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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.[9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

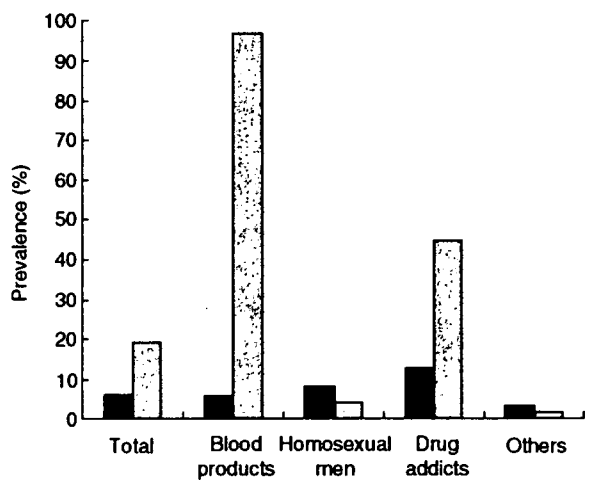

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV-HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV-HBV co-infected patients in the current study. Nonetheless, one-third of HIV-HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV-HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV-HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV-HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV-HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV-HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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Original Article

Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan

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People with human immunodeficiency virus (HIV) infection are frequently infected with hepatitis C virus (HCV), because of the common transmission routes. Since the dissemination of hyperactive antiretrovirus therapy (HAART), the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made HCV-associated liver diseases the leading cause of mortality in Western countries. A similar situation is assumed in Japan, but the status of coinfection with HIV and HCV is unclear. We conducted a nationwide survey to determine the prevalence of coinfection with HIV and HCV by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan. Among 4877 patients reported to be HIV-positive, 935 (19.2%) were also positive for the anti-HCV antibody. Most (84.1%) of the patients coinfected with HIV and HCV were recipients of blood products. These data, for the first time, show the current status of coinfection with HIV and HCV in Japan. A detailed analysis of the progression and severity of liver diseases in the coinfected patients is expected.

Key words: coinfection, hepatitis C, HIV, liver disease

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection and human immunodeficiency virus (HIV) infection are major public health problems worldwide. In the USA, the estimated prevalence of the anti-HCV antibody is 1.8%, with 2.7 million people having HCV-RNA detected in their blood, indicative of ongoing HCV infection.[1] The prevalence of HIV is <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral ones, many people with HIV infection are also infected with HCV.[3] Before the introduction of hyperactive antiretroviral treatment (HAART) in 1996, most people with HIV infection died of HIV-associated opportunistic infections such as *Pneumocystis carinii* (currently called *P. jiroveci*) pneumonia and cytomegaloviral infection. Since the dissemination of HAART, the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made patients coinfected with HIV and HCV faced with the menace of progressive liver diseases due to HCV infection in the United States and Europe.[4,5]

Coinfection with HIV has been shown to increase the HCV load in HCV infection,[6] being a negative prognostic factor for clearance of HCV in anti-HCV therapy using interferon.[7,8] It also accelerates the development of cirrhosis and, eventually, hepatocellular carcinoma. Although still controversial, coinfection with HIV and HCV yields a more rapid progression to acquired immunodeficiency syndrome (AIDS) in some cases.[9,10] Importantly, coinfection with HIV and HCV will increase the morbidity and mortality of HIV-infected patients also in Japan, where the prevalence of HIV infection is increasing in a linear fashion, exceptionally among developed countries.[11] There are more than 10 000 HIV-positive people in Japan as of the end of 2004, according to the AIDS National Survey in Japan,[12] and approximately 1.8 million chronic HCV carriers, according to the estimation by the Ministry of Health, Labor and Welfare (MHLW) of Japan. However, unfortunately, the prevalence of coinfection with HIV and HCV in Japan has been unclarified to date. Therefore, we conducted a nationwide study by distributing an

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email-based questionnaire to the hospitals in the HIV/AIDS Network of Japan.

METHODS

IN THE QUESTIONNAIRE, the following information was obtained from hospitals regarding the number of patients who visited the hospitals at least once between January and December 2003: (1) the number of HIV-positive patients; (2) the number of anti-HCV-positive patients among (1); (3) the number of HCV-RNA-positive patients among (2); (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of anti-HCV-positive patients among (4); (6) the number of HCV-RNA-positive patients among (5); (7) the number of HIV-positive patients among men who have sex with men (MSM); (8) the number of anti-HCV-positive patients among (7); (9) the number of HCV-RNA-positive patients among (8); (10) the number of HIV-positive patients who contracted HIV through intravenous drug use; (11) the number of anti-HCV-positive patients among (10); (12) the number of HCV-RNA-positive patients among (11); (13) the number of HIV-positive patients whose transmission routes were classified as 'others'; (14) the number of anti-HCV-positive patients among (13); and (15) the number of HCV-RNA-positive patients among (14).

The questionnaire was sent to the 366 hospitals in the HIV/AIDS Network of Japan by email. When emails were returned with a failure of delivery, the questionnaire was forwarded by post. Answers were mostly returned by email, and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be browsed at: http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 366 hospitals that were on the list of hospitals in the HIV/AIDS Network of Japan in January 2004. One hundred and seventy-six hospitals (48.1%) responded within the indicated period. A collection rate of 47.8% may appear rather low, particularly considering the number of reported HIV-positive people, 10 000, in 2004 according to the statistics of the MHLW of Japan.[12] However, not all the HIV-positive cases are visiting hospitals, and answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. These factors suggest that not all but a majority of HIV-positive patients in Japan were enrolled in the study.

Table 1 Number of hospitals categorized by the number of patients infected with HIV and those coinfected with HIV and HCV

No. of HIV(+)/HCV(+)	No. of HIV(+)				
	0	1–19	20–49	50+	Total
0	43	52	5	1	101
1–9	0	45	9	3	57
10+	0	2	4	12	18
Total	43	99	18	16	176

There were one or more HIV-positive patients in 133 of 176 (75.6%) hospitals; there were no HIV-positive patients in the remaining 43 hospitals (Table 1). Eighteen of 176 (10.2%) hospitals had 20–49 HIV-positive patients, and 16 (9.1%) hospitals had 50 or more HIV-positive patients. On the other hand, there were one or more patients who were coinfected with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfected patients in 18 (10.2%) hospitals. HIV/HCV coinfected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area (Fig. 1). Of the 16 hospitals with 50 or more HIV-positive patients and of the 18 hospitals with 10 or more HIV/HCV coinfected patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfected patients, but with less than 50 HIV-positive patients had the characteristic that most HIV-positive patients contracted HIV from blood products.

In total, 4877 patients were reported to be HIV-positive. Among these, 935 (19.2%) were positive for anti-HCV (Table 2). Of these 935 patients, 780 were HCV-RNA-positive, although it should be noted that not all the patients underwent HCV-RNA testing.

HCV prevalence when fractionated by routes of transmission was as follows. Among 811 HIV-positive patients who contracted HIV from blood products such as unheated concentrated coagulation factors, 786 (96.9%) were anti-HCV-antibody-positive. Of 20 intravenous drug users, nine (45.0%) were anti-HCV-antibody-positive. Among 2730 HIV-positive patients who were MSM (men who have sex with men), 114 (4.2%) were anti-HCV positive. In the remaining 1316 HIV-positive patients whose routes of HIV transmission

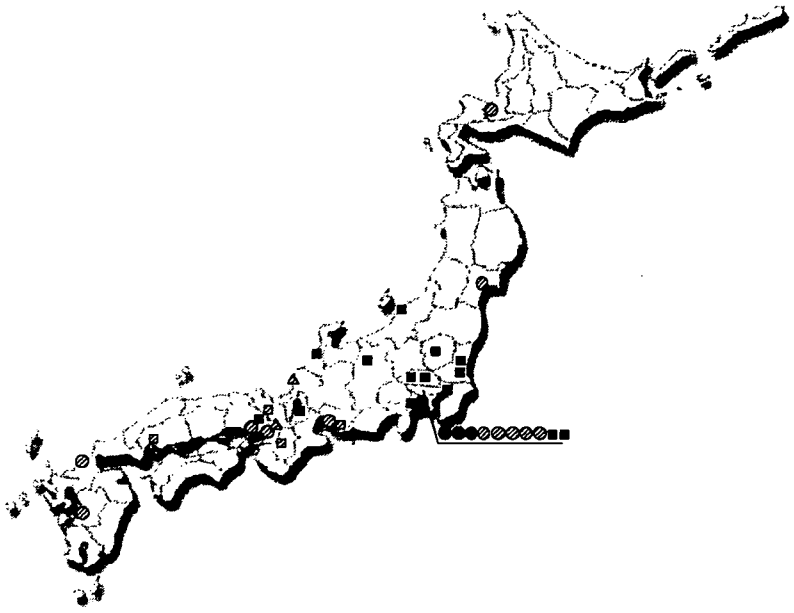

Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (△) hospitals with 1–19 HIV-positive patients; (□) hospitals with 20–49 HIV-positive patients; (○) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfected patients. Closed figures: hospitals with less than 10 HIV/HCV coinfected patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfected patients are omitted from the figure.

were classified as "others", most of whom contracted HIV heterosexually, 26 (2.0%) were anti-HCV-antibody-positive. On the other hand, in HIV/HCV coinfected patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfected patients in Japan are those who contracted HIV, and most likely also HCV, from blood products.

DISCUSSION

ACCORDING TO THE statistics of the MHLW of Japan, the number of reported HIV-positive people was just over 10 000 in 2004.[12] The total number of HIV-positive patients in the current study is approximately half of that. By a simple calculation, there would be about 1900 HIV/HCV coinfected patients in Japan. However, because HIV-positive patients who contracted HIV from blood products are almost all registered in Japan and most of them should have been enrolled in this survey, the number of HIV/HCV coinfected patients is likely smaller than 1900. It is regrettable that not all the patients underwent HCV-RNA testing, but it is unavoidable in this type of questionnaire-based study. In some cases, the existence of a positive anti-HCV antibody indicates a memory of a remote HCV infection.

Almost all of the patients who contracted HIV through blood products were also anti-HCV-antibody-positive, suggesting that both viruses were transmitted through the same route. In MSM patients who were HIV-positive, approximately 4% were anti-HCV-antibody-positive, which is about threefold higher than the prevalence of HCV in Japan.[13] In people aging from 40 to 50 years old in the general Japanese population, whose ages are similar to those of the MSM patients in the current study, the prevalence of HCV is less than 0.5%.[13] Therefore, an HCV prevalence of 4% in MSM

Table 2 Prevalence of HCV infection in HIV-positive patients

Routes of transmission	No. of patients	Anti-HCV-positive	HCV-RNA-positive†
Blood products	811	786 (96.9%)	667
MSM‡	2730	114 (4.2%)	98
Drug addicts	20	9 (45.0%)	8
Others (heterosexual etc.)	1316	26 (2.0%)	7
Total	4877	935 (19.2%)	780

†Not all patients were subjected to HCV-RNA test. ‡MSM, men who have sex with men.

HIV-positive patients is quite high, suggesting the same route of the transmission of HIV and HCV, and a more intensive exposure to HCV or more susceptibility to HCV in these HIV-positive patients. Similarly, an HCV prevalence of 1.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.

To establish measures that decrease the morbidity and mortality of HIV/HCV coinfected patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfected patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfected patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfected with HIV and HCV in Japan. A detailed analysis of the progression and severity of HCV-associated liver diseases is expected.

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CASE REPORT

Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report

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Abstract

We present a case of fetal liver failure caused by the activation of lamivudine-resistant hepatitis B virus (HBV) nine months after lamivudine treatment. A 57-year old man visited our hospital for the treatment of decompensated chronic hepatitis B. Lamivudine was started in December 2001. Subsequently, serum HBV was negative for HBV DNA with seroconversion from HBeAg to anti-HBe and improvement of liver function. However, HBV DNA and HBeAg were again detected in September 2002. He was complicated by breakthrough hepatitis and admitted to our hospital in November for severely impaired liver function. Vidarabine treatment was started and serum HBV DNA and alanine aminotransferase (ALT) decreased transiently. However, after the start of α-interferon treatment, HBV DNA level increased and liver function deteriorated. He died 1 mo after admission. An analysis of amino acid sequences in the polymerase region revealed that rtM204I/V with rtL80I/V occurred at the time of viral breakthrough. After the start of antiviral treatment, rtL180M was detected in addition to rtM204I/V and rtL80I/V, and became predominant in the terminal stage of the disease. HBV clone with a high replication capacity may be produced by antiviral treatment leading to the worsening of liver function. Antiviral therapy for patients with breakthrough hepatitis in advanced liver disease should be carefully performed.

Key words: Hepatitis B virus; Lamivudine; Polymerase; Interferon; Tyrosine-methionine-aspartate-aspartate

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INTRODUCTION

Lamivudine is a nucleoside analogue that interrupts the reverse transcription of hepatitis B viral (HBV) pregenomic RNA. Lamivudine is effective for controlling chronic hepatitis B and currently recommended as the first line of treatment for chronic active hepatitis B[1,2]. Even for patients with decompensated liver cirrhosis, lamivudine improves liver function and extends transplantation free intervals[3-10]. Since more than 10% of patients with chronic HBV infection are estimated to develop liver cirrhosis and may eventually suffer from decompensated liver cirrhosis or hepatocellular carcinoma, the role of lamivudine in the treatment of advanced liver disease caused by chronic HBV infection is large[11-14].

The major problems concerning lamivudine treatment are the viral and biochemical breakthroughs caused by drug resistance. Amino acid mutation in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif can occur six months after treatment and often increases alanine aminotransferase (ALT) level. Although the increase is usually mild, a marked increase in ALT level leading to fatal hepatic failure has been reported[15-17]. Factors other than the YMDD motif mutation that are associated with the worsening of liver function remain to be clarified.

Here, we report a case of fatal hepatic failure caused by lamivudine-resistant HBV. A serial analysis of viral amino acid sequences indicated that the acquisition of mutations outside the YMDD motif might be related to the deteriolation of the patient's condition.

CASE REPORT

A 57-year old man visited our hospital in September 2001 for the treatment of decompensated chronic hepatitis

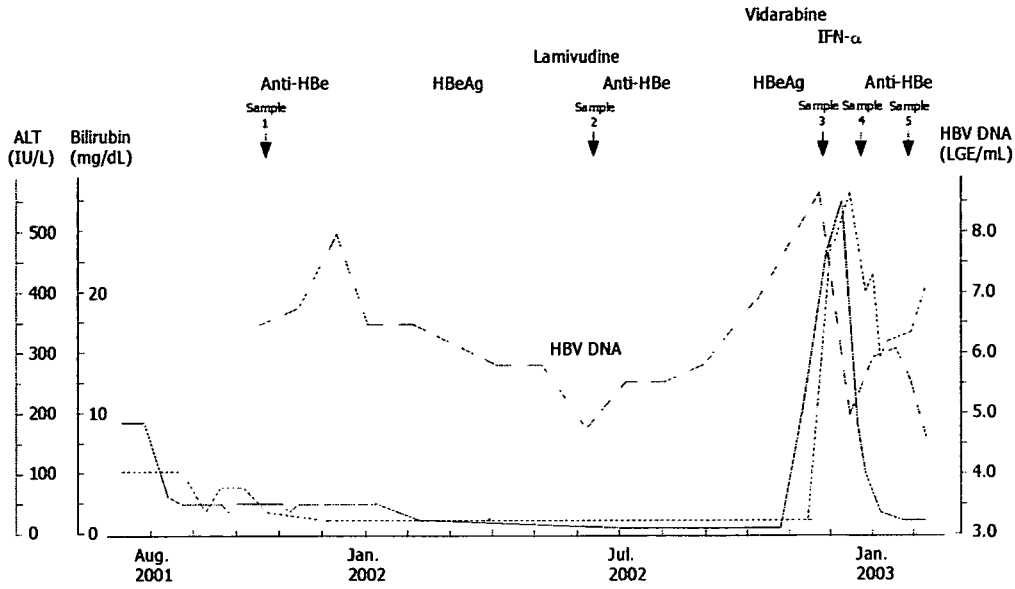

Figure 1 Clinical course of our patient. HBV DNA level was quantified by transcription-mediated amplification assay. The levels of HBV DNA started to increase 8 mo after treatment with reappearance of HBeAg. Breakthrough hepatitis developed 12 mo after treatment. The timing of serum sample analysis for mutations is shown by the arrowhead.

B. In 1978, He was found to be positive for serum HBs antigen (HBsAg). In July 2001, he was admitted to a nearby hospital for ascites where he was diagnosed as having decompensated cirrhosis with exacerbated chronic hepatitis B. The symptomatic control of his ascites improved his general condition. For further treatment, he was referred to our hospital.

On his first visit, he showed no symptoms or signs of worsening hepatic failure or encephalopathy. No ascites or leg edema was observed. His bulbar conjunctiva was slightly jaundiced. Dilated vasculature was observed in his neck and chest. His ALT, total bilirubin and albumin were 50 IU/L, 3.1 mg/dL and 3.7 g/dL, and his prothrombin time was 76%. He was diagnosed as having liver cirrhosis with a Child-Pugh score of 8. He was negative for HBe antigen (HBeAg) and his HBV DNA level measured by transcription-mediated amplification and hybridization protection assay[18] was 106.5 genome copies/mL.

In November 2001, he was found to be positive for HBeAg and showed an increase in HBV DNA level. Because he had a history of decompensated chronic hepatitis B, lamivudine treatment (100 mg/d) was started in December. Figure 1 shows the clinical course. The high serum levels of bilirubin and ALT decreased and normalized within 6 mo after lamivudine treatment was started. The patient became negative for HBV DNA and HBeAg.

However, in September 2002, he was found to be positive for HBeAg again and showed an increase in HBV DNA level. In November 2002, he observed jaundice of his bulbar conjunctiva and was admitted to our hospital. Although he was alert, his bulbar conjunctiva and skin were jaundiced. His ALT, total bilirubin, were 474 IU/L, 11.4 mg/dL and 4.3 g/dL. His HBV DNA level was 108.6 genome copies/mL. He was diagnosed as having breakthrough hepatitis caused by lamivudine-resistant mutants of HBV. HBV with an amino acid substitution in the YMDD motif in the domain C of polymerase region was detected.

Because interferon is not indicated in patients with decompensated cirrhosis, vidarabine, which is effective for the control of active HBV infection[19-21], was administered together with lamivudine under informed consent. Liver function improved transiently with a decrease in HBV DNA within 2 wk. As prolonged vidarabine administration may induce several complications[22], vidarabine was switched to interferon-α. After the start of interferon-α treatment, HBV DNA level increased and liver function worsened. He died of hepatic failure and rupture of esophageal varices 1 mo after his admission.

The histolopathology of the patient's liver after necropsy showed cirrhosis with zonal necrosis. Hepatocyte regeneration was scarce (Figure 2).

To elucidate the viral factors affecting early viral breakthrough and fatal outcome, amino acid sequences of the upstream polymerase region (aa 1-250) of HBV DNA in serum were examined at 5 points as shown in Figure 1. The methods were as follows.

First, DNA was extracted from 100 μL of a serum sample using the QIAamp DNA blood mini kit (Qiagen Inc., Valencia, CA). Three fragments spanning the upper polymerase region of HBV DNA were amplified by nested PCR with the primers shown in Table 1. The first stage of amplification was carried out using a thermal cycler for 40 cycles (94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min) in 100 μL of reaction mixture containing 200 mmol/L dNTPs, 1.0 mmol/L each of the primers and 1 × PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.001% (w/v) gelatin] and 2 units of Ampli-Taq polymerase gold (Perkin Elmer Cetus Corp., CT). Two microliters of the PCR products was subjected to the second stage of amplification under the same conditions as the first stage.

Second, PCR products were purified using Wizard PCR preps DNA purification resin (Promega, WI) and cloned into a plasmid vector using the TA cloning kit (PCR cloning kit Qiagen, CA). Four clones were selected from each plate, from which recombinant plasmid DNA was

Table 1 Primers used for amplification and sequencing of polymerase region of HBV

Region 1		
Outer sense	nt 2222-2241	CTTACTTTTGGAAGAGAAAC
Outer antisense	nt 2490-2509	GGACAGTAGAAGAATAAAG
Inner sense	nt 2222-2241	CTTACTTTTGGAAGAGAAAC
Inner antisense	nt 2478-2497	GAATAAAGCCCAGTAAAGTT
Region 2		
Outer sense	nt 2413-2434	GCGTCGCAGAAGATCTCAATC
Outer antisense	nt 2816-2835	GTTCCCAAGAATATGGTGAC
Inner sense	nt 2434-2452	CTCGGGAATCTCAATGTTAG
Inner antisense	nt 2816-2835	GTTCCCAAGAATATGGTGAC
Region 3		
Outer sense	nt 2490-2509	CTTTATTCTTCTACTGTACC
Outer antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG
Inner sense	nt 2637-2656	ATGCCTGCTAGGTTTTATCC
Inner antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG

purified using a commercially available kit (Plasmid midi kit, Qiagen, Valencia, CA). Nucleotide sequences were determined bidirectionally using the dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, CA) and the PCR primers. Sequencing was performed using an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The determined amino acid sequences in the polymerase region are shown in Figure 3. No amino acid sequence changes were found at the start of lamivudine treatment. At the time of viral breakthrough, rtM204I with rtL80I became dominant. After the start of interferon treatment, rtM204I was replaced by rtM204V and rtL80I by rtL80V. At the final stage of the disease, mutation rtL180M appeared besides rtM204V and rtL80V.

DISCUSSION

Lamivudine monotherapy is effective in suppressing HBV replication and ameliorating liver disease in chronic hepatitis B patients regardless of HBeAg positivity. A one-year study of HBeAg-positive chronic hepatitis B patients showed that 16% of these patients become seroconverted to anti-HBe and 72% of these patients showed normalization of their ALT levels[23]. Furthermore, treatment with lamivudine is associated with histologic improvement not only in terms of necroinflammatory score but also in terms of fibrosis score after long-term treatment[24].

One advantage of lamivudine is that it can be used safely in patients with decompensated cirrhosis[3-10]. In contrast to IFN-α, lamivudine is well tolerated without any significant side effects even in patients with decompensated cirrhosis. Furthermore, lamivudine can improve liver function and survival prognosis.

However, the emergence of a drug-resistant mutant is a big problem in lamivudine treatment. A large-scale Asian study showed that lamivudine resistant HBV infection occurred in 23 % of patients in year one and 65 % of patients in year five. Hepatitis flares, which occurred more commonly in patients with lamivudine resistant mutations, occurred in 10% of patients in year one, and in 18% to 21% of patients in years two to five. Among patients with

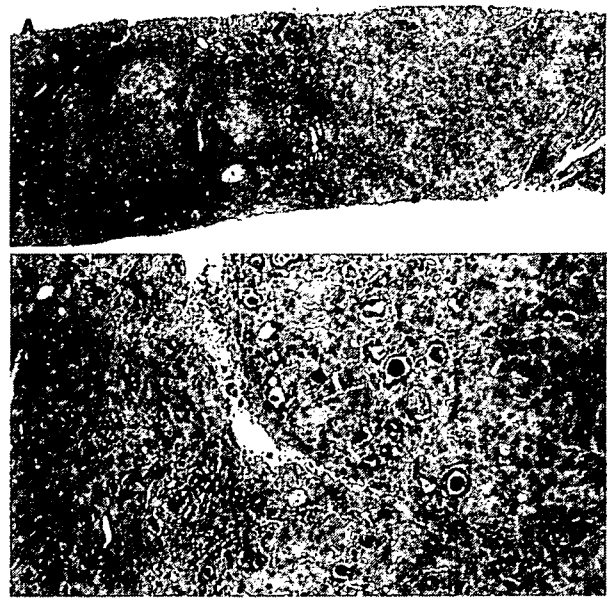

Figure 2 Histopathological findings of liver specimens. Irregularly-shaped parenchymal cells with massive necrosis (**A**) and scarce hepatocyte regeneration (**B**) surrounded by extensive fibrosis (**A**: HE × 20; **B**: HE × 80).

lamivudine resistant HBV infection, occurrence of hepatic decompensation increased significantly in patients with lamivudine resistant HBV infection for more than 4 years (from 0% to 6%)[25]. In this large-scale Asian study, liver-disease-related death occurred in two patients.

The prognosis of patients with lamivudine-resistant HBV infection, particularly those with advanced liver disease, may be determined by the timing and severity of breakthrough hepatitis. However, the viral factors that may influence the severity of this hepatitis remain to be clarified. A recent study indicated that patients with a normal ALT level even after the emergence of a YMDD motif mutant are characterized by HBeAg negativity during pretreatment, HBeAg loss during therapy, a longer duration from the commencement of therapy until the emergence of YMDD mutant, and lack of mixed-type YMDD mutants[26]. In contrast, patients with severely exacerbated hepatitis after the emergence of a YMDD mutant tend to have more substitutions in the reverse transcriptase (rt) region within the polymerase gene at the time of hepatitis exacerbation than those without hepatitis exacerbation[26].

Our patient acquired amino acid mutations in the polymerase region one after the other. Amino acid changes in rtM204/I appeared at the time of viral breakthrough. After the initial treatment with vidarabine, rtM204/V substituted for rtM204/I in one of the four clones. During the interferon treatment, rtM204/V became predominant.

Another mutation observed in our patient was rtL80I/V. Ogata *et al*[27] showed that rtL180M is accompanied with rtM204I in some patients with resistance to lamivudine. Because the mutation at aa position 80 was found at the same time as that at aa position 204 in our patient, it is not clear whether the mutation at aa position 80 affects the clinical course.

At the final stage of the disease with deterioration of

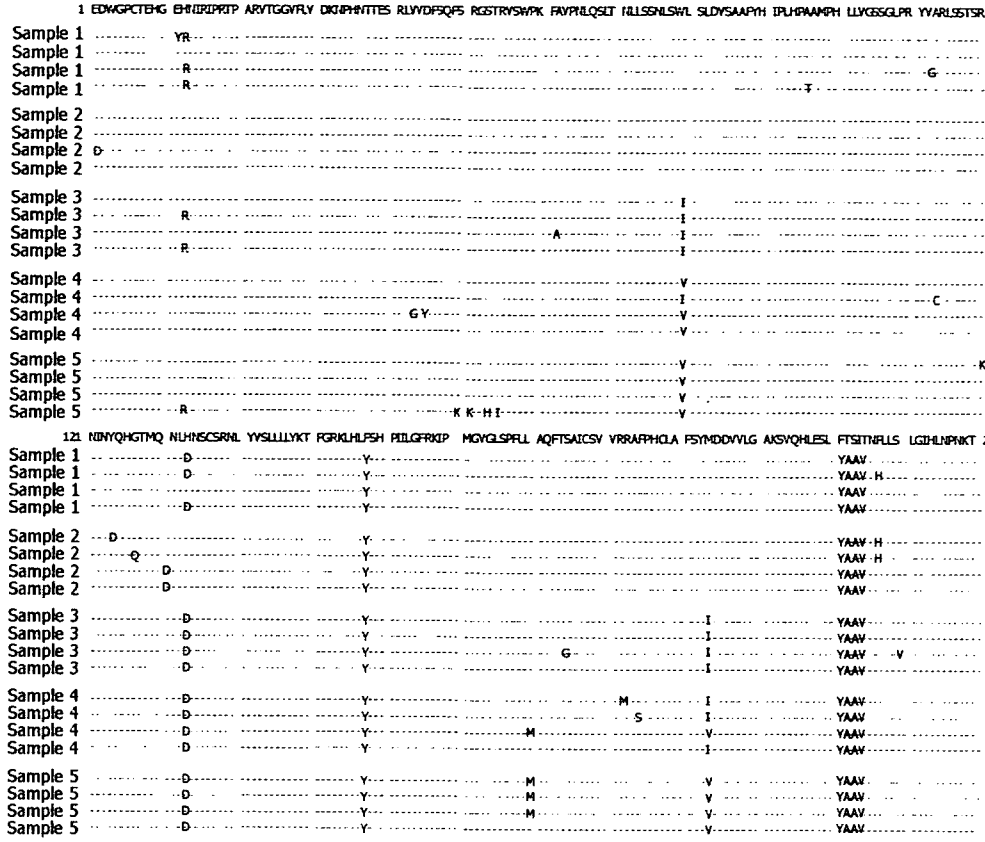

Figure 3 Comparison of amino acid sequences of HBV polymerase gene of isolates before lamivudine treatment (sample 1) and four sequential isolates (samples 2-5) during treatment. A HBV mutant with substitutions of isoleucine for leucine at residue 80 (rtL80I) in combination with isoleucine for methionine at residue 204 (rtM204I) was observed 12 mo after treatment (sample 3). After vidarabine treatment, another HBV mutant with substitutions of valine for leucine at residue 80 (rtL80V) and valine for methionine at residue 204 (rtM204V) was observed (sample 4). These mutations predominated in combination with methionine for leucine at residue 180 (rtL180M) after interferon treatment (sample 5). The published HBV DNA sequence of hepatitis B virus variant (genotype C, AB033550, Okamoto *et al*[28]) was used for comparison

the condition of the patient, rtL180M, rtM204I/V and rtL80I/V became predominant. Natsuizaka *et al*[26] showed that rtL180M and rtM204V are related to the exacerbation of hepatitis. Interestingly, a patient with a marked elevation in HBV DNA level in Ogata's report had rtL180M in addition to rtL80I and rtM204I. Therefore, the acquisitions of rtL80I/V and rt180M in addition to rtM204I/V may be associated with a severe exacerbation of hepatitis. Large-scale studies are necessary to elucidate this hypothesis.

In our patient, vidarabine decreased HBV DNA levels and improved liver function. Although the long-term use of vidarabine is contraindicated because of its possible side effects including irreversible neurotoxicity[27], its short-term use is effective for controlling active HBV infection and herpes simplex viral infection.

Vidarabine was replaced by interferon-α because adefovir dipivoxil was not available in 2002. Serum HBV DNA and bilirubin levels increased again, which led to a fatal outcome, and HBV clones that have rtL180M became predominant, indicating that withdrawal of vidarabine and administration of interferon may be dangerous for the treatment of severe breakthrough hepatitis. Since interferon may potentially precipitate immunological flares and liver failure[29], nuoceoside analogues that are effective for lamivudine-resistant HBV such as adefovir dipivoxil[30-32], entecavir[33-35] and tenofovir[36,37], should be used for the treatment of severe breakthrough hepatitis instead of vidarabine or interferon.

In conclusion, antiviral therapy should be considered in the treatment of patients with hepatic failure after breakthrough hepatitis caused by HBV mutants to lamivudine. The serial acquisition of amino acid mutations outside the YMDD motif in the polymerase region may be associated with severe hepatitis.

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Original Article

Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis

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Background: An increase in the number of acute hepatitis patients with hepatitis B virus (HBV) of non-indigenous genotypes may reduce the efficacy of vaccination against HBV.

Methods: We have determined the amino acid (aa) sequences in the major hydrophilic region (MHR) in the S region of HBV in patients with acute hepatitis B and compared those with the ones from HBV strains used for the production of HBV vaccines commercially available in Japan.

Results: Of 48 patients studied, 11 were infected with genotype A, 11 with genotype B and 26 with genotype C HBV. The aa sequences of the nine genotype A isolates were the same as the aa sequence of J02205 which is used for the production of one of the commercially available recombinant vaccines. The aa sequences of the 11 genotype B isolates differed from the aa sequence of J02205 in two or three amino acids. Of the 26 genotype C isolates, 22 had the same aa sequence as X01587 which is used for the production of another recombinant vaccine. The remaining genotype C isolates had aa substitutions at aa131, which have a potential to alter the hydropathy and the three-dimensional structure of the MHR. The differences among the three current HBV vaccines in aa sequences in the MHR theoretically alter the hydropathy and three-dimensional structure.

Conclusion: Our results suggest that the transmission of HBV isolates with different genotypes or with aa substitutions in the MHR might reduce the efficacy of currently available HBV vaccines in the protection of HBV infections.

Key words: genotype, hepatitis B virus, major hydrophilic region, vaccine

INTRODUCTION

ABOUT 300 MILLION people in the world are chronically infected with hepatitis B virus (HBV). Chronic infection may eventually lead to liver cirrhosis or hepatocellular carcinoma.[1–4] To prevent the transmission of this virus, vaccination has been introduced in many countries. Indeed, universal vaccination has not only reduced the number of infected individuals, but also the number of deaths related to HBV.[5,6]

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In Japan, in 1985, a national project was started to vaccinate children born to HBV-infected mothers. The chances of vertical transmission from HBV-carrying mothers have decreased. Recently, the prevalence of HBV in Japan has decreased to approximately 0.6%.[7]

Because the number of individuals infected with HBV has decreased, the number of patients with acute hepatitis B, mainly caused by horizontal transmission from HBV carriers, should also have decreased. However, in Japan, the number of patients with acute hepatitis B has recently increased (Yatsuhashi H. *et al.*, 2004, unpubl. data).

The increase in the number of patients with acute hepatitis B may, in part, be the result of patients carrying novel HBV genotypes imported from abroad. For

example, in recent years, genotype A HBV has often been detected in patients with acute hepatitis B.[8,9]

Genotype A HBV is transmitted from individuals who live in or have immigrated from other countries to Japan. Its infection is characterized by a high viral load and a long hepatitis B surface antigen (HBsAg) positivity period. The transition of acute hepatitis B with genotype A HBV infection to the chronic state has been reported recently.[8,10] Decreasing the transmission rate of genotype A HBV is therefore important for the control of the disease. Introducing universal vaccination for adolescents or adults is a measure to be considered.

The effectiveness of universal vaccination depends on the reactivity of vaccines against HBV. HBsAg binds antibody to hepatitis B surface antigen (anti-HBs) produced against HBV vaccines mainly via the 'a' determinant region (aa124–aa149). This region contains common antigenic epitopes of all subtypes (adw, adr, ayw, ayr) of HBsAg and lies in the major hydrophilic region (MHR) between aa99 and aa169. Amino acid (aa) substitutions in the MHR, particularly in the 'a' determinant region, can alter B cell epitopes of HBsAg, leading to immunological escape from the host immunity induced by either vaccination or previous infection.[11] Therefore, if HBV prevalent in Japan has aa substitutions in the MHR, the effect of universal vaccination may be reduced.

In Japan, three types of HBV vaccine (Bimmugen, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; Heptavax, Merck & Co., Whitehouse Station, NJ, USA; and Meinyu, Meiji Dairies, Tokyo, Japan) are now available. Efficacy and immunogenicity of vaccines are not always comparable or identical.[12,13] Whether giving a single vaccine effectively prevents the transmission of all genotypes of HBV is an important but still unsolved problem. Elucidating the aa substitutions in the MHR may give a clue to this problem.

The purpose of the present study is to determine the difference of the aa sequences in the MHR of HBV among isolates from patients with acute hepatitis and also the difference of the aa sequences among viral strains used for the production of anti-HBV vaccines, and to find ways to use currently available vaccines as effective prophylaxes.

METHODS

Patients

FROM 1992 TO 2001, serum samples were collected from 48 patients diagnosed with acute hepatitis B in our institutions. Only patients whose serum samples were stored at the onset of hepatitis were included in this study. All the 48 patients ran a self-limited clinical course. No patients subsequently developed fulminant hepatic failure or chronic sequelae.

The criteria for the diagnosis of acute hepatitis B were the following: (i) an acute onset of liver injury without a history of liver dysfunction and positivity for HBsAg in serum; and (ii) immunoglobulin M (IgM) antibody to HBV core antigen (anti-HBc) at a titer of more than 2.5 of cut-off index. Coinfection with a hepatitis A virus or a hepatitis C virus was excluded by serological tests. None of the patients had previously received any vaccination against HBV.

Serum samples from the 48 patients with acute hepatitis B were examined virologically, and the results were examined for correlations with clinical characteristics. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Determination of HBV-DNA

Hepatitis B virus DNA level was determined using transcription-mediated amplification (TMA) and a hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) using the protocol of Kamisango *et al.*[14] The range of detection using TMA was from 3.7 log genome equivalents (LGE)/mL (i.e. $10^{3.7}$ copies/mL corresponding to 5000 copies/mL) to 8.7 LGE/mL ($10^{8.7}$ copies/mL). In seven of 34 studied serum samples, the level of HBV-DNA was lower than 3.7 LGE/mL and these were categorized as 3.7 LGE/mL.

Genotyping HBV

Hepatitis B virus genotypes were determined using commercial enzyme immunoassay kits (Smitest HBV Genotyping kit; Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by polymerase chain reaction (PCR) using three sense primers (i.e. s1: 5′-ACCAACCCTCTGGGATTCTTTCC-3′, s2: 5′-ACCAATCCTCTGGGATTCTTCCC-3′, and s3: 5′-AGCAATCCTCTAGGATTCCTTCC-3′ [nt 2902–2924]) and an antisense primer (i.e. as1: 5′-GAGCCTGA GGGCTCCACCC-3′ [nt 3091–3073]) biotinylated at the 5′ end; their sequences were deduced from conserved sequences in the pre-S1 region of HBV. The biotin-labeled and amplified HBV-DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one of the seven HBV genotypes (A-G) immobilized on wells of a 96-well

microplate. Thereafter, hybridization was detected by staining with the streptavidin-horseradish peroxidase (HRP) conjugate.[15]

Amplifying and sequencing the S region of HBV-DNA

The entire aa sequence of MHR in the S region was amplified by two-stage PCR using genotype-specific primers. The outer primers for the amplification of the first fragment were 5′-TTTCCACCAAGCTCTGCAA-3′ (sense: nt 9–28) and 5′-TTCAGGGAATAACCCCATCT-3′ (antisense: nt 872–853) for genotype A, 5′-CTCCA CCACTTTCCA GACT-3′ (sense: nt 1–22) and 5′-CAACTCCCAATTACATATCCC-3′ (antisense: nt 899–879) for genotype B and 5′-TTACAGGCGGGG TTTTTCTT-3′ (sense: nt 70–89) and 5′-TACAGACTT GGCCCCCAATA-3′ (antisense: nt 771–752) for genotype C. The inner primers were 5′-AGAGTCAGGGGCC TGTATTTT-3′ (sense: nt 35–55) and 5′-AGGGAATAA CCCCATCACTTT-3′ (antisense: nt 869–849) for genotype A, 5′-TTCAAGATCCCAGAGTCAGG-3′ (sense: nt 24–43) and 5′-AGGGAATATCCCCACCTTTTT-3′ (antisense: nt 869–849) for genotype B and 5′-CGGGGTT TTCTTGTTGACA-3′ (sense: nt 77–97) and 5′-CCCAAT ACCACATCATCCATA-3′ (antisense: nt 758–738) for genotype C.

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μL reaction mixture containing 200 mM dNTPs, 1.0 mM each of primers and PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U Ampli-Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). PCR products (2 μL) were subjected to the second stage of amplification under the same conditions as those in the first stage. Standard precautions to avoid contamination were taken during PCR, with a negative control serum sample included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with a Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above-mentioned PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377; PE Applied Biosystems).

The nucleotide sequences of HBV isolates from the patients were compared with those of three reference HBV strains which are used for vaccine production.[16–18]

Phylogenetic trees were constructed with the Mega Program version 2.1 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA) using the Kimura two-parameter matrix and the neighbor-joining method.[19] To confirm the reliability of phylogenetic tree analysis, boot-strap resampling, and reconstruction were carried out 500 times.

Hydrophobicity and secondary structure analysis

The hydrophobicity profile of the MHR of the S region was predicted by computer-assisted Kyte-Doolittle analysis (an estimate of hydrophobicity based on the bulk phase partitioning of side chain hydrophobicity alone)[20] with GENETYX-MAC software (version 10.1; Software Development, Tokyo, Japan).

The secondary structures of the amino acids in the same region were predicted by computer-assisted Robson[21] and Chou-Fasman analyses[22] with the GENETYX-MAC software.

Statistical analyses

Data were analyzed by the chi-squared test for categorical data and Student's *t*-test or the Mann–Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Distribution and clinical characteristics of HBV genotypes

HEPATITIS B VIRUS genotype was determined in the 48 patients with acute hepatitis B. Genotype A was detected in 11 (23%) patients, genotype B in 11 (23%) and genotype C in 26 (54%).

The clinical and demographic backgrounds of the patients with acute hepatitis B who were infected with HBV of different genotypes are shown in Table 1. The mean ages of all the groups were similar. The proportion of male to female patients was higher in genotype A infection than in genotypes B or C infection (100%, 73% and 64%, respectively: A *vs* B, $P = 0.22$; A *vs* C, $P = 0.01$; B *vs* C, $P = 0.16$). The maximum alanine aminotransferase (ALT) levels were lower in patients with genotype A infection than in patients with genotypes B or C infection (1646 ± 1123, 3085 ± 1119 and 2545 ± 981 IU/L, respectively: A *vs* B, $P = 0.01$; A *vs* C, $P = 0.03$; B *vs* C, $P = 0.89$). The maximum HBV-DNA levels were not significantly different between the

Table 1 Demographic and clinical differences among patients with acute hepatitis infected with HBV of distinct genotypes

Features	Genotypes of HBV			Differences (*P*-value)		
	A ($n=11$)	B ($n=11$)	C ($n=26$)	A *vs* B	A *vs* C	B *vs* C
Age (years)	30.6 ± 7.5	28.1 ± 5.1	31.1 ± 9.1	0.41	0.87	0.33
Gender (M:F)	11:0	8:3	15:11	0.22	0.01	0.16
ALT (IU/L)	1646 ± 1123	3085 ± 1119	2545 ± 981	0.01	0.03	0.89
HBV-DNA (LGE/mL)	6.8 ± 1.7	6.6 ± 2.1	5.2 ± 1.2	0.60	0.23	0.06

ALT, alanine aminotransferase; HBV, hepatitis B virus.

genotypes (6.8 ± 1.7, 6.6 ± 2.1 and 5.2 ± 1.2 LGE/mL, respectively: A *vs* B, $P=0.60$; A *vs* C, $P=0.23$; B *vs* C, $P=0.06$).

Amino acid sequence of the S region

The aa sequence of the S region between aa27 and aa203 was determined in the 48 sequences. Figure 1 shows a phylogenetic tree constructed using the 48 sequences and 15 published sequences (four for genotype A, three for genotype B, three for genotype C, one for genotypes D, E, F, G and H). Among the 48 sequences we studied, 11 were classified into genotype A, 11 into genotype B and 26 into genotype C.

The aa sequence of the region between aa101 and aa163 including MHR (aa111-aa156) was compared among 48 sequences and three HBV sequences (X01587, J02205 and huGK-14) currently used for anti-HBV vaccine production. As shown in Figure 2, the aa sequences of X01587 (used for Bimmugen) and J02205 (used for Heptavax) differed in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161). The aa sequence of huGK-14, which is used for the HBV-vaccine Meinyu, differed from that of X01587 in six amino acids and from that of J02205 in two amino acids.

Nine of the 11 isolates classified into genotype A had the same aa sequence as J02205. The remaining two isolates (AB289727 and AB289728) differed from J02205 at aa161 (Fig. 2).

Ten of the 11 isolates classified into genotype B had the same aa sequence as J02205 except for two amino acids (aa114 and aa131). The remaining isolate had another aa substitution at aa112 (Fig. 2).

As shown in Figure 2, 22 of the 26 isolates classified into genotype C had the same sequence as X01587. The remaining four isolates (from patients 10, 24, 30 and 48) had the same sequence as X01587 except for one aa substitution at aa131; the threonine (aa131) of X01587 was substituted with proline for three isolates (AB289714, AB289720 and AB289736) and with alanine for one isolate (AB289701).

Hydrophobicity and secondary structure analysis

As mentioned above, the aa sequences of the MHR from four isolates differed from that of X01587 only at aa131. Furthermore, the aa sequence of the MHR differed between X01587 and J2205 in eight amino acids. We compared the hydropathy and secondary structure of the MHR among J02205, X01587 and two isolates with genotype C (one isolate with proline at aa131 and one with alanine at aa131). The results of Kyte-Doolittle hydropathy analysis based on the hydropathy index are shown in Figure 3. The substitution with alanine-131 was found to alter the patterns on the hydropathy plot, whereas the substitution with proline-131 was found to have little effect. A substitution with alanine-131 could increase the hydrophobicity of the first loop of the MHR, which may affect the antigenicity of HBV.

The secondary structure of our isolate with alanine-131 by Chou-Fasman analysis predicted an α-helix configuration for the region from aa126 to aa135 instead of the β-configuration predicted for the same region of X01587. The predicted secondary structure of our isolate with proline-131 coincided with that of X01587. In contrast, by Robson prediction, the secondary structure of our isolate with alanine-131 coincided with that of X01587; however, that of our isolate with proline-131 was found to have lost a turn structure between aa131 and aa134, which was predicted for X01587.

DISCUSSION

VACCINATION IS THE key to controlling HBV infection. In countries with a high prevalence of HBV infection, universal vaccination is effective not only for controlling viral infections but also for decreasing the incidence of hepatocellular carcinoma.[5,23] Even in

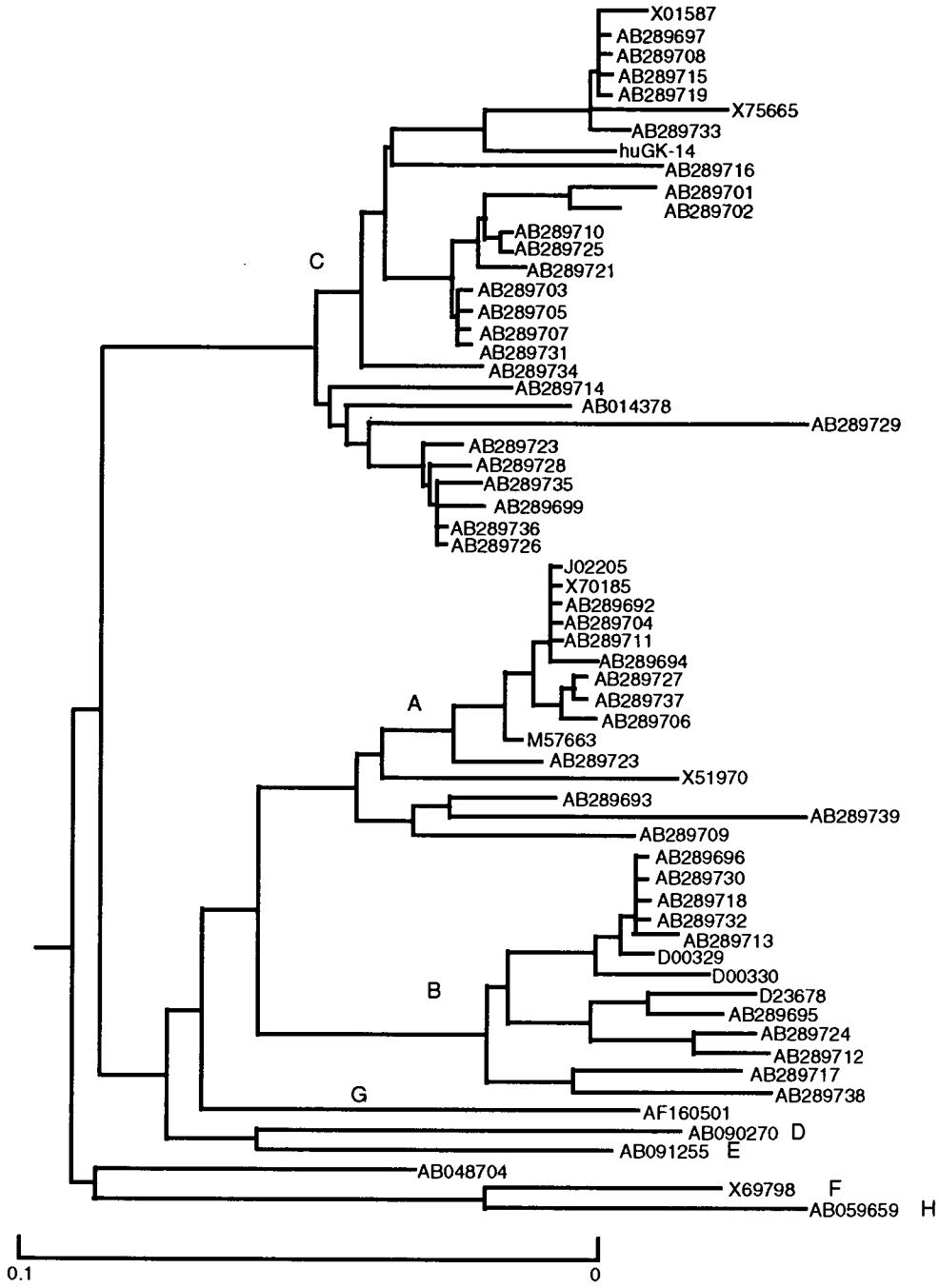

Figure 1 Phylogenetic tree constructed using hepatitis B virus (HBV)-DNA sequences of the *S* gene. The sequences include four with genotype A, four with genotype B, three with genotype C, and those recovered from the serum of 48 patients with acute hepatitis B. J02205 (genotype A) is used for the production of Heptavax and X01587 (genotype C) is used for the production of Bimmugen. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. The accession numbers for the HBV sequences from the 48 patients are also shown.

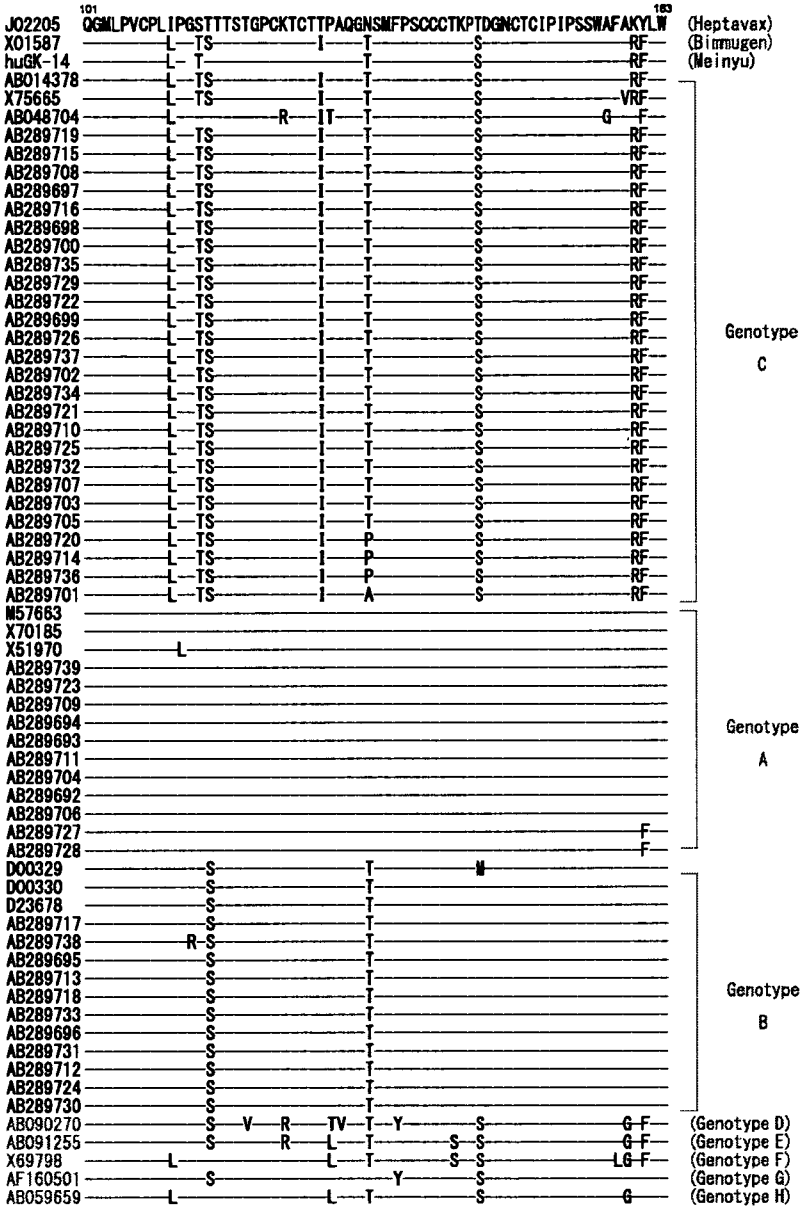

Figure 2 Comparison of amino acid sequences of the major hydrophilic region (MHR) of the *S* gene.

countries with a low prevalence of HBV infection, vaccination is very important for preventing mother-to-child transmission as well as patient-to-staff transmission.

HBV is classified into several genotypes that differ from one another in nucleotide sequence by more than 8% of the entire genome. The aa sequences of their phenotypes also differ among genotypes. The difference in the aa sequence of the 'a' determinant region may alter the three-dimensional structure and antigenicity, and may reduce the protectivity of HBV vaccines.

As mentioned above, the aa sequences of currently available recombinant vaccines differ from each other. J02205 and X01578 differ in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161) between aa101 and aa163. Three (i.e. aa126, aa131 and aa143) of them are included in the MHR and may alter the hydropathy and three-dimensional