Table 3. Multivariate Analysis of Factors Independently Associated With Persistence of HBsAg Positivity Following Acute Hepatitis B

	Persistence of HBsAg More Than 6 Months From AHB		
Factors	Odds Ratio	95% CI	*P* Value
ALT (per 1 IU/L increase)	1.000	0.999-1.000	0.035
HBV DNA (per 1 log copy/mL increase)	1.176	0.931-1.484	0.173
Genotypes			
Non-A	1.00		
A	4.224	1.853-9.631	0.001

95% CI, 95% confidence interval; ALT, alanine aminotransferase; HBV, hepatitis B virus.

in the former group than in the latter group. The percentages of HBV genotype A (48.8% versus 88.9%, $P = 0.018$) and NAs treatment (+) (48.3% versus 88.9%, $P = 0.017$) were significantly higher among patients in whom the HBsAg persisted for more than 12 months.

Factors Independently Associated With Viral Persistence Following AHB. A stepwise logistic regression model was used to perform multivariate analysis which explains relationships between some factors and persistence of HBsAg positivity more than 6 months following AHB. Peak ALT level, peak HBV DNA level, genotype A, and treatment with NAs were retained in the final multivariate logistic model in a backward stepwise manner ($P < 0.1$). For predicting the persistence of HBsAg for more than 6 months, only genotype A was independently associated with progression of AHB to the persistence of HBsAg (odds ratio [OR]: 4.224, $P = 0.001$, Table 3).

Characteristics of Patients Who Progressed to Chronicity That Was Defined as the Persistence of HBsAg for More Than 12 Months Following Acute Hepatitis B. Table 4 shows the clinical and virological characteristics of nine patients who progressed to chronicity defined as the persistence of HBsAg for more than 12 months following AHB. Among the nine patients who progressed to chronicity from AHB, eight (88.9%) were men and eight (88.9%) were HBeAg-positive. In general, among the patients who progressed to chronicity following AHB, the peak HBV DNA levels were high, and the peak total bilirubin and ALT levels were low. In eight (88.9%) patients, entecavir was administered; however, the duration until the onset of NA treatment from AHB onset was long (75-570 days).

Early Onset of Treatment With NAs Was Able to Prevent Viral Persistence After AHB Caused by Genotype A. The cumulative proportion maintaining HBsAg positivity during follow-up, expressed in terms of time after AHB onset, were significantly longer in patients with NAs treatment than in those without NAs treatment ($P = 0.046$, Fig. 2A). Table 5 shows the percentages of patients in whom HBsAg persisted for more than 6 or 12 months among patients categorized based on the period of time (i.e., duration) until the onset of NAs treatment. For patients in whom the onset of NAs treatment was less than 4 weeks from the onset of AHB, 12.7% of the patients showed persistent HBsAg for more than 6 months, while none showed HBsAg positivity for more than 12 months. For patients in whom the onset of NAs treatment was at 5-8 weeks, 37.5% of the patients showed persistent HBsAg for more than 6 months, whereas none showed persistent HBsAg for more than 12 months. For all groups, the period of HBsAg positivity in patients starting NAs treatment within 8 weeks from AHB onset was significantly shorter than that in patients beginning NAs treatment after more than 8 weeks from AHB onset ($P < 0.0001$, Fig. 2B). Patients starting NAs treatment within 8 weeks from AHB onset never progressed to chronicity after AHB caused by genotype A.

Table 4. Characteristics of Patients Who Progressed to Chronicity Following Acute Hepatitis B

Case	Age	Gender	HIV	HBeAg	HBV DNA (log copies/mL)	Total Bilirubin (mg/dL)	ALT (IU/L)	Observation Period (Months)	NAs Treatment	Duration Until NAs Treatment (Days)	Transmission Routes	Genotype
1	23	Male	(−)	(+)	7.6	1.7	1271	26	ETV	570	Heterosexual	A
2	40	Male	(−)	(−)	8.8	1.4	568	13	ETV	240	Heterosexual	A
3	45	Male	(−)	(+)	7.7	0.9	867	57	ETV	135	Heterosexual	A
4	37	Male	(−)	(+)	7.6	3.4	384	29	ETV	75	Unknown	A
5	54	Male	(−)	(+)	9	2	455	17	ETV	155	Homosexual	A
6	45	Male	(−)	(+)	4.8	21.2	512	60	(−)	(−)	Homosexual	A
7	61	Male	(−)	(+)	9.1	1.5	804	17	ETV	88	Unknown	A
8	56	Male	(−)	(+)	9.0	1.1	1820	14	ETV	118	Unknown	A
9	31	Female	(−)	(+)	7.4	0.8	296	66	ETV	150	Blood transfusion	C

HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; NAs, nucleotide analogs; ETV, entecavir.

Table 5. Proportion of Patients in Whom HBsAg Persisted for More Than 6 or 12 Months Among Patients Categorized Based on the Number of Weeks Until the Onset of NAs Treatment

Duration Until Onset of NAs Treatment (Weeks)	Persistence of HBsAg for More Than 6 Months	Persistence of HBsAg for More Than 12 Months	Total Patients
<4 weeks (n, %)	9 (12.7)	0 (0)	71
5-8 weeks (n, %)	6 (37.5)	0 (0)	16
9-12 weeks (n, %)	1 (33.3)	1 (33.3)	3
13-16 weeks (n, %)	4 (100)	1 (25.0)	4
>17 weeks (n, %)	9 (100)	6 (66.7)	9
Total	29	8	103

HBsAg, hepatitis B surface antigen; NAs, nucleotide analogs.

Discussion

A multicenter nationwide study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in Japanese patients who contracted AHB in adulthood. The study was feasible in Japan, where a universal vaccination program for HBV has not been implemented because of the extremely high efficacy of the immunoprophylaxis that is given to babies born to carrier mothers. The implementation of this program has resulted in a decrease in the persistent HBV carrier rate from 1.4% to 0.3%.[19] Selective vaccination means that Japanese are more likely to be infected with HBV by way of horizontal transmission since the percentage of the population possessing anti-HBs is much lower than that in countries in which universal vaccination programs have been established.[20] In addition, Japan is faced with the ever-increasing impacts of globalization: as many as 17 million Japanese travel abroad and over 7 million people visit Japan from overseas each year. This "population mixing" may help to explain the increased prevalence in Japan of AHB due to genotype A, which is transmitted through indiscriminate sexual contact. Consequently, Japan may be the only country in the world where the influences of HBV genotypes, including genotype A (as is predominant in Western countries) and genotypes B and C (as are predominant in Asian countries), on chronic outcomes after AHB can be compared.

Currently, the persistence of HBsAg in serum for more than 6 months is considered to represent a progression to chronic infection.[21] However, our data showed that HBsAg frequently disappeared between 7 to 12 months after the onset of AHB in patients with genotype A (31/107 [29.0%]) and non-A genotypes (9/105 [8.6%]) (Fig. 1). These patients were considered to exhibit prolonged cases of AHB, rather than persistent infection. This finding reflects the higher sensitivity of the most up-to-date assays for HBsAg as compared with previous methods. In the present study, HBsAg was measured by CLIA, which has been reported to be about 150 times more sensitive in the detection of HBsAg than reverse passive hemagglutination (RPHA)-HBsAg, which has been used for the last 30 years in Japan.[22] The use of a more sensitive assay for HBsAg results in a longer period during which HBsAg may be detected. In this study, HBsAg did not disappear in nine patients after remaining continuously detectable for more than 12 months. Therefore, the persistence of HBsAg for more than 12 months, as measured with a highly sensitive method for detecting HBsAg, may be suitable for defining the progression of AHB to chronicity; however, further study is necessary to determine whether this definition is appropriate worldwide.

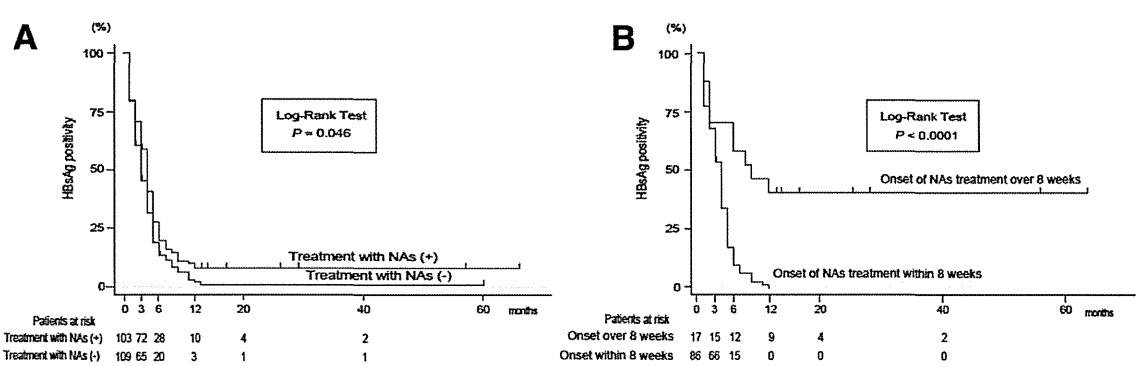

Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. $P = 0.046$, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the cumulative proportion of AHB patients in genotype A maintaining HBsAg positivity between treatment onset with NAs within 8 weeks and treatment onset with NAs over 8 weeks after onset of AHB, as analyzed using the Kaplan-Meier test. $P < 0.0001$, treatment onset with NAs over 8 weeks: red line, treatment onset with NAs within 8 weeks: blue line.

It has been reported that ~10% of patients who contract HBV as adults do not clear HBsAg from their serum and become carriers.[23] Meanwhile, a wide variation has been seen in the rate of persistence after AHB infection in adults. For example, viral persistence following AHB was seen in 0.2% (1/507) of adults in Greece,[24] 7.7% (5/65) of adult Alaskan Eskimos, and 12.1% (7/58) of adults in Germany.[25] The difference in the proportion of patients progressing from AHB to chronicity in different regions may be attributable to virological and host factors. In this study, 4.2% (9/212) of patients progressed to chronicity after AHB: 7.5% (8/107) of those infected with genotype A and 0.9% (1/105) of those infected with non-A genotypes. The non-A genotypes included genotypes B, C, D, F, and H (n = 25, 77, 1, 1, and 1, respectively). Genotypes B and C are predominant in eastern Asian countries, where the majority of those infected with HBV acquired the virus during the perinatal period by way of vertical transmission.[26] On the other hand, genotype A is predominant in Western countries, where the main route is horizontal transmission later in life.[26,27] Because HBeAg persists long after the infection in the genotype C as compared to other genotypes, this genotype has been shown to be a risk factor for perinatal and horizontal transmission in newborns and children.[28] The predominance of genotype A in Western countries may be attributable to a higher chronicity rate following AHB by way of horizontal transmission in adults.

In this study the characteristics of AHB associated with genotype A were a higher peak level of HBV DNA and a lower peak level of ALT. These findings were similar to those for patients with HBV-HIV coinfection.[29] Such characteristics of genotype A or coinfection with HIV are assumed to be attributable to milder hepatitis associated with weaker cellular immune responses. More slowly replicating viruses have been reported to evoke weaker cellular responses, enhancing the likelihood of persistence.[30] Indeed, our prior study showed that the replication of genotype A was significantly slower than that of genotype C in immunodeficient, human hepatocyte chimeric mice.[31] Moreover, variation among genotypes in the expression pattern of HBeAg may affect the progression of AHB to chronicity. Another previous study of ours revealed that a single form of HBeAg was detected by western blot analysis in serum samples from patients infected with genotypes B through D, but that two additional larger forms of HBeAg were detected in patients with genotype A.[32] Milich and Liang[33] reported that HBeAg may modulate the host immune response as a tolerogen to promote chronicity. Therefore, the different expression pattern of HBeAg by genotype A HBV may contribute to chronicity following AHB.

Early NAs initiation appeared to enhance the viral clearance across genotypes, although treatment with NAs did not show any overall benefit in duration of HBsAg. Previous studies examining the efficacies of NAs for preventing progression to chronic infection after AHB have reported conflicting results. Some small-scale studies have suggested the efficacy of lamivudine and entecavir in preventing the progression of AHB to chronic hepatitis.[34,35] Another study showed a lower seroconversion rate of HBsAg in lamivudine users.[36] Further, a randomized placebo-controlled trial showed no significant difference in clinical outcomes.[37] However, these previous studies did not mention the prevalence of HBV genotypes in the respective study populations. Although this was a retrospective study, our study included data on the prevalence of HBV genotypes. Additionally, our findings suggested that larger prospective randomized studies for every HBV genotype should be performed to determine whether early treatment with NAs prevented the progression of AHB to a chronic state.

In conclusion, in Japan genotype A was an independent risk factor for progression to chronic infection following AHB in adults. Confirmation of this association in patients with AHB in other countries is desirable and may provide insight into the pathogenetic mechanisms underlying this association. Early NA treatment appeared to reduce the likelihood of chronicity but this potentially important intervention needs to be prospectively studied before recommendations can be made.

Appendix

Members of the Japanese AHB Study Group include Yasuharu Imai (Ikeda Municipal Hospital), Norie Yamada, Hideaki Takahashi (St. Marianna University School of Medicine), Koji Ishii (Toho University School of Medicine), Hideyuki Nomura (Shin-Kokura Hospital), Jiro Nishida (Tokyo Dental Collage Ichikawa General Hospital), Shigeru Mikami (Kikkoman Hospital), Tsuneo Kitamura (Juntendo University Urayasu Hospital), Akihito Tsubota (Kashiwa Hospital Jikei University School of Medicine), Noritomo Shimada (Shinmatsudo Central General Hospital), Tetsuya Ishikawa (Nagoya University Graduate School of Medicine), Yoshiyuki Ueno (Tohoku University Graduate School of Medicine), Tomoyoshi Ohno (Social Insurance Chukyo Hospital), Etsuro Orito (Nagoya

Daini Red Cross Hospital), Michihiro Suzuki (Kawasaki Municipal Tama Hospital), Hitoshi Takagi (Gunma University Graduate School of Medicine), Eiichi Tomita (Gifu Municipal Hospital), Kumada Takashi (Ogaki Municipal Hospital), Toshihiko Mizuta (Saga University Faculty of Medicine), Tetsuya Mine (Tokai University School of Medicine), Jong-Hon Kang (Teine-Keijinkai Hospital), Katsuji Hirano (Juntendo University Shizuoka Hospital), Hirohito Tsubouchi (Kagoshima University Graduate School of Medical and Dental Sciences), Akito Nozaki (Yokohama City University Medical Center), Akito Sakai (Kanazawa University Graduate School of Medical Science), Shuhei Nishiguchi (Hyogo College of Medicine), Akihiro Tamori (Osaka City University Graduate School of Medicine), Satoru Hagiwara (Kinki University School of Medicine), Takahide Nakazawa (University of Kitasato East Hospital), Michio Sata (Kurume University School of Medicine), Toshiro Kamoshida (Hitachi General Hospital) Atsushi Takahashi (Fukushima Medical University School of Medicine), Satoshi Kakizaki (Gunma University Graduate School of Medicine), Yoshimasa Kobayashi (Hamamatsu University School of Medicine), Shigeru Sasaki (Sapporo Medical University), Tadashi Ikegami (Tokyo Medical University Ibaraki Medical Center), Yoichi Hiasa (Ehime University Graduate School of Medicine), Kenji Nagata (University of Miyazaki), Tomoyuki Kubota (Saiseikai Niigata Daini Hospital), Hiroshi Mitsui (Tokyo Teishin Hospital), Norihiko Yamamoto (Mie University School of Medicine), Masataka Tsuge (Hiroshima University), Shuichi Sato (Shimane University Hospital), Yoshito Ito (Kyoto Prefectural University of Medicine), Wataru Sato (Akita University School of Medicine), Shigeharu Uchida (Japanese Red Cross Society), Yuki Tada (National Institute of Infectious Diseases), Toshiaki Mizuochi (National Institute of Infectious Diseases), Norihiro Furusho (Kyushu University), and Shuhei Hige (Hokkaido University Graduate School of Medicine).

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MAJOR ARTICLE

High Levels of Hepatitis B Virus After the Onset of Disease Lead to Chronic Infection in Patients With Acute Hepatitis B

Hiroshi Yotsuyanagi,[1,a] Kiyoaki Ito,[2,5,a] Norie Yamada,[1,3,4] Hideaki Takahashi,[3] Chiaki Okuse,[3] Kiyomi Yasuda,[4] Michihiro Suzuki,[3] Kyoji Moriya,[1] Masashi Mizokami,[2] Yuzo Miyakawa,[6] and Kazuhiko Koike[1]

[1]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Bunkyo; [2]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa; [3]Division of Gastroenterology and Hepatology, Department of Internal Medicine, St Marianna University School of Medicine, Kawasaki; and [4]Department of Internal Medicine, Center for Liver Diseases, Kiyokawa Hospital, Suginami, [5]Department of Microbiology and Immunology, Aichi Medical University School of Medicine, and [6]Miyakawa Memorial Research Foundation, Minato, Tokyo, Japan

Background. Some patients with acute hepatitis B virus (HBV) infection develop chronic infection. However, the method for identifying these patients has not been established.

Methods. We followed 215 Japanese patients with acute HBV infection until the clearance of hepatitis B surface antigen (HBsAg) or the development of chronic infection. Levels of HBsAg and HBV DNA were serially monitored from the onset.

Results. Of the 215 patients, 113 (52.5%) possessed HBV genotype A, 26 (12.0%) genotype B, and 73 (34.0%) genotype C. Twenty-one of the 215 (9.8%) developed chronic infection, with the persistence of HBsAg for >6 months. The rate of chronicity of genotype A, B, and C was 12.4%, 3.8%, and 8.2%. Of the 21 patients, only 6 (2.8%) patients, including 5 with genotype A, failed to clear HBsAg within 12 months. Levels of HBsAg at 12 weeks and HBV DNA at 4 weeks were useful for distinguishing the patients who became chronic from those who did not ($P < .001$ and $P < .001$, respectively). Likewise, the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks were useful for discriminating between the patients who lost HBsAg within 12 months and those who did not ($P < .01$ and $P < .05$, respectively).

Conclusions. In acute HBV infection, clearance of HBV may happen between 6 and 12 months from the onset. Only those who fail to clear HBV within 12 months from the onset may develop chronic infection.

Keywords. hepatitis B virus antigen; hepatitis B virus; genotype.

The clinical outcome of acute hepatitis B is self-limited in the majority of immunocompetent adults. However, some patients run a prolonged or even chronic course, or are complicated by acute liver failure. Several factors are implicated in different clinical courses.

Hepatitis B virus (HBV) genotypes and subtypes are known to influence the clinical outcome of acute hepatitis B. For instance, HBV subgenotype B1 is associated with fulminant hepatic failure in acute hepatitis B [1]. On the other hand, genotype A is associated with chronic sequelae [2–5]. Furthermore, patients with subgenotype C2 are more likely to develop chronic infection than those with subgenotype B2 [6]. These characteristics may reflect viral kinetics in acute HBV infection that would differ among HBV infections with distinct genotypes/subgenotypes, but little is known about them.

Quantitation of hepatitis B surface antigen (HBsAg), in addition to HBV DNA, has been introduced to analysis of viral kinetics in patients with chronic hepatitis B in recent years. HBsAg levels are also useful for estimating

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[a]H. Y. and K. I contributed equally to this work.

Correspondence: Hiroshi Yotsuyanagi, MD, Department of Internal Medicine, Graduate Institute of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo, Tokyo 113-8655, Japan (hyotsu-tky@umin.ac.jp).

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viral loads and predicting the response to antiviral treatments [7–9], and for determining the natural history of chronic hepatitis B [10, 11]. Therefore, HBsAg and HBV DNA would be instrumental in foretelling the outcome of acute hepatitis B. However, the clinical utility of these markers in patients with acute hepatitis B is largely unknown.

Therefore, the aim of the present study was to examine differences in viral kinetics among patients with acute hepatitis B, who were infected with HBV of different genotypes, and evaluate the usefulness of quantifying HBsAg and HBV DNA for predicting the clinical outcome.

PATIENTS AND METHODS

Patients

This was a retrospective study of patients who were diagnosed with acute hepatitis B in our institutions during 1994 through 2010. Criteria for the diagnosis of acute hepatitis B were (1) acute onset of liver injury without a previous history of liver dysfunction; (2) detection of HBsAg in the serum; (3) immunoglobulin M (IgM) antibody to HBV core (anti-HBc) in high titers (detectable in serum samples diluted 10-fold) [3]; (4) absence of a past or family history of chronic HBV infection; and (5) exclusion of coinfection with hepatitis A virus, hepatitis C virus, or other hepatotropic viruses by serologic testing. Among the 232 patients who met these criteria, 215 patients (159 men and 56 women with a mean age of 31.8 ± 10.0 years) whose serum samples were available for virologic analyses were included in the study. No patient developed liver failure.

No patient received antiviral treatment. Of the 215 patients, 159 (74.0%) patients could be regularly followed up until the confirmation of clinical outcomes. Based on the duration of HBsAg (defined as the interval between the onset [defined by the first visit] and the last visit with detectable HBsAg), we classified the 159 patients into the following 4 groups (the duration of HBsAg is indicated in parentheses): group 1 (<3 months); group 2 (3–6 months); group 3 (>6–12 months); and group 4 (>12 months). Changes in virologic parameters were analyzed in relation with clinical characteristics. The study was approved by the ethics committees of our institutions, and written informed consent was obtained from each patient.

Quantification of Serologic Markers for HBV Infection and HBV DNA

HBsAg had been measured quantitatively by chemiluminescent enzyme-linked immunosorbent assay (ELISA; Sysmex JAPAN Co, Ltd, Kobe, Japan) every 2–4 weeks, until the clinical outcome was known. It has a dynamic range of 0.03–2, 500 IU/mL. Serum samples scaling out from this range were diluted so as to contain them within it. Antibody to hepatitis B s antigen (anti-HBs), hepatitis B e antigen (HBeAg), and IgM anti- HBc were determined by ELISA (Abbott JAPAN Co, Ltd, Tokyo, Japan). Levels of HBV DNA were determined using the COBAS TaqMan HBV v.2.0 kit (Roche Diagnostics, Basel, Switzerland), which has a dynamic range over 2.1–9.0 log copies/mL.

HBV Genotyping

The HBV genotype was determined by a genotype-specific probe assay (Smitest HBV genotyping Kit, Genome Science, Fukushima, Japan) as previously reported [12].

Molecular Evolutionary Analyses

HBV genotype A started to prevail in Japan merely several years ago, suggesting that it was imported to Japan only recently [3, 13]. Therefore, genomic sequences of HBV genotype A (HBV/A), recovered from sera of patients with acute HBV infection, would be closely related to one another and with those reported from abroad. To evaluate this possibility, 20 HBV/A samples were selected randomly and sequenced by the method reported previously [14].

The number of nucleotide substitutions per site was estimated by the 6-parameter method [15], and a phylogenetic tree was constructed by the neighbor-joining method [16] based on the numbers of substitutions. To confirm the credibility of phylogenetic analyses, bootstrap resampling tests were carried out 1000 times [17].

Statistical Analyses

Categorical variables were compared by χ^2 test or Fisher exact test, and continuous variables by the Mann-Whitney U test. $P < .05$ was considered statistically significant. Receiver operating characteristic (ROC) analysis was performed to compute the area under the ROC curves for viral markers to determine cutoff points for predicting the outcome.

RESULTS

Distribution of HBV Genotypes in Patients With Acute Hepatitis B

HBV genotypes were determined in 215 of the 232 (93%) patients with acute hepatitis B. Of the 215 patients, genotype A was detected in 113 (52%), B in 26 (12%), C in 73 (33%), D in 1 (1%), E in 1 (1%), and F in 1 (1%). The distribution of genotypes was compared among 4 periods during 1994 through 2010 (Table 1). The proportion of patients with genotype A gradually increased to 65.9% in 2007–2010; it was higher than those in the earlier periods (34.4% in 1994–1998 [$P = .002$], 36.8% in 1999–2002 [$P = .002$], and 51.9% in 2003–2006 [$P = .093$]).

Phylogenetic Relationship Among HBV Strains of Genotype A

We randomly selected 11 HBV/A strains sampled in 2007–2010 and 9 of those in 2001–2006, and constructed a molecular evolutionary tree (Figure 1). All 20 samples had similar nucleotide sequences with a concordance of 99%. They were close to previously

Table 1. Prevalence of Hepatitis B Virus Genotypes in Patients With Acute Hepatitis B During 4 Chronologic Periods

Period	Genotype A	Genotype B	Genotype C	Others
1994–1998 (n = 32)	11[a] (34.4%)	3 (9.3%)	18 (56.3%)	0
1994–1998 (n = 38)	14[b] (36.8%)	4 (10.5%)	20 (52.7%)	0
1994–1998 (n = 54)	28[c] (51.9%)	6 (11.1%)	19 (35.1%)	1 (1.9%)
1994–1998 (n = 91)	60[a,b,c] (65.9%)	13 (14.3%)	16 (17.6%)	2 (2.2%)
Total (N = 215)	113 (52.5%)	26 (12.0%)	73 (34.0%)	3 (1.5%)

[a] $P = .0032$.

[b] $P = .0014$.

[c] $P = .02$.

reported genotype A2 sequences from Western countries. The results support the possibility that HBV/A was imported to Japan only recently and has been spreading throughout the country.

Clinical Features Among Patients Infected With HBV of Different Genotypes

Clinical features of patients with acute hepatitis B of different genotypes are compared in Table 2. The mean age was no different among patients infected with HBV of different genotypes. The proportion of men was higher in genotype A or B than C infection (93.8% or 80.7% vs 39.7% [A vs C, $P < .001$; B vs C, $P < .001$]).

The maximum alanine aminotransferase (ALT) level was lower in patients with genotype A than in those with genotype C (2126 ± 938 vs 2857 ± 1668 IU/L, $P = .002$). The maximum bilirubin level was higher in patients with genotype A (7.1 ± 6.4 mg/dL) or C (9.0 ± 7.5 mg/dL) than in those with genotype B (4.8 ± 3.3 mg/dL) (A vs B, $P = .003$; B vs C, $P < .001$). Regarding viral markers, the peak HBV DNA level was higher in patients with genotype A than in those with genotype C (6.3 ± 1.7 vs 4.9 ± 1.5 log copies/mL, $P < .001$). HBeAg was detected in 95 of the 121 (77.3%) patients with genotype A, 24 of the 28 (88.5%) with genotype B, and 37 of the 58 (65.5%) with genotype C (A vs C, $P = .036$). Men who have sex with men were more frequently represented among patients with genotype A than B or C (31.4% vs 4.8% or 11.3% [A vs B, $P = .017$; A vs C, $P = .002$]). Antibody to human immunodeficiency virus (anti-HIV) was examined in 72 of the 113 (63.7%) patients with genotype A, 7 of the 26 (26.9%) with genotype B, 58 of the 73 (79.5%) with genotype C, and 1 with genotype E. Anti-HIV was detected in 7 of the 72 (9.7%) patients with genotype A, and the other 96 patients tested for anti-HIV showed negative results. All of the 7 patients with anti-HIV cleared HBsAg from the serum within 6 months without antiviral treatment.

Among the 215 patients whose HBV genotypes were determined, 159 could be followed until the confirmation of clinical outcomes. The distribution of HBsAg-positive period is compared among patients with different genotypes. Group 1 (HBsAg persisting for <3 months) comprised 84 patients; group 2 (3–6 months) comprised 54 patients; group 3 (>6–12 months) comprised 15 patients; and group 4 (>12 months) comprised 6 patients. HBsAg remained >6 months in 21 of the 215 (9.8%) patients, including 14 of the 113 (12.4%) with genotype A, 1 of the 26 (3.8%) with genotype B, and 6 of the 73 (8.2%) with genotype C. Among the 21 patients, 15 (71.4%) cleared HBsAg within 12 months from the onset, and were classified into group 3. The remaining 6 (5 with genotype A and 1 with genotype B) who failed to clear HBsAg within 12 months were classified into group 4. All of the 6 were negative for anti-HIV. The proportion of group 4 was 6.0% in the patients with genotype A, 4.0% in those with genotype B, and 0% in those with genotype C.

The mean duration of HBsAg was 13.9 ± 8.7 weeks in patients with genotype A, 7.1 ± 5.3 weeks in those with genotype B, and 9.6 ± 7.6 weeks in those with genotype C, presuming the duration of HBsAg in group 4 at 12 months. The duration was longer in patients with genotype A than in those with B or C (A vs B, $P < .001$; A vs C, $P = .04$).

Prediction of the Outcome by the Duration of HBsAg

Table 2 shows that the duration of HBsAg among patients with genotype A varied to a higher extent than that among those with other genotypes. Therefore, we determined HBsAg and HBV DNA levels serially, and evaluated them for the ability to predict the outcome of acute hepatitis B in patients with genotype A.

Serial changes in HBsAg levels are shown in Supplementary Figure 1*A*. HBsAg levels declined more slowly in group 2 than group 1, as well as in group 3 than group 2. In group 4, HBsAg reelevated at 12 weeks after the onset. Figure 2 compares HBsAg levels among groups 1–4 at different intervals from the onset. HBsAg at 8 weeks from the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBsAg at 12 weeks from the onset was helpful for discriminating among groups 2, 3, and 4.

Prediction of the Outcome by HBV DNA

We also studied serial changes of HBV DNA in patients with genotype A, and examined if they also were useful for predicting the clinical outcome of acute hepatitis B. Supplementary Figure 1*B* shows serial changes in HBV DNA levels in patients in 4 groups. Although the reelevation of HBV DNA was not observed, the decline of HBV DNA was quite slow in group 4. Figure 3 compares HBV DNA levels among groups 1–4 at different intervals from the onset. HBV DNA at 4 weeks from

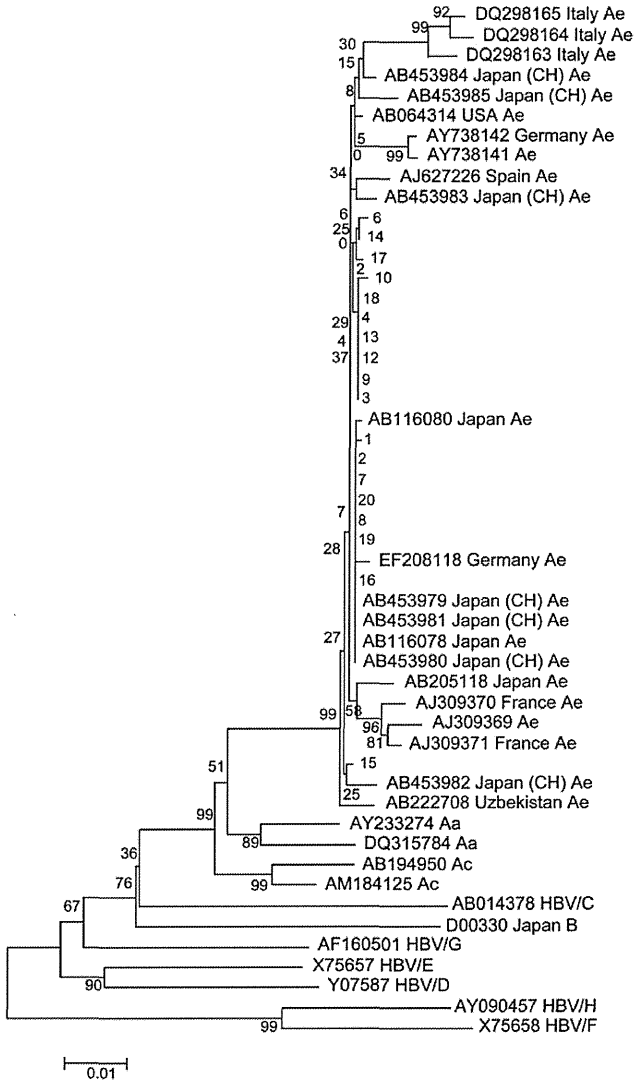

Figure 1. Evolutionary relationships of 86 hepatitis B virus genotype A taxa, including 20 from the present cases. The evolutionary history, inferred using the neighbor-joining method, shows that all 20 samples had similar nucleotide sequences close to previously reported genotype A2 sequences from Western countries.

the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBV DNA levels at 8 weeks from the onset were useful for discriminating between group 4 and group 3, as well as for distinguishing group 3 or 4 from group 1 or 2.

Levels of HBsAg and HBV DNA for Predicting Persistent Infection

As the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks from the onset were useful for distinguishing group 4 from the other groups, we evaluated the appropriate levels for predicting persistent infection in patients with genotype A. When we set the cutoff value of HBsAg at 1000 IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (98.1%). Likewise, when we set the cutoff value of HBV DNA at 10^6 log IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (96.4%). Therefore, HBsAg at 12 weeks >1000 IU/mL or HBV DNA at 8 weeks >10^6 log copies/mL is useful for predicting persistent infection.

DISCUSSION

In Japan, as shown in Table 1, the dominant HBV in acute hepatitis has been shifting from genotype C to A [3, 5, 14, 18]. The fact that nucleotide sequences of HBV/A isolates from patients

Table 2. Baseline Characteristics and the Duration of Hepatitis B Surface Antigen in Patients With Acute Hepatitis B Virus With Different Hepatitis B Virus Genotypes

	HBV Genotypes					
Features	A (n = 113)	B (n = 26)	C (n = 73)	D (n = 1)	E (n = 1)	F (n = 1)
Age, y	30.8 ± 9.5	32.3 ± 9.5	33.3 ± 10.9	27	26	58
Male	106 (93.8%)[a]	21 (80.7%)[b]	29 (39.7%)[a,b]	0	0	1 (100%)
Transmission routes Identified	102 (90.2%)	21 (80.8%)	53 (72.6%)	1 (100%)	1 (100%)	1 (100%)
Heterosexual	70 (68.6%)	19 (90.4%)	47 (88.7%)	1 (100%)	1 (100%)	1 (100%)
MSM	32 (31.4%)[c,d]	1 (4.8%)[c]	6 (11.3%)[d]	0	0	0
ALT, IU/L	2126 ± 938[e,*]	2394 ± 820	2857 ± 1668[e]	4180	1175	1533
Bilirubin, mg/dL	7.1 ± 6.4[f,*]	4.8 ± 3.3[f,g]	9.0 ± 7.5[g]	6.8	3.9	3.5
HBV DNA, log copies/mL	6.3 ± 1.7[h,*]	5.5 ± 2.3	4.9 ± 1.5[h]	5.2	7.4	4.8
HBeAg	95/121 (77.3%)[i,*]	24/28 (88.5%)	37/58 (65.5%)[i]	1/1 (100%)	1/1 (100%)	1/1 (100%)
Anti-HIV	7/72 (9.7%)	0/7 (0%)	0/23 (0%)	Not tested	0/1 (0%)	Not tested
Duration of HBsAg*						
Group (mo)						
1 (<3)	35 (42.2%)	16 (64.0%)	31 (64.6%)	0	1	1
2 (3–6)	34 (41.0%)	8 (32.0%)	11 (22.9%)	1	0	0
3 (>6–12)	9 (10.8%)	0	6 (12.5%)	0	0	0
4 (>12)	5 (6.0%)	1 (4.0%)	0	0	0	0

Abbreviations: ALT, alanine aminotransferase; anti-HIV, antibody to human immunodeficiency virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

[a] $P < .001$.

[b] $P < .001$.

[c] $P = .017$.

[d] $P = .002$.

[e] $P = .002$.

[f] $P = .003$.

[g] $P < .001$.

[h] $P < .001$.

[i] $P = .036$.

* Data from anti-HIV-positive patients are excluded.

with acute hepatitis B in this study were very close to one another suggests that most HBV/A strains were imported recently and have spread rapidly, which may be attributed to the features of HBV/A in transmission routes and viral kinetics. We have reported that patients with genotype A tend to have multiple sexual partners [5]. Consequently, chances of secondary transmission of HBV/A would be higher than those of other genotypes, which may increase the number of patients who contract HBV/A infections. On the other hand, HBsAg persisted longer in patients with genotype A than B or C, which is consistent with the in vivo experiment using chimera mice carrying human hepatocytes showing that proliferation of HBV starts later and lasts longer in genotype A than in B or C infection [19].

Our results have shown that 6% of the patients with genotype A develop persistent infection. Because liver cirrhosis or hepatocellular carcinoma can develop in a substantial population of HBV carriers [20, 21], it is important to distinguish the patients in whom HBV infection becomes chronic, and follow them carefully. Although polymorphisms in host genes may be useful for identifying patients who are prone to develop chronic HBV infection [22], simple surrogate markers for the outcome have not been reported. Our data indicate that it would be difficult to predict the clinical outcome based on serum levels of viral markers at the first visit alone. This is understandable, because the dose of infecting virus, as well as the interval between infection and the first visit, can vary widely. Hence, we set out to analyze changes in serum levels of viral markers.

As seen in Figure 2, HBsAg levels at 12 weeks from the onset were most useful for discriminating among groups 2, 3, and 4 in the genotype A infection. Therefore, the outcome of acute hepatitis B may be predictable at this time point. Of note is the reelevation of HBsAg observed in group IV (Supplementary Figure 1*A*). Reelevation of viral markers suggests prolonged viral proliferation in the liver, and may be useful to identify the patients who may develop chronic infection.

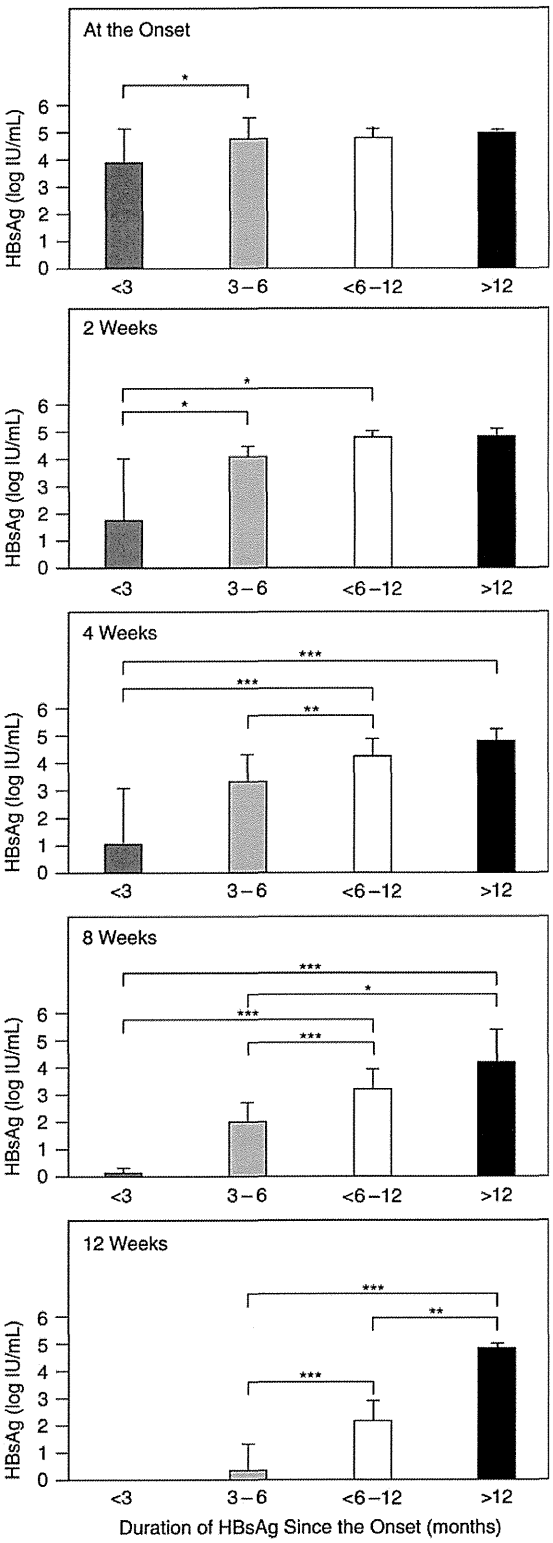

Figure 2. Levels of hepatitis B surface antigen in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviation: HBsAg, hepatitis B surface antigen.

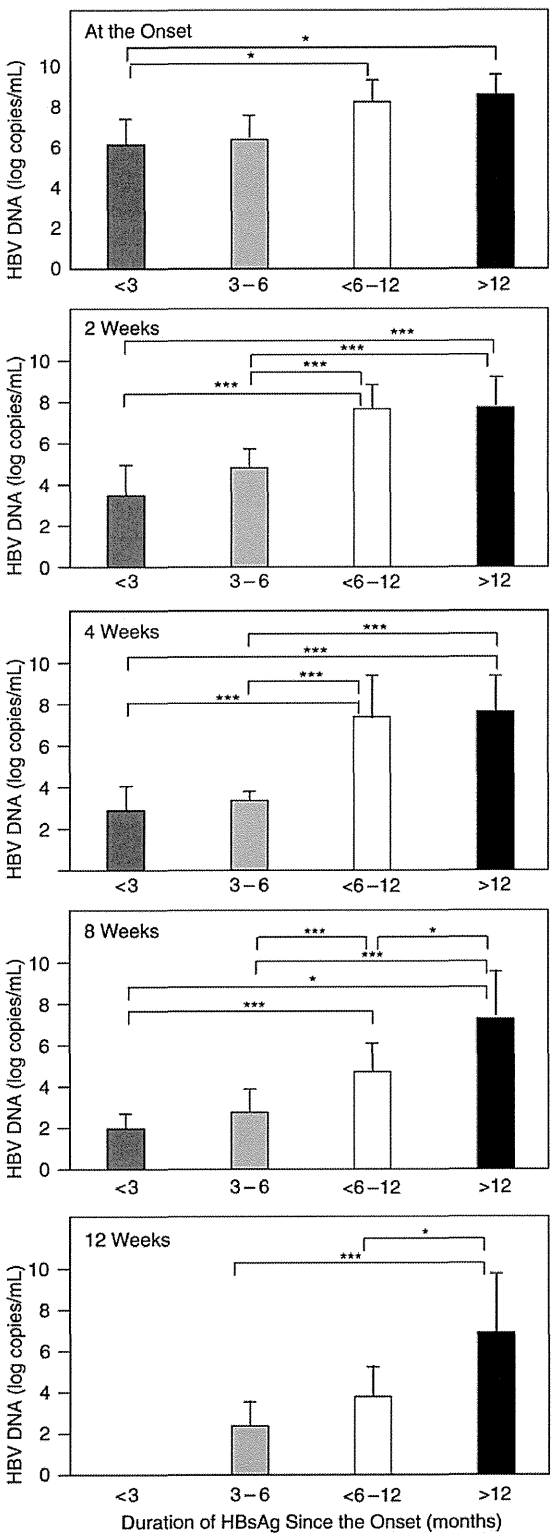

Figure 3. Levels of hepatitis B virus DNA in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A. *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

As shown in Figure 3, HBV DNA levels at 4 weeks from the onset can discriminate groups 1/2 from groups 3/4. Furthermore, HBV DNA levels at 8 weeks from the onset can distinguish group 4 from group 1, 2, or 3. Therefore, the combination of HBV DNA levels at weeks 4 and 8 would be useful for predicting the outcome. For the prediction of a chronic outcome, HBV DNA level at 8 weeks from the onset is a useful surrogate marker of the outcome as well as HBsAg level at 12 weeks. There were differences in viral kinetics among groups 1, 2, 3, and 4.

Our present study showed that 15 of the 215 patients (7.0%) cleared HBsAg from >6 to 12 months after the onset. Sixty percent of the 15 patients had HBV/A. Although these patients met the criteria of chronic infection, they finally cleared HBsAg from the sera. Therefore, we would like to propose that transition to chronic infection in acute hepatitis B be judged at 12 months from onset in patients with genotype A; further studies in larger cohorts are necessary. One reason for our proposal is the indication of antiviral treatment. Antiviral treatment in patients with acute hepatitis B is not indicated because previous studies failed to show the efficacy of antiviral treatments in the patients with acute hepatitis B [23, 24]. However, if patients who actually develop chronic infection can be identified and treated by antiviral treatment, the number of those who develop secondary infection may be markedly reduced. Evaluation of the efficacy of antiviral treatments by prospective studies, based on surrogate markers for the outcome, should be conducted as the next step. HBeAg, which was reported to be useful as a surrogate marker for chronicity, should also be assessed as a surrogate marker [25, 26].

Our study has some limitations. First, the lack of data in early stages made it difficult to study viral kinetics precisely. Second, viral kinetics in the infection with each HBV genotype were obtained from a restricted number of patients, not large enough to establish the usefulness of changes in viral markers in earlier stages of HBV infection. Third, anti-HIV was not checked in all patients due to the lack of informed consent. Fourth, HBsAg and HBV DNA were not determined 24 weeks after onset when discrimination between groups 3 and 4 may be possible more easily. Fifth, the maximum levels of ALT and bilirubin may be affected by the time of blood test. Validation studies in larger cohorts are necessary to evaluate the feasibility of our hypotheses.

In conclusion, we have shown that viral kinetics and the clinical outcome are different among patients with acute hepatitis B who are infected with HBV of distinct genotypes. HBsAg levels at 12 weeks and HBV DNA at 8 weeks after the onset would be useful to predict the clinical outcome of patients with acute hepatitis B.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter NTCP

Koichi Watashi[1*], Ann Sluder[2], Takuji Daito[1, 2], Satoko Matsunaga[3], Akihide Ryo[3], Shushi Nagamori[4], Masashi Iwamoto[1], Syo Nakajima[1], Senko Tsukuda[1, 5], Katyna Borroto-Esoda[2], Masaya Sugiyama[6], Yasuhito Tanaka[7], Yoshikatsu Kanai[4], Hiroyuki Kusuhara[8], Masashi Mizokami[6], Takaji Wakita[1]

[1]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, [2]SCYNEXIS, Inc., Durham, North Carolina, USA, [3]Department of Microbiology, Yokohama City University School of Medicine, Yokohama, Japan, [4]Osaka University Graduate School of Medicine, [5]Micro-signaling Regulation Technology Unit, RIKEN Center for Life Science Technologies, Wako, Japan, [6]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan, [7]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medicinal Sciences, Nagoya, Japan, [8]The University of Tokyo Graduate School of Pharmaceutical Sciences, Tokyo, Japan.

E-mail address: Koichi Watashi, kwatashi@nih.go.jp; Ann Sluder, Ann.Sluder@scynexis.com; Takuji Daito, daitot@wudosis.wustl.edu; Satoko Matsunaga, smatsu02@yokohama-cu.ac.jp; Akihide Ryo, aryo@yokohama-cu.ac.jp; Shushi Nagamori, nagamori@pharma1.med.osaka-u.ac.jp; Masashi Iwamoto, miwamoto@nih.go.jp; Syo Nakajima, s-nakaji@nih.go.jp; Senko Tsukuda, stsukuda@nih.go.jp; Katyna Borroto-Esoda, Katyna.Borroto-Esoda@scynexis.com; Masaya Sugiyama, m.sugiyama@hospk.ncgm.go.jp; Yasuhito Tanaka, ytanaka@med.nagoya-cu.ac.jp; Yoshikatsu Kanai, ykanai@pharma1.med.osaka-u.ac.jp; Hiroyuki Kusuhara, kusuhara@mol.f.u-tokyo.ac.jp; Masashi Mizokami, mmizokami@hospk.ncgm.go.jp; Takaji Wakita, wakita@nih.go.jp

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***Contact Information:** Koichi Watashi, Ph.D.
Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan
Tel: +81-3-5285-1111; Fax: +81-3-5285-1161
E-mail: kwatashi@nih.go.jp

Abbreviations: HBV, hepatitis B virus; CsA, cyclosporin A; NTCP, sodium taurocholate cotransporting polypeptide; IFN, interferon; PPIase, peptidyl prolyl cis/trans-isomerase; CyPs, cyclophilins; CN, calcineurin; MDR, multidrug resistance; MRP, MDR-related protein; HCV, hepatitis C virus; PHH, primary human hepatocytes; HBs, viral envelope protein; LHBs, large envelope protein; MHBs, middle envelope protein; SHBs, small envelope protein; HCVpp, HCV pseudoparticles; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid

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Abstract

Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. Although nucleos(t)ide analogs inhibiting viral reverse transcriptase are clinically available as anti-HBV agents, emergence of drug resistant virus highlights the need for new anti-HBV agents interfering with other targets. Here we report that cyclosporin A (CsA) can inhibit HBV entry into cultured hepatocytes. The anti-HBV effect of CsA was independent of binding to cyclophilin and calcineurin. Rather, blockade of HBV infection correlated with the ability to inhibit the transporter activity of sodium taurocholate cotransporting polypeptide (NTCP). We also found that HBV infection susceptible cells, differentiated HepaRG cells and primary human hepatocytes expressed NTCP, while non-susceptible cell lines did not. A series of compounds targeting NTCP could inhibit HBV infection. CsA inhibited the binding between NTCP and large envelope protein in vitro. Evaluation of CsA analogs identified a compound with higher anti-HBV potency, having an $IC_{50} < 0.2$ μM. This study provides a proof of concept for the novel strategy to identify anti-HBV agents by targeting the candidate HBV receptor, NTCP, utilizing CsA as a structural platform.

Introduction

Hepatitis B virus (HBV) infection is a substantial public health problem, affecting approximately 350 million people worldwide (1-3). HBV-infected patients have an elevated risk for developing liver cirrhosis and hepatocellular carcinoma. Currently, clinical treatment for HBV infection includes interferon (IFN)α and nucleos(t)ide analogs. IFNα therapy yields long-term clinical benefit in only less than 40% of patients and can cause significant side effects. Nucleos(t)ide analog treatment can suppress HBV replication and is accompanied by substantial biochemical and histological improvement, however, it may select for drug resistant viruses, which limit the efficacy of long term treatment. To overcome these problems, the development of new anti-HBV agents targeting a different step of the HBV life cycle is urgently needed.

As HBV has only one viral gene encoding an enzymatic activity, the polymerase, there is no apparent strategy to develop a new class of antiviral agents other than polymerase inhibitors. Hence, it is important to define alternative molecular targets for anti-HBV agents as well as to identify potential anti-HBV compounds (3, 4). Myrcludex-B is a peptide mimicking pre-S1, which is crucial for virus-cell membrane interaction. Pretreatment with this peptide has been shown to prevent virus entry and spread of virus infection (5, 6). Phenylpropenamide derivatives and heteroaryl-pyrimidines (HAP) suppressed HBV replication through capsid disassembly (7-10). Although the development of the former was discontinued because of significant toxicity (3), HAP exhibited anti-HBV efficacy in the absence of robust toxicity (8, 10). Deoxynojirimycin derivatives are iminosugars that inhibit alpha-glucosidases. Although treatment with these compounds suppressed HBV secretion in both cell culture and mouse models (11, 12), further investigation will be required to assess their anti-HBV efficacy and the specificity to HBV. Thus, it is an attractive strategy to identify a cellular factor that is specifically involved in HBV infection and relevant for the development of anti-HBV agents.

Cyclosporin A (CsA) is an immunosuppressant clinically used for suppression of the immunological failure of xenograft tissues. CsA primarily targets cellular peptidyl prolyl cis/trans-isomerase (PPIase) cyclophilins (CyPs) (13). The resultant CsA/CyP complex subsequently binds to and inhibits calcineurin (CN), a phosphatase that dephosphorylates nuclear factor of activated T cell (NF-AT) to allow nuclear translocation and transactivation of

downstream genes. This CN inhibition contributes to the suppression of immune responses. In addition, CsA is known to inhibit the transporter activity of membrane transporters, including the multidrug resistance (MDR) and MDR-related protein (MRP) families (14). Previously, we demonstrated that CsA and its non-immunosuppressive derivatives suppress hepatitis C virus (HCV) replication (15, 16), with the anti-HCV activity being mediated by the inhibition of CyPs (17-19). Currently, a series of drugs classified as CyP inhibitors are in clinical development for treatment of HCV-infected patients (20, 21).

In this study, we report that CsA and its analogs inhibited HBV entry through a CyP-independent mechanism. We established a screening system that can identify small molecules inhibiting HBV entry. Screening in this system revealed that CsA blocked HBV entry. The anti-HBV activity of CsA was not correlated with binding to CyPs and CN. CsA inhibited the transporter activity of NTCP, a recently reported candidate for the HBV entry receptor (22), and interrupted the binding between NTCP and large envelope protein in vitro. Other NTCP inhibitors also blocked HBV infection. Analog testing identified CsA-related compounds with higher anti-HBV potency than CsA. Thus, CsA and NTCP inhibitors can be used as a platform to develop a novel class of anti-HBV agents.

Experimental Procedures

Cell culture

HepaRG (Biopredic), HepAD38 (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center), and primary human hepatocytes (PHH) were cultured as described previously (23).

HBV preparation and infection

HBV used in this study was mainly derived from the culture supernatant of HepAD38 cells. HBV infection was performed as described previously (23). More detailed procedures are given in the Supporting Information.

Indirect immunofluorescence analysis, real time PCR, Southern blot analysis, MTT assays, and reporter assays

Indirect immunofluorescence analysis, real time PCR, Southern blot analysis, MTT assays, and reporter assays were performed essentially as described (23). More detailed procedures are given in the Supporting Information.

Detection of HBs and HBe antigens

HBs antigen was quantified by ELISA as described previously (23). HBe antigen was detected by a Chemiluminescent Immuno-Assay (Mitsubishi Chemical Medience).

HCV pseudoparticle assay

The HCV pseudoparticles (HCVpp), which reproduce HCV envelope-mediated entry, were generated by transfecting the expression plasmids for MLV Gag-Pol, HCV E1E2, and a luciferase that can be packaged into the virion (kindly provided by Dr. Francois-Loic Cosset at University of Lyon) into 293T cells. HCVpp recovered from the culture supernatant of transfected cells were used in a HCV entry assay as described previously (24).

Transporter assay

Transporter activity of NTCP was assayed essentially as described (25) using 293 and HepG2 cells permanently overexpressing human NTCP (Sekisui Medical). Briefly, the cells were preincubated with compounds at 37°C for 15 min and then incubated with radiolabeled substrate, [^{3}H]taurocholic acid (TCA), at 37°C for 5 min to allow substrate uptake into the cells. The cells were then washed and lysed to measure the accumulated radioactivity. In this assay, we did not observe cytotoxic effects of compounds at any of the concentrations tested. More detailed procedures are given in the Supporting Information.

AlphaScreen assay

Recombinant NTCP and HBs proteins, which were tagged with 6xHis and biotin, respectively, were synthesized using a wheat cell-free protein system as described previously (26). Protein-protein interactions were detected using the AlphaScreen IgG (ProteinA) detection kit (PerkinElmer) according to the manufacturer's instruction. Briefly, the recombinant tagged proteins were incubated with streptavidin-coated donor beads and anti-6xHis antibody-conjugated acceptor beads that generate a luminescence signal when

brought into proximity by binding to interacting proteins. Luminescence was analyzed with the AlphaScreen detection program of an Envision spectrophotometer (PerkinElmer). More detailed procedures for the AlphaScreen assay are described in the Supporting Information.

Additional experimental procedures are included in the Supporting Information.

Results

Cyclosporin A blocked HBV infection

We focused on HBV entry and established a cell culture system to evaluate this step in HBV infection. To identify small molecules inhibiting HBV entry, we pretreated HepaRG cells (27) with compounds for 2 h, then added a HBV inoculum and continued incubation with compounds for 16 h (Fig. 1A). After washing out free HBV and compounds, the cells were cultured for an additional 12 days in the absence of compounds (Fig. 1A). For robust chemical screening, HBV infection was monitored by the viral envelope protein (HBs) level secreted from the infected cells at 12 days postinfection by ELISA. This assay could identify heparin, an HBV attachment inhibitor (28, 29), and bafilomycin A1, a v-type H^+ ATPase inhibitor that blocks acidification of vesicles and HBV entry (30), but not lamivudine, a reverse transcriptase inhibitor (31), as compounds reducing HBs protein level in the medium (Fig. 1B). In addition, use of an anti-HBs antibody to neutralize viral entry, but not use of an anti-FLAG antibody, reduced viral protein secreted from the HBV-infected cells (Fig. 1B). Thus, this system is likely to evaluate the effect of compounds on the early phase of the HBV life cycle, including attachment and entry, but not effects on HBV replication. A chemical screen with this system revealed that CsA reduced HBs secretion from HBV-infected cells (Fig. 1B). Treatment with CsA significantly decreased HBc protein expression (Fig. 1C) and HBV DNA as well as cccDNA (Fig. 1D) in the cells and HBe in the medium (Fig. 1E), without causing cytotoxicity (Fig. S1A). This effect of CsA was not limited to infection of HepaRG cells as we observed a similar anti-HBV effect of CsA for primary human hepatocytes (Fig. 1F). The anti-HBV effect of CsA was also observed on HBV infection of primary human hepatocytes in the absence of PEG8000 (Fig. S1B), indicating that the effect of CsA did not depend on PEG8000, which was normally included in