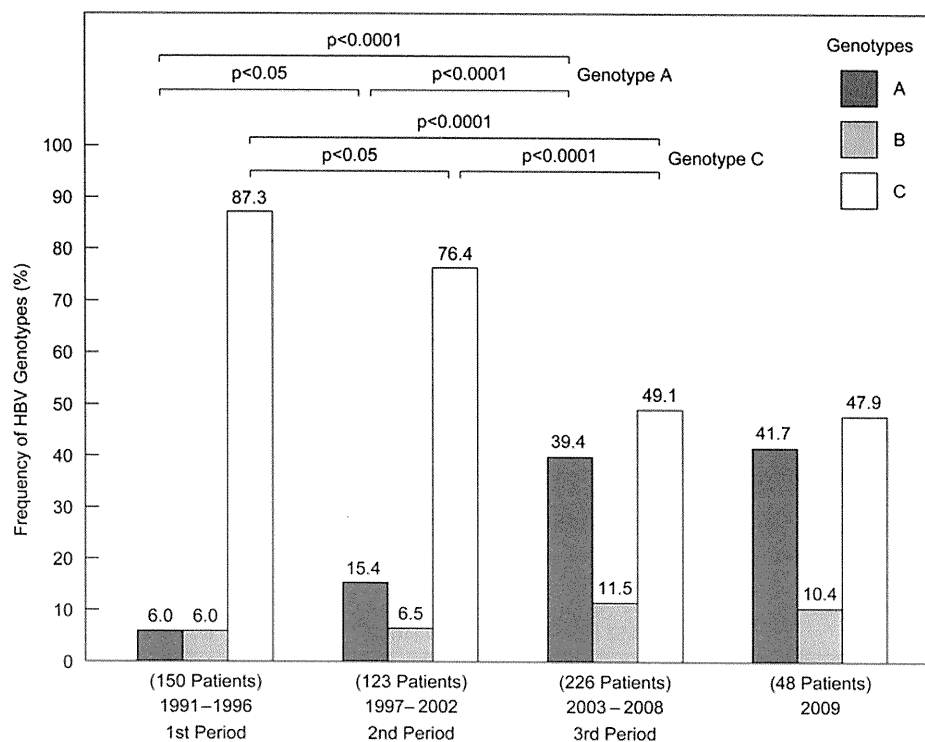
Subgenotypes of genotype A

Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype A isolates retrieved from the database (figure 3).

Of the 114 isolates in this study, 101 (88.6%) were subgenotype A2, and the remaining 13 (11.4%) were subgenotype A1. In a pair-wise comparison, the sequence divergence among the 101 subgenotype A2 isolates was 0–1.3%, and that among the 13 subgenotype A1 isolates spanned 0% to 2.3%. The sequence divergence between subgenotype A2 and A1 isolates ranged from 2.6% to 4.7%.

A sequence of 1203 nucleotides was possessed in common by three of the 101 (3%) isolates of subgenotype A2. For convenience, the group comprising these three isolates was labelled 'identical group I'. Likewise, an additional six 'identical groups' were found, and numbered from 'II' to 'VII'. They comprised 35 (35%), seven (7%), two (2%), three (3%), 12 (12%) and three (3%) of the 101 isolates of subgenotype A2. In contrast, only one identical group, designated 'VIII', was constructed by three of the 13 (23%) isolates of subgenotype A1.

Some isolates of subgenotype A1 and A2 were obtained from patients who had travelled to foreign countries in the recent past (5/13 (38.5%) patients with A1 to Africa, Philippines, Myanmar and China; and 5/101 (5.0%) patients with A2 to Europe, Thailand, Brazil and the USA).

221

Figure 2 Distribution of hepatitis B
virus (HBV) genotypes in three periods.

Subgenotypes of genotype B

Of the 48 isolates of genotype B, subgenotyping was feasible in
43 (90.0%). A phylogenetic tree was constructed on preS1/S2/S-
gene sequences from these 43 isolates, along with those from 25
isolates of genotype B retrieved from the database (figure 4). Of
the 43 isolates in this study, 10 (23.3%) were subgenotype B1, 28
(65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4.
In a pair-wise comparison, the sequence divergence among 10
subgenotype B1 isolates ranged from 0.4% to 1.4%, and that
among 28, two and three isolates of subgenotypes B2, B3 and
B4 spanned 0—1.7%, 0.5% and 0.6—0.8%, respectively. The
inter-subgenotype divergence among B1—B4 ranged from 0.6%
to 4.4%.

One 'identical group' made up of five isolates was detected
among the 28 of subgenotype B2; it was named 'IX'. In contrast,
no 'identical group' was found in 10, two or three isolates of
subgenotype B1, B3 or B4.

Some isolates of subgenotypes B2, B3 and B4 were obtained
from patients who had travelled to foreign countries in the
recent past (7/28 (25.0%) patients with B2 to China and other
countries; 1/2 (50.0%) patients with B3 to a country unknown;
and 1/3 (33.3%) patients with B4 to Vietnam). However, none of
the 10 subgenotype B1 isolates was associated with travel to
foreign countries.

Identical groups

The proportion of isolates that shared a sequence in identical
groups was higher for subgenotype A2 (64.4%) than for A1, B1,
B2, B3 or B4 (23.1%, 0%, 17.9%, 0% or 0%, respectively (A2 vs
A1, $p<0.001$; A2 vs B1, $p<0.0001$; A2 vs B2, $p<0.0001$)).

Homosexual activity was more common in patients belonging
to the seven identical groups than the non-identical group of
subgenotype A2 (17/65 (26.2%) vs 3/36 (8.3%), $p<0.05$). Among
the isolates in the seven identical groups of subgenotype A2,
those in groups I, III and VII clustered locally during short
periods of 2—7 years. In contrast, subgenotype A2 isolates in
groups II and VI were scattered widely over longer periods of
11—16 years.

DISCUSSION

In Japan, as in most Asian countries, the persistent HBV carrier
state had been established mainly through perinatal trans-
mission from mother to baby and horizontal infection during
infancy. In 1986, a national prevention programme was
launched in Japan with selective vaccination of babies born to
carrier mothers with hepatitis B e antigen (HBeAg). In 1995, this
was extended to babies born to HBeAg-negative carrier mothers.
As a result, the prevalence of HBsAg among younger people born
since 1986 has decreased dramatically.[28][29] However, there are an

Table 2 Changes in the distribution of genotype A compared between the capital region and other regions over three periods

Area	n	1st Period (1991—1996)	2nd Period (1997—2002)	3rd Period (2003—2008)	2009
Capital region	65/183 (35.5%)†***	2/42 (4.8%)‡* §***	12/41 (29.3%)†* §*	42/84 (50.0%)†*	9/16 (56.3%)
Other regions	72/364 (19.8%)	7/108 (6.5%)§***	7/82 (8.5%)§***	47/142 (33.1%)	11/32 (34.4%)
Total	137/547 (25.0%)	9/150 (6.0%)‡* §***	19/123 (15.4%)§***	89/226 (39.4%)	20/48 (41.7%)

Statistical analysis of the differences between the capital and other regions was performed, as well as through the 1st, 2nd and 3rd periods.
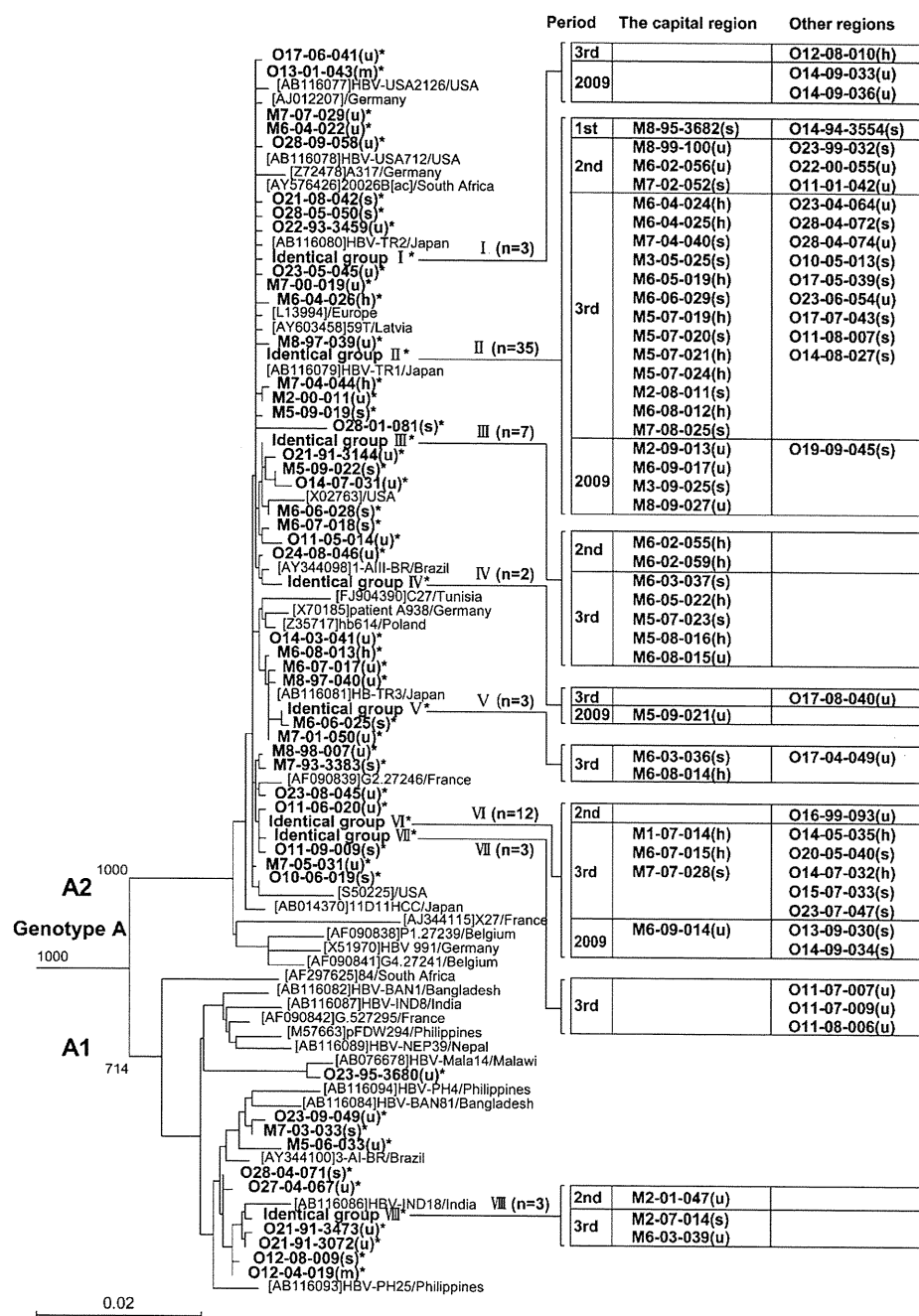†Significantly different compared with other regions.
‡Significantly different compared with the 2nd period.
§Significantly different compared with the 3rd period.
*$p<0.05$, ***$p<0.0001$.

222

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1–M8 and those in other regions O9–O28 (corresponding to those in figure 1). Year of onset is indicated by the last two digits after the first hyphen. Numbers after the second hyphen represent the identification numbers of patients in each year (not always consecutive). Transmission routes are shown in lower-case letters in parentheses: h, homosexual; s, heterosexual; m, medical procedure; o, others; and u, undetermined. Isolates with identical sequences are bracketed in 'Identical groups I through VIII' on the tree. Each bracket is divided by areas and periods. Reference hepatitis B virus (HBV) isolates, including 12 of subgenotype A1 and 22 of subgenotype A2, were obtained from the database and specified by their accession numbers, isolate names and countries of origin. Bootstrap values are indicated on major phylogenetic branches.

Phylogenetic tree tip labels (subgenotype A2 and A1 clades):

O17-06-041(u)*; O13-01-043(m)*; [AB116077]HBV-USA2126/USA; [AJ012207]/Germany; M7-07-029(u)*; M6-04-022(u)*; O28-09-058(u)*; [AB116078]HBV-USA712/USA; [Z72478]A317/Germany; [AY576426]20026B[ac]/South Africa; O21-08-042(s)*; O28-05-050(s)*; O22-93-3459(u)*; [AB116080]HBV-TR2/Japan — Identical group I * — I (n=3); O23-05-045(u)*; M7-00-019(u)*; M6-04-026(h)*; [L13994]/Europe; [AY603458]59T/Latvia; M8-97-039(u)* — Identical group II * — II (n=35); [AB116079]HBV-TR1/Japan; M7-04-044(h)*; M2-00-011(u)*; M5-09-019(s)*; O28-01-081(s)* — Identical group III* — III (n=7); O21-91-3144(u)*; M5-09-022(s)*; O14-07-031(u)*; [X02763]/USA; M6-06-028(s)*; M6-07-018(s)*; O11-05-014(u)*; O24-08-046(u)*; [AY344098]1-AIII-BR/Brazil — Identical group IV* — IV (n=2); [FJ904390]C27/Tunisia; [X70185]patient A938/Germany; [Z35717]hb614/Poland; O14-03-041(u)*; M6-08-013(h)*; M6-07-017(u)*; M8-97-040(u)*; [AB116081]HB-TR3/Japan — Identical group V* — V (n=3); M6-06-025(s)*; M7-01-050(u)*; M8-98-007(u)*; M7-93-3383(s)*; [AF090839]G2.27246/France; O23-08-045(u)*; O11-06-020(u)* — Identical group VI* — VI (n=12); Identical group VII* — VII (n=3); O11-09-009(s)*; M7-05-031(u)*; O10-06-019(s)*; [S50225]/USA; [AB014370]11D11HCC/Japan; [AJ344115]X27/France; [AF090838]P1.27239/Belgium; [X51970]HBV 991/Germany; [AF090841]G4.27241/Belgium; [AF297625]84/South Africa; [AB116082]HBV-BAN1/Bangladesh; [AB116087]HBV-IND8/India; [AF090842]G.527295/France; [M57663]pFDW294/Philippines; [AB116089]HBV-NEP39/Nepal; [AB076678]HBV-Mala14/Malawi; O23-95-3680(u)*; [AB116094]HBV-PH4/Philippines; [AB116084]HBV-BAN81/Bangladesh; O23-09-049(u)*; M7-03-033(s)*; M5-06-033(u)*; [AY344100]3-AI-BR/Brazil; O28-04-071(s)*; O27-04-067(u)*; [AB116086]HBV-IND18/India — Identical group VIII* — VIII (n=3); O21-91-3473(u)*; O21-91-3072(u)*; O12-08-009(s)*; O12-04-019(m)*; [AB116093]HBV-PH25/Philippines

A2 — 1000; Genotype A — 1000; A1 — 714

Scale: 0.02

Period	The capital region	Other regions
3rd		O12-08-010(h)
2009		O14-09-033(u) · O14-09-036(u)
1st	M8-95-3682(s)	O14-94-3554(s)
2nd	M8-99-100(u) · M6-02-056(u) · M7-02-052(s)	O23-99-032(s) · O22-00-055(u) · O11-01-042(u)
3rd	M6-04-024(h) · M6-04-025(h) · M7-04-040(s) · M3-05-025(s) · M6-05-019(h) · M6-06-029(s) · M5-07-019(h) · M5-07-020(s) · M5-07-021(h) · M5-07-024(h) · M2-08-011(s) · M6-08-012(h) · M7-08-025(s)	O23-04-064(u) · O28-04-072(s) · O28-04-074(u) · O10-05-013(s) · O17-05-039(s) · O23-06-054(u) · O17-07-043(s) · O11-08-007(s) · O14-08-027(s)
2009	M2-09-013(u) · M6-09-017(u) · M3-09-025(s) · M8-09-027(u)	O19-09-045(s)
2nd	M6-02-055(h) · M6-02-059(h)	
3rd	M6-03-037(s) · M6-05-022(h) · M5-07-023(s) · M5-08-016(h) · M6-08-015(u)	
3rd		O17-08-040(u)
2009	M5-09-021(u)	
3rd	M6-03-036(s) · M6-08-014(h)	O17-04-049(u)
2nd		O16-99-093(u)
3rd	M1-07-014(h) · M6-07-015(h) · M7-07-028(s)	O14-05-035(h) · O20-05-040(s) · O14-07-032(h) · O15-07-033(s) · O23-07-047(s)
2009	M6-09-014(u)	O13-09-030(s) · O14-09-034(s)
3rd		O11-07-007(u) · O11-07-009(u) · O11-08-006(u)
2nd	M2-01-047(u)	
3rd	M2-07-014(s) · M6-03-039(u)	

estimated one million HBV carriers in Japan at present.[30] Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and no national surveillance data are available in Japan, the incidence has not been determined accurately. In the USA, the incidence of AHB has decreased markedly since the adoption of a comprehensive immunisation strategy in 1991.[31 32]

In the present study over 1991–2009, we conducted a nationwide, sentinel surveillance on AHB in Japan. In the 547 patients recruited over 19 years, genotype C was the most prevalent (65.6%), followed by genotype A (25.0%) and genotype B (8.8%). Demographic and clinical differences were observed among patients with genotypes A, B and C (table 1).

The proportion of men reached 94.2% for genotype A infection, higher than that for genotype B (79.2%) or C (56.0%) infection. In the analysis of the route of transmission, homosexual activity was reported by 21.2% of patients with genotype A; all were male. In general, sexual activity tends to be higher in men than women. The predominance of genotype A in men may be attributable to a high frequency of homosexual activity among men.

Although adult-acquired HBV infection persists at a high frequency of ~10% in European countries and the USA,[33] it rarely, if ever, becomes chronic in Japan. Recent studies suggest that the chance of a chronic outcome of AHB may differ by HBV genotype[21 34]; it is more common for genotype A than other genotypes.[22 35 36] In the present study, HBV infection persisted in 4.1% of patients with genotype A, in comparison with 0% of

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 reference isolates of subgenotype B1 and 13, two and two of those of B2, B3 and B4, respectively, were retrieved from the database; they are specified as in figure 3.
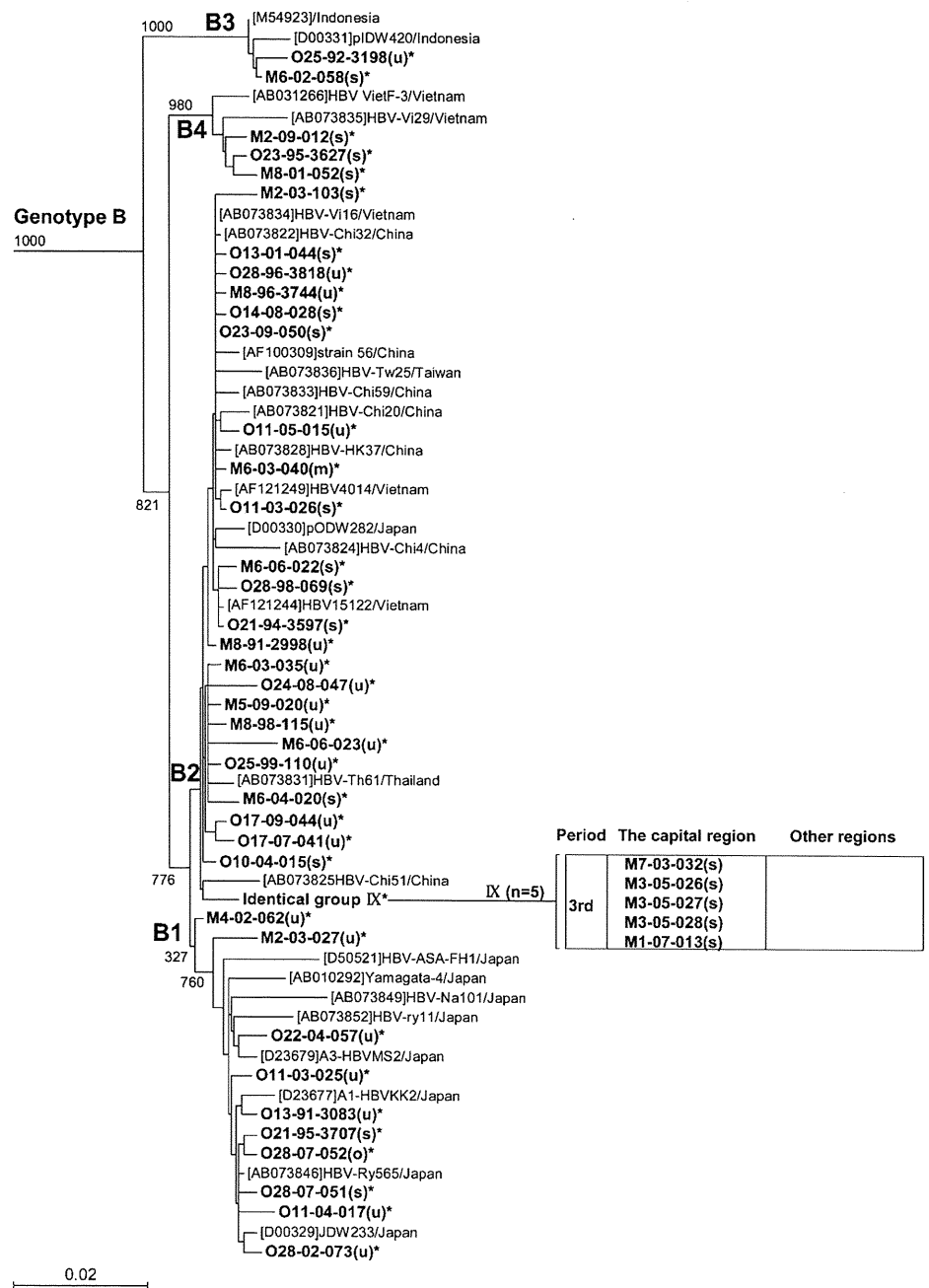
those with genotype C. Remarkably, all five patients with AHB who acquired chronic infection possessed HBV genotype A, either alone (four patients) or together with HBV genotype G (one). Increasing genotype A infections may have changed the genotype distribution in patients with AHB and those with chronic HBV infection. In Japanese patients with chronic hepatitis B, the proportion of genotype A has doubled, from 1.7% in 1999–2000 to 3.5% in 2005–2006.[37]

The genotype was A in 29 of the 32 (91%) homosexual men. Of the 29 homosexuals with genotype A, 10 gave consent to anti-HIV testing, and four of these (40%) were found to be positive. Of the five patients who acquired chronic HBV infection, anti-HIV was tested in four (three with genotype A and one with genotypes A and G), and one with genotype A was found to be positive. There is a possibility that co-infecting HIV in this patient with genotype A may have promoted chronic

HBV infection; HIV is known to prolong and aggravate HBV infection by compromising immune responses.[38]

Patients with FH in this study were infected with either HBV genotype B (1/48 (2.1%)) or C (8/359 (2.2%)); no patients with genotype A developed FH. PreC and/or CP mutations were significantly less common in genotype A (1/109 (3.7%)) than B (6/39 (15.4%)) or C (279/310 (5.5%)) infection. The single patient with genotype A who had PreC mutation was simultaneously infected with HBV genotype G. There is a possibility that the PreC mutation in this patient was from HBV genotype G.[26] FH did not develop in any patients with genotype A, which may be attributable, at least in part, to the lack of PreC mutation in genotype A infections.[39]

Previous reports have shown that genotype A is common in patients with AHB in Metropolitan Tokyo,[20] [21] [40] as well as around Aichi located in the middle of Mainland Japan.[22]

Yotsuyanagi et al[23] reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi et al[41] found that, in patients with AHB, the proportion with genotype A has increased over time. The present study indicates that the number of patients with AHB in Japan would not have decreased. We found that the proportion of patients with genotype A infection is increasing in the 28 national hospitals in Japan (6.0% in the 1st period, 15.4% in the 2nd, and 39.4% in the 3rd (figure 2)), with the prevalence much higher in the capital than other regions (35.5% vs 19.8% (table 2)).

In this study, there was a time lag in the increase in genotype A infection between the capital region and other regions of Japan (table 2). In the capital region, the prevalence of genotype A started to increase in the late 1990s, and kept increasing through the early 2000s (4.8% in the 1st period, 29.3% in the 2nd, 50.0% in the 3rd, and 56.3% in 2009). In other regions, by contrast, the frequency of genotype A did not change during the late 1990s, and increased significantly in the 2000s (6.5% in the 1st period, 8.5% in the 2nd, 33.1% in the 3rd, and 34.4% in 2009). Thus infiltration of genotype A infection into other regions occurred 5—6 years behind the epidemic in the capital region. This indicates that genotype A infection originated in the capital region and then spread to other areas of Japan.

Some genotypes are classified into several subgenotypes, and they have distinct geographical distributions.[42] Hence, subgenotypes are useful in tracing the route of HBV infection. By phylogenetic analysis (figures 3 and 4), 88.6% of genotype A isolates had the European—American type (A2), and the remaining 11.4% possessed the Asian—African type (A1). Likewise, 76.7% of genotype B isolates had Asian types (B2—B4), and the remaining 23.3% possessed the type endemic to Japan (B1). Of the 157 HBV isolates of genotype A or B, 147 (93.6%) had subgenotypes foreign to Japan. They are thought to have been transmitted from foreign sex workers, and spread among certain populations who share particular sexual behaviours in Japan.[41]

Of note, some HBV isolates of distinct subgenotypes possessed an identical sequence in the preS1/S2/S gene. The isolates of subgenotype A2 were prominent in this regard, and more often had the same sequence than those of other subgenotypes, such as A1, B1 and B2. The high prevalence of subgenotype A2 isolates with an identical sequence would not have been caused by cross-contamination. If cross-contamination had occurred, it would have affected isolates of all subgenotypes, and not influenced subgenotype A2 isolates preferentially. As many as 35% of subgenotype A2 isolates had an identical sequence, and those with the same sequence increased to 56.3% in the recent 2009 survey in Metropolitan Tokyo. Furthermore, some subgenotype A2 isolates in groups I, III and VII clustered locally within short periods, whereas others in groups II and VI were scattered widely over a long period of time. On the basis of these results, it is tempting to speculate that some subgenotype A2 strains would have been transmitted from person to person without undergoing mutations for many years.

In summary, the present study indicates the following. (1) AHB in the 28 national hospitals in Japan has not decreased, because genotype A infections are increasing. (2) Genotype A infections started to increase in the capital region, and then spread to local areas 5—6 years later. (3) Approximately 90% of genotype A in patients with AHB is subgenotype A2. (4) Subgenotype A2 strains with an identical sequence are spreading among younger generations with high sexual activity. (5) On the basis of the results obtained, AHB in Japan is not decreasing, because HBV of subgenotype A2 is prevailing in particular

subpopulations at high risk. Finally, in order to prevent further increases in AHB in Japan, universal vaccination of young people deserves consideration.

Author affiliations
[1]Clinical Research Center, NHO Nagasaki Medical Center, Nagasaki, Japan
[2]Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[3]National Center for Global Health and Medicine, Tokyo, Japan
[4]NHO Kyushu Medical Center, Fukuoka, Japan
[5]NHO Osaka National Hospital, Osaka, Japan
[6]NHO Yokohama Medical Center, Kanagawa, Japan
[7]NHO Sagamihara Hospital, Kanagawa, Japan
[8]NHO Oita Medical Center, Oita, Japan
[9]NHO Nagoya Medical Center, Aichi, Japan
[10]NHO Osaka-Minami Medical Center, Osaka, Japan
[11]NHO Kokura Medical Center, Fukuoka, Japan
[12]NHO Sendai Medical Center, Miyagi, Japan
[13]NHO Kyoto Medical Center, Kyoto, Japan
[14]NHO Tokyo Medical Center, Tokyo, Japan
[15]NHO Kure Medical Center and Chugoku Cancer Center, Hiroshima, Japan
[16]NHO Kanazawa Medical Center, Ishikawa, Japan
[17]NHO National Disaster Medical Center, Tokyo, Japan
[18]Miyakawa Memorial Research Foundation, Tokyo, Japan

REFERENCES
1. **Arauz-Ruiz P,** Norder H, Robertson BH, et al. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;**83**:2059—73.
2. **Norder H,** Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;**198**:489—503.
3. **Okamoto H,** Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;**69**:2575—83.
4. **Olinger CM,** Jutavijittum P, Hubschen JM, et al. Possible new hepatitis B virus genotype, southeast Asia. Emerg Infect Dis 2008;**14**:1777—80.
5. **Stuyver L,** De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;**81**:67—74.
6. **Tran TT,** Trinh TN, Abe K. New complex recombinant genotype of hepatitis B virus identified in Vietnam. J Virol 2008;**82**:5657—63.
7. **Tatematsu K,** Tanaka Y, Kurbanov F, et al. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J Virol 2009;**83**:10538—47.

8. **Chu CJ,** Lok AS. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002;**35**:1274—6.

9. **Kao JH.** Hepatitis B viral genotypes: clinical relevance and molecular characteristics. *J Gastroenterol Hepatol* 2002;**17**:643—50.

10. **Miyakawa Y,** Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003;**46**:329—38.

11. **Kimbi GC,** Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004;**85**:1211—20.

12. **Kramvis A,** Weitzmann L, Owiredu WK, *et al*. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol* 2002;**83**:835—9.

13. **Sugauchi F,** Kumada H, Acharya SA, *et al*. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* 2004;**85**:811—20.

14. **Sugauchi F,** Kumada H, Sakugawa H, *et al*. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. *Clin Infect Dis* 2004;**38**:1222—8.

15. **Sugauchi F,** Orito E, Ichida T, *et al*. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 2003;**124**:925—32.

16. **Tanaka Y,** Orito E, Yuen MF, *et al*. Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. *Hepatol Res* 2005;**33**:216—24.

17. **Akuta N,** Suzuki F, Kobayashi M, *et al*. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. *J Hepatol* 2003;**38**:315—21.

18. **Tanaka Y,** Hasegawa I, Kato T, *et al*. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004;**40**:747—55.

19. **Orito E,** Ichida T, Sakugawa H, *et al*. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001;**34**:590—4.

20. **Kobayashi M,** Arase Y, Ikeda K, *et al*. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. *J Med Virol* 2002;**68**:522—8.

21. **Ogawa M,** Hasegawa K, Naritomi T, *et al*. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. *Hepatol Res* 2002;**23**:167—77.

22. **Takeda Y,** Katano Y, Hayashi K, *et al*. Difference of HBV genotype distribution between acute hepatitis and chronic hepatitis in Japan. *Infection* 2006;**34**:201—7.

23. **Yotsuyanagi H,** Okuse C, Yasuda K, *et al*. Distinct geographic distributions of hepatitis B virus genotypes in patients with acute infection in Japan. *J Med Virol* 2005;**77**:39—46.

24. **Kato H,** Orito E, Sugauchi F, *et al*. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. *J Virol Methods* 2003;**110**:29—35.

25. **Orito E,** Mizokami M, Sakugawa H, *et al*. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001;**33**:218—23.

26. **Kato H,** Orito E, Gish RG, *et al*. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. *Hepatology* 2002;**35**:922—9.

27. **Sugiyama M,** Tanaka Y, Sakamoto T, *et al*. Early dynamics of hepatitis B virus in chimeric mice carrying human hepatocytes monoinfected or coinfected with genotype G. *Hepatology* 2007;**45**:929—37.

28. **Koyama T,** Matsuda I, Sato S, *et al*. Prevention of perinatal hepatitis B virus transmission by combined passive-active immunoprophylaxis in Iwate, Japan (1981—1992) and epidemiological evidence for its efficacy. *Hepatol Res* 2003;**26**:287—92.

29. **Noto H,** Terao T, Ryou S, *et al*. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980—1994. *J Gastroenterol Hepatol* 2003;**18**:943—9.

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

31. **Daniels D,** Grytdal S, Wasley A. Surveillance for acute viral hepatitis—United States, 2007. *MMWR Surveill Summ* 2009;**58**:1—27.

32. **Goldstein ST,** Alter MJ, Williams IT, *et al*. Incidence and risk factors for acute hepatitis B in the United States, 1982—1998: implications for vaccination programs. *J Infect Dis* 2002;**185**:713—19.

33. **Sherlock S.** The natural history of hepatitis B. *Postgrad Med J* 1987;63 Suppl 2:7—11.

34. **Mayerat C,** Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 1999;**6**:299—304.

35. **Hayashi K,** Katano Y, Takeda Y, *et al*. Association of hepatitis B virus subgenotypes and basal core promoter/precore region variants with the clinical features of patients with acute hepatitis. *J Gastroenterol* 2008;**43**:558—64.

36. **Ozasa A,** Tanaka Y, Orito E, *et al*. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006;**44**:326—34.

37. **Matsuura K,** Tanaka Y, Hige S, *et al*. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. *J Clin Microbiol* 2009;**47**:1476—83.

38. **Bodsworth NJ,** Cooper DA, Donovan B. The influence of human immunodeficiency virus type 1 infection on the development of the hepatitis B virus carrier state. *J Infect Dis* 1991;**163**:1138—40.

39. **Li JS,** Tong SP, Wen YM, *et al*. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J Virol* 1993;**67**:5402—10.

40. **Suzuki Y,** Kobayashi M, Ikeda K, *et al*. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J Med Virol* 2005;**76**:33—9.

41. **Sugauchi F,** Orito E, Ohno T, *et al*. Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan. *Hepatol Res* 2006;**36**:107—14.

42. **Norder H,** Courouce AM, Coursaget P, *et al*. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004;**47**:289—309.

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Changes in the serum level of hepatitis B virus (HBV) surface antigen over the natural course of HBV infection

Akihiro Matsumoto · Eiji Tanaka · Susumu Morita ·
Kaname Yoshizawa · Takeji Umemura ·
Satoru Joshita

Abstract

Background Despite its status as a potential biomarker of hepatitis B virus (HBV) response to interferon treatment, the changes in hepatitis B surface antigen (HBsAg) levels over the natural course of HBV carriers have not been analyzed sufficiently.

Methods A total of 101 HBV carriers were followed prospectively from 1999 to 2009. HBsAg level was measured yearly during the followed period.

Results HBsAg levels at baseline ranged from −1.4 to 5.32 log IU/ml, with a median value of 3.2 log IU/ml. Lower HBsAg levels were significantly associated with higher age and lower HBV replication status. The rate of change of HBsAg levels showed two peaks, with a cut-off value of −0.4 log IU/year. Based on this, patients were tentatively classified into rapid decrease (rate of change <−0.4 log IU/year) and non-rapid decrease groups. All baseline levels of HBsAg, HB core-related Ag, and HBV DNA were lower in the rapid decrease group than in the non-rapid decrease group. Patients with persistently positive HBeAg were all classified into the non-rapid decrease group. In patients with persistently negative HBeAg, HBV DNA levels were significantly ($P = 0.028$) lower in the rapid decrease group than in the non-rapid decrease group.

Conclusions Lower baseline HBsAg levels were significantly associated with older age and lower viral activity. Both a loss of HBeAg detection as well as inactive replication of HBV are suggested to be fundamental factors contributing to a rapid decrease in HBsAg over the natural course of HBV infection.

Abbreviations

HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
IU	International unit
HBcrAg	Hepatitis B virus core-related antigen
HCC	Hepatocellular carcinoma
NA	Nucleos(t)ide analogue
HBeAg	Hepatitis B e antigen
CLEIA	Chemiluminescent enzyme immunoassay
Da	Dalton
HR	Hazard ratio
cccDNA	Covalently closed circular DNA

A. Matsumoto · E. Tanaka (✉) · S. Morita · K. Yoshizawa ·
T. Umemura · S. Joshita
Department of Medicine, Shinshu University School
of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan
e-mail: etanaka@shinshu-u.ac.jp

K. Yoshizawa
Department of Gastroenterology, National Hospital Organization
Shinshu Ueda Medical Center, 1-27-21 Midorigaoka,
Ueda 386-8610, Japan

Introduction

With an estimated 350–400 million cases of chronic infection, hepatitis B virus (HBV) infection is a major worldwide health problem [1]. Chronic infection of HBV often leads to chronic hepatitis and eventually to liver cirrhosis and hepatocellular carcinoma [2, 3]. During infection, hepatitis B surface antigen (HBsAg), which is a component of the virion envelope, is secreted into the

Springer

bloodstream in large amounts as subviral particles. Thus, serum HBsAg is routinely used as a marker for detection of HBV infection.

Recently, several groups have reported that HBsAg levels can be used as an indicator of the response to peg-interferon in chronic hepatitis B similarly to the conventional markers of HBV DNA level and hepatitis B (HB) e antigen/antibody status [4, 5]. Since HBV carriers who clear HBsAg usually have a better prognosis than those who do not [6–8], it may be worthwhile to monitor HBsAg levels in the natural disease course of HBV infection. However, such changes need to be clarified more thoroughly to validate their clinical significance. In the present study, we analyzed the changes in HBsAg levels in a cohort of HBV carriers who were followed prospectively and compared them with those of HBV DNA and HB core-related antigens (HBcrAg) levels.

Patients and methods

Patients

A total of 101 HBV carriers were followed prospectively from 1999 to 2009. Patients were selected consecutively between 1997 and 1999 and met the following conditions: (1) HBsAg was positive in at least two examinations performed over 1 year apart; (2) no complications of hepatocellular carcinoma (HCC) or signs of hepatic dysfunction, such as jaundice or ascites, were observed; (3) nucleos(t)ide analogues (NAs) were not administered at the start of follow-up; and (4) patients were negative for hepatitis C and human immunodeficiency virus antibodies. The clinical and virological characteristics of our cohort are shown in Table 1.

The 101 patients consisted of 57 men and 44 women with a median age of 50 years (range 15–83 years). Hepatitis B e antigen (HBeAg) was positive in 38 (38%) patients and negative in 63 (63%). Of the 38 patients with HBeAg, 15 remained positive and 23 became negative during the follow-up period. Alanine aminotransferase (ALT) level flares of over 1,000 IU/L were observed in four (17%) of the 23 patients with HBeAg loss, but in none of the 15 patients with persistent HBeAg ($P = 0.138$). HBV genotype distribution was A in three (3%) patients, B in nine (9%), C in 87 (86%), and undetermined in two (2%). All patients were seen at Shinshu University Hospital or one of its affiliated hospitals. Our cohort tended to have a higher prevalence of cirrhosis (19%) and HCC (14%). These tendencies may be attributed to the higher age distribution in our cohort than that in other cohorts of HBsAg studies [6, 9, 10].

Patients were seen at least once a year during the 10 years of follow-up. The presence of cirrhosis was judged by histological findings and/or typical findings seen in cirrhosis, such as esophageal varices and splenomegaly. Screening for HCC was done using ultrasonography (US), computed tomography (CT), and/or magnetic resonance (MR) imagining at least once a year. The presence of complicating HCC was judged by evidence of characteristic hepatic masses on liver CT, MRI, and/or hepatic angiography. Serum samples were collected on a yearly basis and immediately stored at −20°C or below until assayed. This study was approved by the Ethics Committee of Shinshu University.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and HBe antibody, were tested using commercially

Table 1 Clinical and virological characteristics of patients with respect to HBeAg status

Characteristic	Overall ($n = 101$)	HBeAg-positive ($n = 38$)	HBeAg-negative ($n = 63$)	P
At baseline				
Age (years)[a]	50 (15 to 83)	42 (15 to 72)	53 (25 to 83)	<0.001
Male[b]	57 (56%)	22 (58%)	35 (56%)	>0.2
With cirrhosis[b]	19 (19%)	10 (26%)	9 (14%)	0.188
ALT (IU/L)[a]	31 (10 to 447)	47 (13 to 447)	29 (10 to 81)	0.002
HBV genotype (A:B:C:UD)	3:9:87:2	1:0:36:1	2:9:51:1	0.144
HBsAg (log IU/ml)[a]	3.2 (−1.4 to 5.3)	3.7 (1.6 to 5.3)	2.9 (−1.4 to 4.3)	<0.001
HBcrAg (log U/ml)[a]	3.8 (<3.0 to >6.8)	6.8 (<3.0 to >6.8)	3.1 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	4.7 (neg. to >9.5)	7.4 (2.4 to >9.5)	3.6 (neg. to 8.3)	<0.001
During follow-up				
Followed period (years)[a]	5 (1 to 10)	6 (1 to 10)	5 (1 to 10)	>0.2
Clearance of HBsAg[b]	20 (20%)	3 (8%)	17 (27%)	0.022
Complication of HCC[b]	14 (14%)	8 (21%)	6 (10%)	0.139
Introduction of NAs[b]	23 (23%)	11 (29%)	12 (19%)	>0.2

UD undetermined

[a] Data are expressed as median (range)

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

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

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

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

Statistical analyses

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

Results

Follow-up of patients

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

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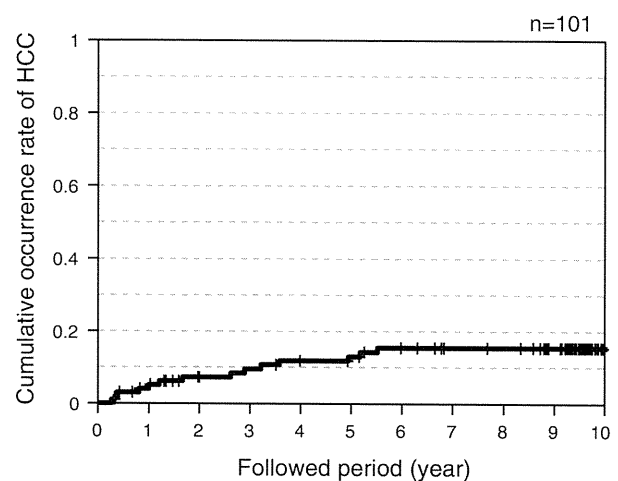

Fig. 1 Changes in the cumulative occurrence rate of HCC during the follow-up period

 Springer

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

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

HBsAg levels at baseline and during clinical course

Baseline HBsAg levels ranged from −1.4 to 5.32 log IU/ml, with a median value of 3.2 log IU/ml (Fig. 2). Table 2
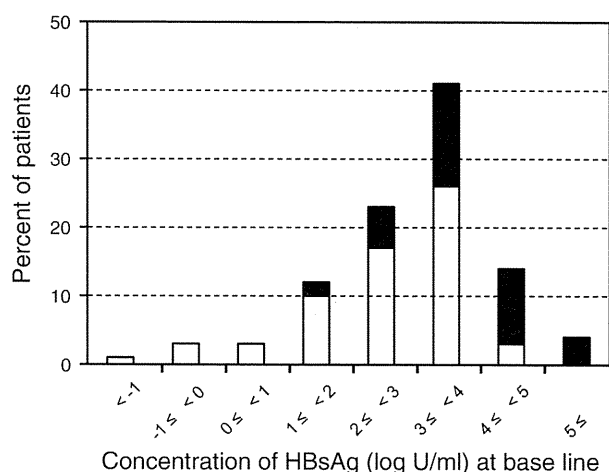

Fig. 2 Distribution of HBsAg concentration at baseline. *Closed bars* indicate patients with detectable HBeAg and *open bars* indicate those without

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

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

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

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

UD undetermined

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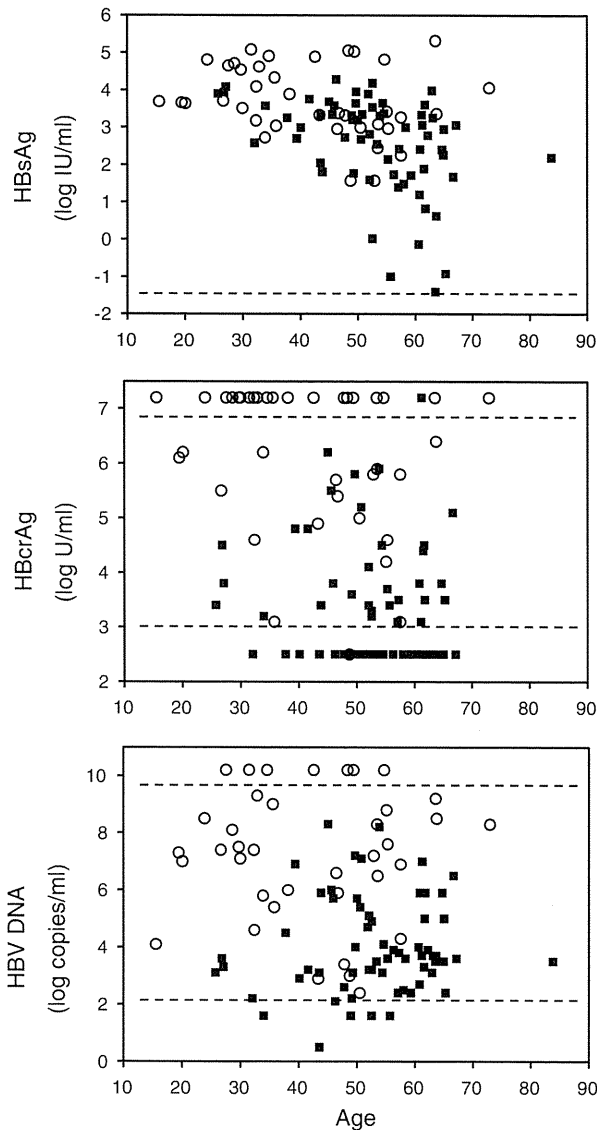
[b] Data are expressed as positive number (%)

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

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

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

Changes in HBsAg levels during the follow-up period

Positivity for HBsAg decreased gradually over the follow-up period (Fig. 4). A total of 20 patients cleared HBsAg during the follow-up period, for a disappearance rate of 2.1% per year. Clinical and virological backgrounds were compared between patients with and without clearance of HBsAg in Table 3. Patients losing HBsAg positivity were
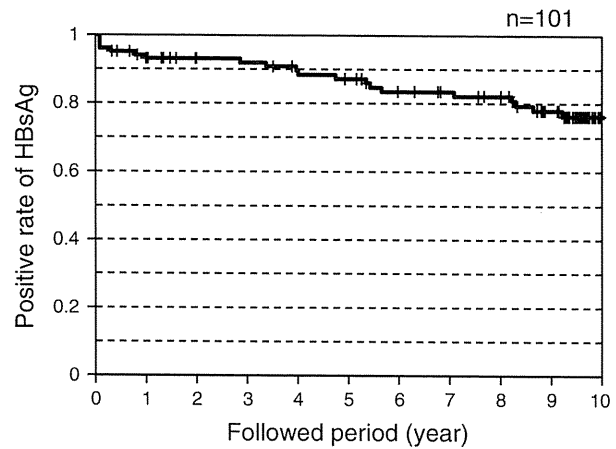
significantly older than those who did not. Baseline levels of HBsAg, HBcrAg, and HBV DNA were significantly lower in these patients as well. Clearance of HBsAg was significantly associated with HBV DNA (HR 3.6, 95% CI 1.1–11.4, $P = 0.033$) and HBcrAg (HR 4.0, 95% CI 1.1–14.9, $P = 0.036$) levels at baseline by multivariate analysis. Of the 20 patients who cleared HBsAg, seven were positive for HBV DNA (range, positive 3.0 log copies/ml) and three were positive for HBcrAg (range 3.0–3.2 U/ml).

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

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

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

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

UD undetermined

[a] Data are expressed as median (range)

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

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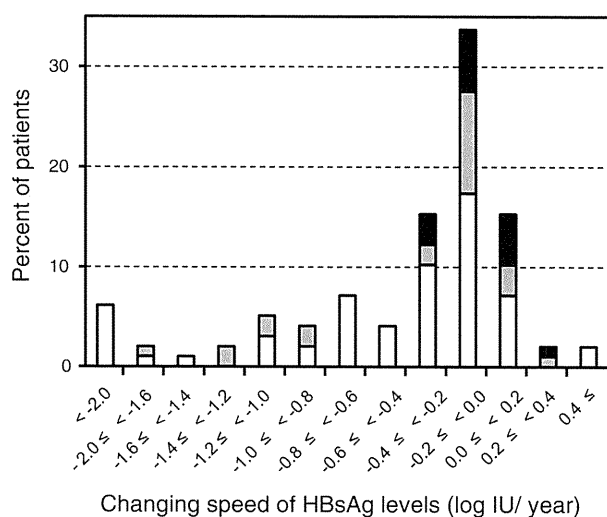
Fig. 5 Distribution of patients classified according to rate of change of HBsAg levels (log IU/year) during follow-up period. *Closed bars* indicate patients with persistent HBeAg-positive status. *Shaded bars* indicate patients who became negative for HBeAg during follow-up period. *Open bars* indicate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

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

Discussion

During the natural course of HBV infection, HBsAg levels showed almost normal distribution, making a sharp peak at a median value of 3.2 log IU/ml. Lower HBsAg levels were

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

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