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Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001

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Background. Because genotype A of hepatitis B virus (HBV) is not indigenous, there have been only few data on infection with it in Japan. ***Methods.*** We examined clinical and virological features of the 66 Japanese patients who admitted Toranomon Hospital in Tokyo, Japan, between 1976 and 2001, who were found to have HBV/A infection. HBV genotype A was classified into subtype A (European type) and A′ (South African type) by phylogenetic analysis of the preS1 and preS2 regions, and the S gene sequences. ***Results.*** Of the 66 patients infected with HBV/A, 14 (21%) were asymptomatic carriers, 26 (39%) presented with acute hepatitis, 22 (33%) with chronic hepatitis, and 4 (6%) with liver cirrhosis. HBV/A infection persisted for more than 6 months in 5 of the 26 (19%) patients with acute hepatitis. The frequency of acute hepatitis in patients infected with HBV/A was higher after than before 1991 (2/22 [9%] vs 24/44 [55%]; $P < 0.0001$). The frequency of nucleotide 1858 of T was higher in asymptomatic carriers than in patients with acute hepatitis in whom infection was resolved (5/14 [36%] vs 0/21 [0%]; $P = 0.008$). Of the 57 patients for whom subtypes of genotype A were determined, subtype A was identified in 53 (93%) and subtype A′ in only 4 (7%). All patients infected with subtype A′ were persistently infected with HBV. ***Conclusions.*** HBV/A infection has become more frequent during recent years, predominantly presenting as acute hepatitis, and subtype A′ is uncommon in the Tokyo metropolitan area.

Key words: genotypes, hepatitis B virus, hepatitis B e antigen, subtypes

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Introduction

Hepatitis B virus (HBV) is classified into seven genotypes by a sequence divergence in the entire genome of at least 8%, and named by capital alphabet letters from A to G.[1–3] HBV genotypes have distinct geographic distributions.[4,5] HBV Genotype A is prevalent in Northwestern Europe, North America, and South Africa, and was not reported in any of the Asian HBV genomes isolated until 1990.[1,6]

In 1997, Bowyer et al.[7] identified a unique subgroup of genotype A, based on comparison of the large S gene sequence, and named it A′. Of South African HBV/A isolates, 59% were classified into subgroup A′ and clustered to form a segment discrete from the original genotype A isolates. Amino-acid differences that set A′ isolates apart from the rest of group A tend to cluster in the pre-S2 region (amino acids 7, 10, 32, 35, 47, 48, 53, and 54), with a few changes in the major surface antigen (amino-acid sites 207 and 209). HBV isolates of subgroup A′ were separated from those in Northwestern Europe genotype A by sequence differences ranging from 4.1% to 6.2% in the preS2/S region,[7] as well as by phylogenetic differences in the entire genomic sequence.[8]

To our knowledge, there are no large-scale studies on Japanese patients infected with HBV of genotype A or subgroup A′ with clinical and virological characterizations. In the present study, we examined 68 Japanese patients infected with HBV/A who visited Toranomon Hospital in the Tokyo metropolitan area during 1976 through 2001, and we examined their clinicopathological characteristics and virological features. Further, HBV/A isolates were classified into subtypes A and A′ by phylogenetic analysis. The nucleotide (nt) 1858 of T or C was determined, also, which influences the development of the precore stop-codon mutation at nt from G to A.[9,10]

Patients and methods

Patients

Among the patients who admitted the Department of Gastroenterology, Toranomon Hosital, in metropolitan Tokyo during 1976 through 2001, 2022 were infected with HBV and received liver biopsy. Genotypes of HBV were determined in these patients, and genotype A was detected in 68 (3.4%), B in 239 (11.8%), C in 1649 (81.6%), D in 5 (0.2), and F in 2 (0.1%) of them; HBV isolates from the remaining 59 (2.9%) patients were untypeable. The 68 patients infected with HBV/A were examined medically for the diagnosis of liver disease, and HBV DNA in their sera were studied virologically. The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital, and an informed consent was obtained from each of the patients.

These 68 patients infected with HBV/A were classified into four clinical groups. An asymptomatic carrier state was diagnosed in 14 (21%) patients. They had serum alanine aminotransferase (ALT) levels consistently within the normal range (≤50 IU/l) and ultrasonographic findings of a "normal liver" or "fatty liver" at the initial visit. Chronic hepatitis was diagnosed in 22 (32%) patients, based on histopathological examination of liver biopsy specimens obtained under laparoscopy. Four (6%) patients had already developed liver cirrhosis that was diagnosed by findings on laparoscopy and imaging modalities, including ultrasonography and computed tomography.

The remaining 28 (41%) patients were diagnosed with acute hepatitis. They had all contracted HBV infection in adulthood, and possessed antibody to hepatitis B core antigen of IgM class in high titers (2.2–11.7) in serum for the diagnosis of acute hepatitis B. De-novo HBV infection in them was confirmed by the lack of hepatitis B surface antigen (HBsAg) at health check-ups they had received before they came down with acute hepatitis.

Serum markers of HBV infection

HBsAg was determined by hemagglutination (MyCell; Institute of Immunology, Tokyo, Japan) and radioimmunoassay (AUSRIA II-125; Dinabot, Tokyo, Japan). Hepatitis B e antigen (HBeAg) and the corresponding antibody (anti-HBe) were determined by radioimmunoassay (AUSRIA-II 125; Dinabot). HBV DNA was determined by transcription-mediated amplification and hybridization assay (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed as log genome equivalents (LGE) per milliliter of serum, with detection limits ranging from 3.7 LGE/ml (corresponding to 5000 copies/ml) to 8.7 LGE/ml. Genotypes of HBV were determined, in sera from the patients at presentation, by commercial enzyme-linked immunosorbent assay (ELISA) kits (HBV Genotype EIA; Institute of Immunology), using monoclonal antibodies against epitopes on the pre-S2-region products.[11,12] Serotypes of HBsAg were determined by ELISA, using commercial kits (HBs Antigen Subtype EIA; Institute of Immunology).

Determination of subtypes A and A′ of genotype A

Nucleic acids were extracted from serum (100 µl), using the Smitest EX & R kit (Genome Science, Tokyo, Japan) and stored at −80°C. Nucleotide sequences spanning the large S gene (preS1 and preS2 regions and the S gene) were amplified in extracted nucleic acids by polymerase chain reaction (PCR) with nested primers. The first-round PCR was performed with BGF1 (sense, 5′-CTG TGG AAG GCT GGC ATT CT-3′ [nt 2757–2776]) and BGR2 (antisense, 5′-GGC AGG ATA GCC GCA TTG TG-3′ [nt 1050–1079]) primers, and the second-round PCR with PLF5Bm (sense, 5′-TGT GGA TCC TGC ACC GAA CAT GGA GAA-3′ [nt 136–162] and BR112 (Antisense, 5′-TTC CGT CGA CAT ATC CCA TGA AGT TAA GGG A-3′ [nt 163–864]), as well as BGF5 (sense, 5′-TGC GGG TCA CCA TAT TCT TG-3′ [nt 2811–2830]) and BGR6 (antisense, 5′-AGA AGT CCA CCA CGA GTC TA-3′ [nt 2831–248]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, Foster City CA, USA). Then, they were purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences of the large S gene in HBV DNA samples from patients were analyzed phylogenetically, along with reference sequences of subtypes A and A′,[7] by six-parameter[13] and neighbor-joining methods.[14]

Mutations in the core promoter and precore region

For determination of the wild-type or mutants in the core promoter and precore region, nucleic acids extracted from serum were amplified by PCR with nested primers. The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ [nt 1580–1599]) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ [nt 2295–2276]) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAC CAC CGT GAA C-3′ [nt 1606–1625]) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ [nt 2014–1995]) for 35 cycles

Table 1. Comparison of 66 patients who were infected with HBV genotype A and presented with various hepatic diseases

Features	ASC (n = 14)	AH (n = 26)	CH (n = 22)	LC (n = 4)	Differences
Male	10 (71%)	26 (100%)	19 (86%)	3 (75%)	NS
Age (years)[a]	28 (18–62)	33 (21–56)	36 (12–55)	43 (22–66)	NS
Follow-up (years)[a]	5 (0.6–15)		9 (3–20)	8 (0.3–21)	NS
Family history[b]	5 (36%)		3 (14%)		NS
Father	3 (21%)				
Mother			1 (5%)		
Siblings	2 (14%)		2 (9%)		
Transfusion	0	0	0	1 (25%)	NS
HCV RNA	0	0	1 (5%)	1 (25%)	NS

ASC, asymptomatic carriers; AH, acute hepatitis; CH, chronic hepatitis; LC, liver cirrhosis
[a] Median values are shown, with ranges in parentheses
[b] Family member with HBV-associated liver disease according to statements of patients

each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis, purified, and sequenced as described above. Mutations interfering with translation or transcription of HBeAg were searched for in the precore region and core promoter. They included a G-to-A mutation at nt 1896 in the precore region (A1896) and a double mutation in the core promoter, converting codon 1762 from A to T, and codon 1764 from G to A (T1762/A1764). Also examined was nt 1858 of T or C in the precore sequence.

Statistical analysis

Nonparametric procedures were used for the analysis, including the Mann-Whitney *U*-test, Fisher's exact test, and Bonferroni test. A P value of less than 0.05 in two-tailed analysis was considered significant.

Results

Characteristics of patients

Table 1 compares demographic features, history of transfusion, and infection with hepatitis C virus (HCV) in the 68 patients who were infected with HBV/A and presented with various liver diseases at the first hospital visit. They were all positive for HBsAg of serotype *adw* in serum. There were no differences in sex, age, duration of follow-up, family members infected with HBV, and history of blood transfusion among them. There was only one mother positive for serum HBsAg in the patients with chronic hepatitis. Her genotype of HBV was B, different from genotype A in her son, who was diagnosed with chronic hepatitis. Homosexual contacts were reported by 11 of the 28 (39%) patients with acute hepatitis. HBV infection persisted in 4 of the 11 (36%) patients who had contracted HBV/A infection through homosexual activity. HCV RNA was detected in 1 patient with chronic hepatitis and 1 with liver cirrhosis.

Of particular note, HBV/A infection persisted for longer than 6 months in 5 of the 26 (19%) patients who presented with acute hepatitis. On liver biopsies undertaken 6–96 months after the estimated time of infection, necroinflammatory changes and fibrosis were detected in all the 5 patients for the diagnosis of chronic hepatitis B.

For the purpose of analyzing trends of liver disease that may change with time, patients were divided into two groups according to the time of the first visit to the Gastroenterology Department at Toranomon Hospital, i.e., before or after 1991, when the serological diagnosis of HCV infection had become nationwide. There were 22 patients in 1976–1990 and 44 patients in 1991–2001. Figure 1 compares the proportions of patients who presented with four categories of clinical diagnosis between the two groups of patients.

There was an apparent increase in the proportion of patients who presented with acute hepatitis in the period 1991–2001 compared to 1976–1990 (2/22 [9%] vs 24/44 [55%]; $P < 0.0001$). By contrast, the proportions of asymptomatic carriers, as well as those of patients with chronic hepatitis and liver cirrhosis, decreased after 1991 (27% vs 17%, 55% vs 22%, and 9% vs 4%, respectively; $P < 0.0001$ for all).

Many patients diagnosed as asymptomatic carriers and those diagnosed with chronic hepatitis or liver cirrhosis in 1976–1990 were less than 40 years of age. However, in 1991–2001, there were many patients diagnosed with chronic hepatitis or liver cirrhosis who were 40 years or older (Fig. 2). Thus, of asymptomatic carriers, those aged less than 40 years significantly increased in frequency from 1976–1990 to 1991–2001. In a mirror image, the frequency of patients younger than 40 years significantly decreased in those with chronic hepatitis or liver cirrhosis. Patients with acute hepatitis were excluded from the comparison, because only three (11%) of them were 40 years or older at presentation.

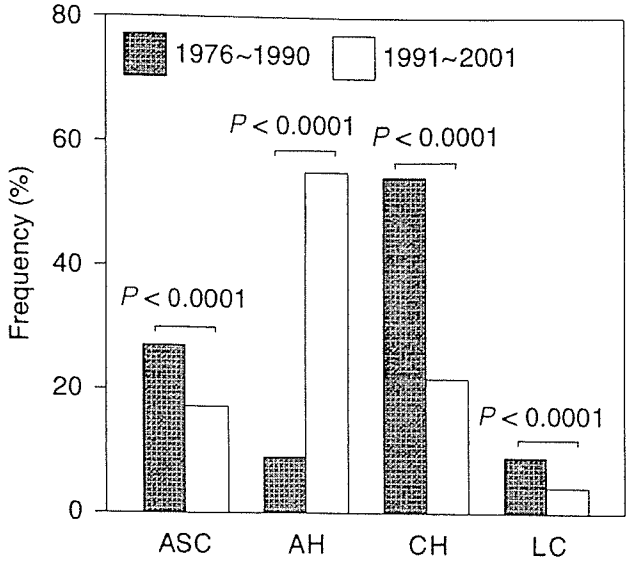

Fig. 1. Comparison of the proportions of liver diseases in patients infected with hepatitis B virus (HBV) genotype A who visited Toranomon Hospital between 1976 and 1990 and between 1991 and 2001. *ASC*, asymptomatic carriers; *AH*, acute hepatitis; *CH*, chronic hepatitis; *LC*, liver cirrhosis

Wild-types in the core promoter and precore region in patients who were infected with HBV genotype A

Table 2 compares the frequency of the wild-type in the core promoter and precore region among patients infected with HBV/A who presented with various liver diseases. The wild-type precore region with G1896 was detected in 61 of the 66 (92%) patients for whom the analysis was possible. All the 14 asymptomatic carriers were negative for HBeAg in serum, and the wild-type core promoter with A1762/G1764 was detected in only 43% of them. The wild-type core promoter was found in all 20 patients with acute hepatitis who had serum HBeAg at presentation and in whom HBV infection was resolved. Of the 5 patients with acute hepatitis who went on to chronicity, all of whom had serum HBeAg, 4 (80%) were infected with HBV/A of the wild-type in the core promoter, a frequency comparable to that in 10 of the 14 (71%) patients with HBeAg-positive chronic hepatitis. Patients with HBeAg-negative chronic hepatitis possessed the wild-type core promoter (38%) and precore region (88%) somewhat less frequently than the others.

The 1858th nt of T or C in patients infected with HBV/A at the time of presentation is shown in Table 3. T1858 was not detected in any patients with acute hepatitis in whom HBV infection was resolved, but it was found in one of the 5 (20%) patients who acquired persistent infection, as well as in 24%–32% of those with the other categories of liver disease. T1858 was

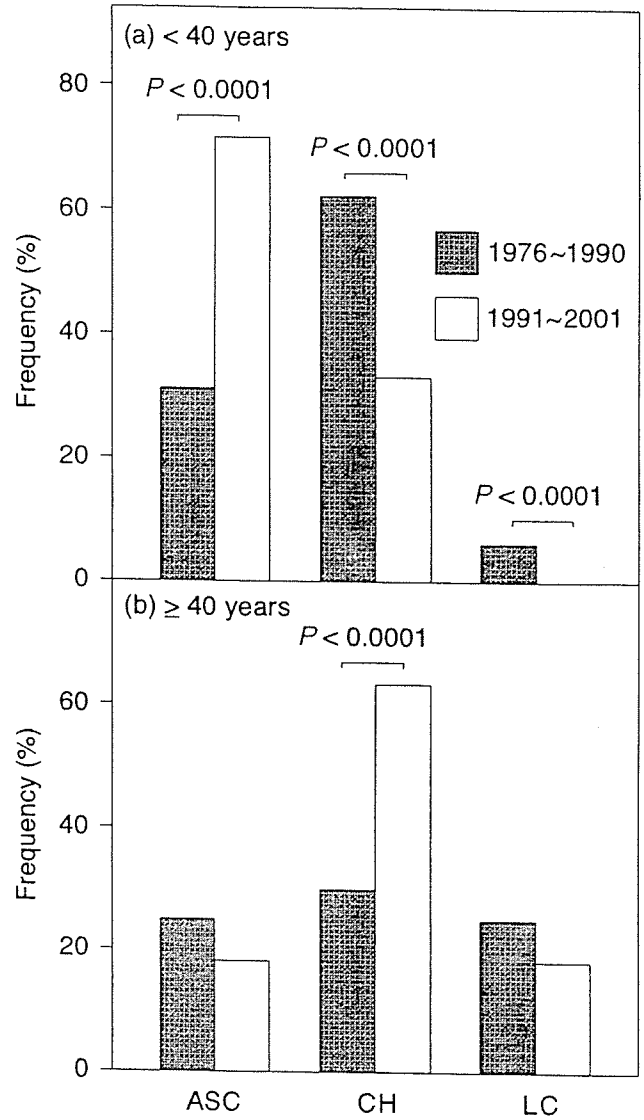

Fig. 2a,b. Comparison of the proportions of patients infected with HBV genotype A aged less than 40 years (**a**) and aged 40 years or older (**b**) who visited Toranomon Hospital between 1976 and 1990 and between 1991 and 2001

detected significantly more frequently in asymptomatic carriers than in patients with acute hepatitis (36% vs 4%; $P = 0.002$), and the difference was even more prominent between asymptomatic carriers and patients with acute hepatitis in whom HBV infection was resolved (36% vs 0%; $P = 0.008$).

Subtypes A and A′ of genotype A

HBV DNA sequences of the large S gene were determined in nucleic acids extracted from 68 patients infected with HBV/A, and subtypes A and A′ were analyzed phylogenetically (Table 4). Subtype A was detected in 53 (80%) patients, and subtype A′ in 4 (6%);

Table 2. Wild-types of core promoter and precore sequence in HBV DNA from patients infected with HBV genotype A at presentation

Liver disease	Wild-type core promoter (A1762/G1764)	Wild-type precore region (G1896)
ASC		
HBeAg (−) (n = 14)	6 (43%)	13 (93%)
AH: Resolved		
HBeAg (+) (n = 20)	20 (100%)	20 (100%)
HBeAg (−) (n = 1)	ND	ND
AH: Persisted for >6 months		
HBeAg (+) (n = 5)	4 (80%)	5 (100%)
CH		
HBeAg (+) (n = 14)	10 (71%)	14 (100%)
HBeAg (−) (n = 8)	3 (38%)	7 (88%)
LC		
HBeAg (−) (n = 2)	1 (50%)	2 (100%)

ND, not detectable

Table 3. Nucleotide 1858 in HBV DNA samples from patients who were infected with HBV genotype A and presented with various liver diseases

Nucleotide at position 1858	ASC (n = 14)	AH: Resolved (n = 21)	AH: Persisted (n = 5)	CH (n = 22)	LC (n = 4)	Total (n = 66)
	P = 0.008					
T	5 (36%)	0	1 (20%)	7 (32%)	1 (25%)	14 (21%)
C	6 (43%)	18 (86%)	4 (80%)	15 (68%)	1 (25%)	44 (67%)
Deletion	3 (21%)	0	0	0	0	3 (5%)
Not detectable	0	3 (14%)	0	0	2 (50%)	5 (8%)

Table 4. Subtypes in patients infected with HBV genotype A

Subtypes	ASC (n = 14)	AH: Resolved (n = 21)	AH: Persisted (n = 5)	CH (n = 22)	LC (n = 4)	Total (n = 66)
A	11 (79%)	18 (86%)	4 (80%)	18 (82%)	2 (50%)	53 (80%)
A′	1 (7%)	0	0	3 (14%)	0	4 (6%)
Untypeable	2 (14%)	3 (14%)	1 (20%)	1 (4%)	2 (50%)	9 (14%)

distinction between A and A′ was not possible in the remaining 9 (14%) patients. Of the 57 patients in whom subtypes were determined, A was detected in 53 (80%) and A′ in the remaining 4 (6%). The 53 patients infected with subtype A, for whom subtyping was feasible, included 11 of the 13 (85%) asymptomatic carriers, 22 of the 22 (100%) with acute hepatitis, 18 of the 21 (85%) with chronic hepatitis, and both patients with liver cirrhosis. Of the 4 patients infected with subtype A′, 1 presented with the asymptomatic carrier state and the remaining 3 with chronic hepatitis.

Table 5 lists the demographic, histopathological, and virological features of the four patients infected with subtype A′. They comprised two males and two females, and two of them were in their twenties and the remaining two in their forties. Two patient with chronic hepatitis and infected with subtype A′ (patient 3 and 4) cleared HBsAg from serum during about 3 years of follow up.

Table 5. Characteristics of the four patients who were infected with HBV of subtype A′

Features	Patient 1	Patient 2	Patient 3	Patient 4
Sex	M	F	F	M
Age (years)	26	27	43	47
Family history		Father Brother		
Diagnosis	ASC	CH	CH	CH
Liver histology	A0/F0	A1/F1	A1/F1	A1/F2
HBV DNA (LGE/ml)	<3.7	≥8.7	<3.7	≥8.7
HBeAg	±	±→±	±	±→±
Nucleotide 1858	C	C	T	C
Wild-type				
Core promoter	No	Yes	Yes	No
Precore sequence	Yes	Yes	Yes	Yes

A, grade of necroinflammatory activity; F, stage of fibrosis; LGE, log genome equivalent

Discussion

In Japan, by far the greatest number of carriers contracted their HBV infection perinatally through mother-to-bady transmission decades ago.[15,16] By contrast, individuals in Europe and the United States acquire HBV infection in adulthood. There are remarkable differences in the distribution of HBV genotypes between Japan and western countries. Genotypes B and C are prevalent in Japan, in contrast to genotypes A and D common in European countries,[4,5] while all seven HBV genotypes are found in the United States, with the distribution dependent on the ethnicity of carriers.[17]

We previously reported that the proportion of patients with acute hepatitis infected with HBV/A in the Tokyo metropolitan area was significantly higher ($P < 0.0001$) than that in those with chronic hepatitis who visited the same hospital.[18] In the present study, a mother with HBV infection was not reported by any of the patients infected with HBV/A who presented with acute hepatitis. Only one patient infected with HBV/A, with chronic hepatitis, was found to have a mother with HBV infection. Her genotype was B, however, thereby excluding mother-to-baby transmission in this patient. These results indicate that, at least in Tokyo, most infection with HBV/A occurs horizontally rather than perinatally, in corroboration with our previous findings.[18]

It may be surprising that 5 of the 26 (19%) patients with acute hepatitis who were infected with HBV/A failed to clear infection. They had all contracted de-novo HBV infection, because they did not test positive for HBsAg in health check-ups before they came down with acute hepatitis. In Japan, acute HBV infection in adulthood evolves into chronicity in only fewer than 1%, unlike that in western countries, where it persists in approximately 10%.[19] It appears that primary infection with HBV of genotype A would tend to be chronic more frequently than those of the other genotypes, even when it is contracted in adulthood.

There is evidence in support of a role of homosexuality in HBV/A infection. A cluster of HBV/A strains was identified in men having sex with men in Amsterdam.[20] In the present study, the most common route of infection with HBV/A in patients with acute hepatitis was homosexual activity, as reported by 11 of 28 patients (39%). HBV/A infection persisted in 4 of the 11 (36%) who had contracted infection by homosexual contacts. There are increasing numbers of young male homosexuals in the Tokyo metropolitan area, some of whom may be at increased risk of infection with HBV/A as well as human immunodeficiency virus type 1.[21] It is worrying that the persistence of acute HBV/A infection will increase in Japan in the future. For preventing the chronicity of HBV/A infection, hepatitis B vaccine would be effective in persons at risk of contracting infection by sexual contacts, in particular.

HBV/A isolates have the nucleotide 1858 of C (C1858) that makes a pair with T1896. Therefore, the G-to-A point mutation in the precore region creating the stop codon is prevented in HBV/A isolates, because A1896 is incompatible with C1858 and destroys the conformation of the pregenome encapsidation signal.[9,10] Hence, the precore mutation (A1896) in HBV/A isolates always accompanies C-to-T mutation at nt 1858. T1858 in HBV/A infection was detected in 5 of the 14 (36%) asymptomatic carriers (all of whom were without serum HBeAg), this being significantly more frequent than in none of the 21 patients in whom acute hepatitis had resolved ($P = 0.008$); 20 of them (95%) presented with HBeAg in serum.

HBV/A isolates in South Africa are phylogenetically different from those in Europe and the United States, and most of them are of subtype A′.[7,22] Subtyping of HBV/A isolates into A (the original genotype A) and

A′ was feasible in 58 (85%) of the 68 patients in the present study. The subtype was A in 53 of these 57 (93%) HBV/A isolates, and A′ in the remaining 4. Where the HBV/A strains of subtype A′ came from, and how they have spread in Tokyo, is not certain, and these questions need to be sorted out in future epidemiological studies. All HBV/A infections of subtype A′ occurred in persistent HBV infection and occurred in 1 asymptomatic carrier and 3 patients with chronic hepatitis, of whom 1 cleared infection during 3 years of follow-up.

How these four patients contracted HBV infection of subtype A′ is a matter of conjecture. Although they all had chronic HBV infection, a possibility remains that there had been evolution of acute infection with subtype A′, which may have been acquired through sexual contacts. Special care will have to be devoted to them, because infection with subtype A′ in Africa is associated with a high incidence of hepatocellular carcinoma,[23] although its development would be accelerated by afratoxin there.

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Interferon monotherapy for patients with chronic hepatitis C and normal serum aminotransferase levels at commencement of treatment

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Background. Approximately 30% of patients with chronic hepatitis C have normal serum alanine amino transferase (ALT) levels. While interferon (IFN) monotherapy is approved for patients with chronic hepatitis C infection, the effectiveness of such therapy for chronic hepatitis C patients with normal ALT levels at commencement of treatment remains poorly understood. ***Methods.*** Ninety-four individuals (M/F, 54:40; median age, 46 years) with normal ALT levels (< 50 IU/l) at the commencement of treatment who were positive for both anti-hepatitis C virus (HCV) and serum HCV-RNA were studied. Among this group, 18 individuals (M/F, 9:9; median age, 50 years) had had persistently normal ALT levels for at least 3 months prior to treatment. All patients received their first course of IFN therapy in this study. ***Results.*** Forty-three (45.7%) of 94 individuals had lost serum HCV-RNA at 6 months after cessation of therapy (complete response; CR). The proportion of patients with genotype 2a and HCV-RNA level over 1 Meq/ml who showed CR was significantly lower in those with normal ALT levels than in those with elevated ALT levels (23.8% vs 55.6%; $P = 0.0189$). Two patients who had persistently normal ALT levels and HCV-RNA level over 1 Meq/ml were non-responders (NR) and had ALT flare-ups after IFN therapy. Patients with HCV-RNA levels of less than 1 Meq/ml did not show differential responses based on ALT levels. ***Conclusions.*** Our data suggest that IFN therapy is effective for patients with normal ALT levels and less than 1 Meq/ml HCV-RNA. Thus, such patients should be considered for curative IFN therapy.

Key words: interferon, chronic hepatitis C, normal ALT

Introduction

Antiviral treatment for patients with chronic hepatitis C virus (HCV) infection has generally been limited to those with significantly abnormal serum transaminase activity. Results of interferon (IFN) monotherapy in patients with normal serum alanine amino transferase (ALT) levels were the basis for the conclusions of the 1997 Consensus Conference that these patients should not be treated.[1] Thirty of 52 patients treated in seven studies, for whom data were available, had de novo elevations of ALT levels during therapy, and some patients continued to have ALT elevation after IFN was discontinued.[2–8] Approximately 30% of patients with chronic hepatitis C have normal ALT levels.[9] It is true that if ALT levels are persistently normal, the possibility of significant and progressive liver disease tends to be low, but a significant proportion of patients with persistently normal ALT levels show some histological signs of fibrosis—the degree of which is usually mild but is sometimes more marked—and in rare cases, cirrhosis may be present.[1] IFN treatment is effective for patients with chronic hepatitis C, reducing ALT levels, improving histological activity,[10,11] and eliminating HCV-RNA.[12–15] However, the effectiveness of IFN therapy for patients with normal ALT remains poorly understood. Few studies have compared patients with elevated ALT levels and matched patients with normal ALT levels with respect to assessing sustained virological response rates.[1] Moreover, the changes in ALT levels after IFN therapy in patients with normal ALT levels before treatment is not clear.

The aim of this retrospective study was to determine the incidence of sustained virological response after IFN therapy and the changes in ALT levels after therapy in patients with normal ALT levels at commencement of therapy.

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Patients and methods

Patients

Between July 1992 and August 2001, all anti-HCV-positive patients treated at Toranomon Hospital, Tokyo, Japan, were recruited for the present study, and assessed by routine work-up. Routine evaluation of patients included medical history, physical examination, complete blood count, and measurement of ALT and markers for viral hepatitis. Patients coinfected with hepatitis B virus (HBV); those with autoimmune diseases; those with previous IFN treatment or medication for hepatitis; history of heavy alcohol abuse; liver cirrhosis or hepatocellular carcinoma on ultrasonography; or coexisting cardiac, renal, pulmonary endocrine conditions or psychiatric conditions, were excluded from this study. We retrospectively identified 791 patients who were first treated with IFN (Table 1). Normal ALT level was defined at ALT less than 50 IU/l at the commencement of IFN therapy. Of the 791 patients, 94 had normal ALT levels (M/F, 54:40; median age, 46 years) at the commencement of IFN therapy. Among this group, 18 patients (M/F, 9:9; median age, 50 years) had had persistently normal ALT levels for a mean period of 8 months (range, 3–91 months) before IFN therapy. In all patients, ALT level was examined every month before treatment, during treatment, and during the subsequent 6-month follow-up period.

Histopathological examination of liver biopsy specimens

Patients underwent liver biopsy within 6 months prior to the commencement of IFN therapy. The baseline liver histology of chronic hepatitis was classified into four stages according to the extent of fibrosis: stage 0 (F0), no fibrosis; stage 1 (F1), periportal expansion; stage 2 (F2), portoportal septa; and stage 3 (F3), portocentral linkage or bridging fibrosis.[16] No patients with liver cirrhosis (F4) were included in this study.

Serum HCV-RNA marker

Qualitative analysis of HCV-RNA was performed using a branched DNA probe assay (bDNA probe assay, version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan). HCV genotype was classified by polymerase chain reaction (PCR), using a mixture of primers for six subtypes known to exist in Japan, as reported previously.[17]

Interferon (IFN) treatment and definition of response to IFN therapy

The IFN treatment consisted of 6 to 10 million units (MU) of natural IFN alpha or natural IFN beta. IFN treatment was administered daily for 2 months or daily for 2 months and then three times a week for 4–5 months. After discontinuation of the therapy, all patients were followed-up for at least an additional 6-month period. A complete response (CR) was defined as negative HCV-RNA by PCR at two time points, 3 and 6 months after the completion of IFN therapy. Incomplete response (IR) was defined as normalization of serum ALT, but positive HCV-RNA by PCR at two time points, 3 and 6 months after cessation of IFN therapy. All patients other than those with CR and IR were considered nonresponders (NR).

Table 1. Comparison of patients with chronic hepatitis C virus (HCV) infection who had elevated or normal serum alanine aminotransferase (ALT) levels

	Elevated ALT (n = 697)	Normal ALT (n = 94)	P
Age (years)[a]	51 (17–73)	46 (20–68)	0.022
Sex (male/female)	436/261	54/40	0.40
Liver histology (F0/F1/F2/F3/N)[b]	3/446/180/48/20	1/82/5/1/5	<0.0001
HCV genotype (1b/2a/2b)	466/176/55	38/43/13	<0.0001
HCV-RNA (≤1 Meq/>1 Meq) (Meq/ml, bDNA probe, version II)	235/459	48/46	0.0016
Serum ALT (IU/l)[a]	102 (51–683)	36 (11–50)	<0.001
Alpha/Beta IFN (alpha %)	541/156 (77.6)	69/25 (73.4)	0.95
Total dose of IFN (MU)[a]	624 (261–1040)	627 (318–1040)	0.924
Duration of IFN therapy (weeks)[a,c]	24 (8–30)	24 (8–28)	0.483

[a] Data values are expressed as medians with ranges in parentheses unless indicated otherwise
[b] Liver fibrosis assessed on a five-point scale: F0, no fibrosis; F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; N, liver biopsy not performed
[c] IFN therapy was administered daily for 2 months or daily for 2 months and three times/week for 4 months

Statistical analysis and ethical considerations

Differences between groups were examined for statistical significance, using the Mann-Whitney test (*U*-test) and the χ^2 test where appropriate. A two-tailed *P* value of less than 0.05 was considered statistically significant. Independent predictive factors associated with CR to IFN treatment were studied using stepwise Cox regression analysis. Potential predictive factors for CR to IFN treatment that were assessed included the following nine variables: age (≤51 vs >51 years), sex, severity of liver disease (F0 and F1 vs F2 and F3), ALT (≤50 vs >50 IU/l), type of IFN (alpha vs beta), period of IFN therapy (≤24 vs >24 weeks), total dose of IFN (≤624 vs >624 MU), HCV genotype (1 vs other than 1), and HCV RNA level (≤1 vs >1 Meq/ml). All factors found to be at least marginally associated with CR to IFN treatment ($P < 0.15$) were entered into a multivariate multiple logistic regression. The odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relative-risk confidence. All analyses described above were performed using the SPSS program (version 7.5, SPSS, Chicago, IL, USA).

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Results

Clinical and virological features of patients with normal ALT

Table 1 lists the demographic and clinical characteristics of patients with elevated ALT levels and those with normal ALT levels. There were 94 patients with normal ALT levels (group N), with a median age of 46 years (range, 20–68 years). Liver biopsy was performed in all but 5 patients, and 82 patients (87.3%) were assessed as F1 stage. Liver histology in group N tended to be milder compared with that in group E (patients with elevated ALT). HCV genotype 2a tended to be more prevalent in group N than in Group E. Among the patients in group N, 18 had persistently normal ALT levels (median age, 50 years), and were assessed as showing histological stage F1.

Virological response to IFN therapy

The overall CR rate in patients in group N was 45.7% (43/94 patients). Table 2 shows the virological response to IFN therapy in patients with chronic HCV infection for group E and group N. Each HCV genotype and HCV-RNA level were compared between the two groups. No patients in group N with genotype 1b and HCV-RNA level over 1 Meq/ml showed a CR to IFN therapy. Among patients with genotype 2a and HCV-RNA levels more than 1 Meq/ml, the CR rate was significantly lower in group N than in group E (23.8% vs 55.6%; $P = 0.0189$). However, there was no difference between group N and group E among patients of all genotypes with HCV-RNA levels 1 Meq/ml or less. Moreover, in patients with HCV-RNA level over 1 Meq/ml, there were no differences in CR rate and the severity of liver fibrosis between the two groups. After IFN therapy, 26.6% (25/94) of patients in group N had HCV-RNA levels that returned to pretreatment values and had ALT flare-ups during follow-up.

The virological response to IFN therapy in the 18 patients with chronic HCV infection and persistently normal ALT levels is shown in Table 3. The overall CR rate was 38.9% (7/18 patients). Evaluation according to genotype was difficult, as there were few patients with genotype 2b. Among patients with HCV-RNA levels of 1 Meq/ml or less with genotypes 1b and 2a, the effects of IFN therapy were the same as for those in group E. However, the rate of CR to IFN therapy in patients with HCV-RNA levels of more than 1 Meq/ml tended to be low.

Table 2. Proportion of patients with chronic hepatitis C virus infection who showed complete response (CR) to interferon (IFN) therapy, according to HCV genotype and HCV-RNA level

	Elevated ALT group ($n = 697$)	Normal ALT group ($n = 94$)	*P*
Genotype 1b			
≤1 Meq/ml	57/125 (45.6)	15/22 (68.1)	0.085
>1 Meq/ml	36/341 (10.6)	0/16 (0)	0.3442
Genotype 2a			
≤1 Meq/ml	74/95 (77.9)	17/22 (77.3)	1.0
>1 Meq/ml	45/81 (55.6)	5/21 (23.8)	0.0189
Genotype 2b			
≤1 Meq/ml	12/15 (80)	3/4 (75)	1.0
>1 Meq/ml	9/37 (24.3)	3/9 (33.3)	0.897

Complete response (CR): sustained loss of serum HCV RNA was observed for 3 and 6 months after treatment
Data values in parentheses show percentages of patients

Table 3. Proportion of patients with chronic hepatitis C virus infection and persistently normal serum alanine aminotransferase (ALT) levels who showed complete response (CR) to interferon (IFN) therapy, stratified according to HCV genotype and HCV-RNA level

	HCV RNA	
HCV genotype	≤1 Meq/ml	>1 Meq/ml
1b	2/3 (66.6%)	0/3 (0%)
2a	2/3 (66.6%)	2/8 (25%)
2b	No participant	1/1 (100%)

Data values in parentheses show percentages of patients

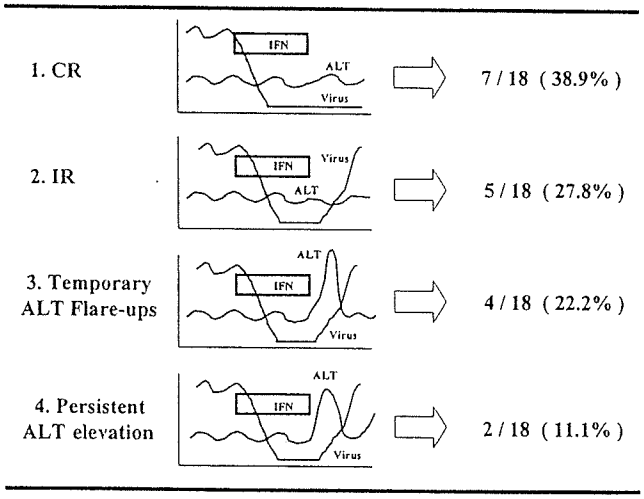

Fig. 1. Virological response and serum alanine aminotransferase (*ALT*) changes during and after interferon (*IFN*) therapy in 18 patients with hepatitis C virus (HCV) infection and persistently normal ALT levels before treatment. *CR*, complete response; *IR*, incomplete response. Data *on the right* show the number of patients showing the particular response/total number of patients in that group, with percentages of patients shown in *parentheses*

Changes in ALT levels after IFN therapy in patients with persistently normal ALT levels

Eighteen patients with persistently normal ALT levels were followed-up after IFN therapy, for a mean duration of 39 months (range, 11–65 months). Figure 1 shows the virological response and ALT changes during and after IFN therapy. Five patients had an IR (27.8%), and four had temporary ALT flare-ups after IFN therapy (22.2%). The five patients with IR had two types of genotype; three patients were 1b and two were 2a. Four patients with IR had an HCV-RNA level over 1 Meq/ml at the commencement of IFN therapy, and their HCV RNA became undetectable during IFN therapy. In the remaining patient, HCV RNA was detectable during therapy. The ALT levels of these five patients were normalized during and after therapy. The four patients with temporary ALT flare-ups had two types of genotype; one was 1b and three were 2a. Three patients with temporary ALT flare-ups had an HCV-RNA level over 1 Meq/ml at the commencement of IFN therapy, and in two of these, HCV RNA levels became undetectable during therapy. However, HCV RNA in another patient was detectable, while it was not examined during therapy in the remaining patient. ALT levels in these patients were normalized during and after therapy. These temporary ALT flare-ups occurred at a mean time point of 3 months posttreatment (range, 2–4 months). Finally, two patients showed persistent ALT elevation after IFN therapy (11.1%); one patient was genotype 1b while the other was 2a. HCV RNA levels in one patient (genotype 2a) were 2.7 Meq/ml at 3 months pretreatment and had increased to 9.2 Meq/ml at the commencement of treatment. HCV RNA in this patient was undetectable during therapy. The HCV RNA level in the other patient (genotype 1b) was 36 Meq/ml at the commencement of treatment and was always detectable during therapy.

Multivariate analysis of predictive factors for response to IFN treatment

We explored the predictive factors for response to IFN therapy in patients with normal ALT levels. Among the nine factors examined in the univariate analysis, only HCV RNA level significantly influenced the response to IFN therapy ($P < 0.0001$). In comparison, HCV genotype showed borderline significance, with a higher chance of sustained response ($P = 0.072$). As these two variables were mutually correlated, multivariate analysis was performed. In the last step, the following variables were entered into the model and could not be removed: HCV RNA level ($P < 0.0001$) and HCV genotype ($P = 0.022$; Table 4).

Finally, we explored the predictive factors for response to IFN therapy in all patients in both groups. On univariate analysis, the following six factors significantly influenced the response to IFN therapy: HCV RNA level ($P < 0.0001$), HCV genotype ($P < 0.0001$), total IFN dose ($P < 0.0001$), severity of liver disease ($P = 0.0005$), ALT ($P = 0.0087$), and age ($P = 0.0285$). As the variables were mutually correlated, multivariate analysis was performed. In the last step, the following variables were entered into the model and could not be removed: HCV RNA level ($P < 0.0001$), HCV genotype ($P < 0.0001$), age ($P = 0.0023$) and total IFN dose ($P = 0.0084$; Table 4).

Discussion

The clinical effectiveness of IFN monotherapy for chronic hepatitis C associated with normal serum ALT levels has been reported in numerous studies.[1–8] At the 1997 National Institutes of Health Consensus Development Conference on "Management of Hepatitis C," the Consensus Panel concluded that alpha IFN treatment could not be recommended for patients with persistently normal ALT levels and that the therapy might actually worsen the course of the disease.[18] The basis of this conclusion regarding IFN monotherapy in patients with normal ALT levels was that 30 of 52 treated patients in seven studies, for whom data were available, had had de novo elevations of ALT levels during therapy, and some patients continued to exhibit ALT

Table 4. Independent variables contributing to complete response to IFN therapy, on multivariate analysis

Variable	Multivariate odds ratio	95% Confidence interval[a]	*P* Value
Multivariate analysis of patients with normal ALT levels			
HCV RNA level (≤1 vs >1 Meq/ml)	0.047	0.015–0.15	<0.0001
Genotype (1 vs other than 1)	3.81	1.21–11.99	0.022
Multivariate analysis of all patients (both normal and elevated ALT levels)			
HCV RNA level (≤1 vs >1 Meq/ml)	0.17	0.12–0.25	<0.0001
Genotype (1 vs other than 1)	4.49	3.13–6.46	<0.0001
Age (≤51 vs >51 years)	0.57	0.39–0.82	0.0023
Total dose of IFN (≤624 vs >624 MU)	1.62	1.13–2.32	0.0084

[a] Values are the odds of having a sustained response to interferon

elevations after discontinuation of IFN.[2–8] In the seven studies, the overall CR rate was only 19%. However, in comparison, the CR rate for patients with elevated ALT levels receiving IFN monotherapy for 24 weeks was only 12%. Although the CR rate for these patients with normal ALT levels was comparable to or even higher than that for patients with elevated ALT levels, few studies had matched these two groups for other important variables, such as HCV genotype, patient age, or underlying disease severity in assessing CR rates.[1] To elucidate the efficacy of IFN therapy for these patients, considering HCV genotype and HCV-RNA level, we evaluated 94 consecutive patients with normal ALT levels at commencement of therapy in our study. We found that no patient with normal ALT levels, genotype 1b, and HCV-RNA levels over 1 Meq/ml showed a CR to IFN therapy. Moreover, among patients with genotype 2a and HCV-RNA levels over 1 Meq/ml, the CR rate was significantly lower in patients with normal ALT levels than in those with elevated ALT levels.

Why did patients, especially those with genotype 2a, with normal ALT levels and HCV-RNA levels over 1 Meq/ml, have a poorer response to IFN monotherapy? Although the effect of IFN therapy is influenced by HCV-RNA levels and/or genotypes, other factors, such as the host immune response to IFN, may influence the overall response. In patients with normal ALT levels, there may be an equilibrium between HCV replication and the host immune response.[18] Under this condition, the efficacy of IFN may decrease. However, the exact reason for this finding is not clear at present, and further studies are required at a genetic level. On the other hand, when HCV-RNA levels were less than 1 Meq/ml, there was no difference in the CR rate of patients between those with normal ALT levels at commencement of treatment and those with elevated ALT levels, independent of HCV genotype. Ohmiya et al.[19] also reported that the HCV-RNA level was associated with CR to natural IFN alpha in patients with normal ALT levels. Moreover, our univariate and multivariate analyses revealed that HCV RNA level was the most important factor in the response to IFN therapy among patients with normal ALT levels. Therefore, a low HCV-RNA level may be a favorable marker for CR in patients with normal ALT. On the other hand, univariate analysis showed that an elevated ALT level (>50 IU); odds ratio [OR], 1.76 (95% confidence interval [CI], 1.14–2.74); $P = 0.011$] was associated with a high probability of CR. However, multivariate analysis showed that ALT level did not independently or significantly influence the outcome of IFN therapy (Table 4; ALT not shown, because of lack of significance). Other factors, such as HCV RNA level and genotype, were more important.

Current evidence suggests that the treatment of patients with persistently normal ALT levels is sometimes not beneficial, and may actually induce liver enzyme abnormalities,[1] which may persist after completion of IFN treatment. The percentage of patients with ALT flare-ups after IFN therapy was reported to be 40% to 70% among patients with normal ALT levels before treatment.[2,20–22] In our study, 6 of 18 (33.3%) IFN-treated patients with persistently normal ALT levels before treatment showed ALT flare-ups posttreatment. Although virological relapse accompanied by increased ALT activity has been reported previously, the enzyme levels returned to normal values within 6 months.[20] It was speculated that these cases might reflect IFN-induced activation of a cytolytic response against infected hepatocytes.[23] In line with this hypothesis, we found that ALT activity returned to normal values after IFN withdrawal. In our study, when temporary ALT flare-ups appeared in patients who had had persistently normal ALT levels, the duration of the rise was, on average, 3 months (range, 2–4 months). However, in two patients, the ALT level remained elevated after more than 1 year (12 and 15 months, respectively) after the therapy had been finished. HCV RNA levels in these patients were high. We speculate that the IFN therapy may have destroyed the equilibrium between

HCV replication and the host immune response in these two patients. In these two patients, persistently active hepatitis may have been induced by the IFN therapy.

Since the advent of combination therapy using IFN and ribavirin, and, more recently, combination therapy using pegylated IFN (peginterferon) and ribavirin, the CR rates for HCV infection have shown a dramatic improvement.[24,25] In all studies of chronic hepatitis C patients with normal or near-normal ALT levels, the CR rates with the combination therapies have been similar to the rates reported in patients with abnormal ALT levels.[26–30] These more potent treatments may, therefore, be beneficial for patients with normal ALT levels before treatment. However, the side effects of these combination therapies have become a topical issue, and may preclude some patients from receiving combination therapy. For these patients, IFN monotherapy may be necessary, and the timing of the IFN therapy is, therefore, important.

In conclusion, we investigated the effect of IFN monotherapy in 94 patients with chronic hepatitis C infection who had had normal ALT levels at the commencement of therapy, including 18 patients with who had had persistently normal ALT levels. Among patients with genotype 2a and HCV-RNA levels over 1 Meq/ml, the CR rate was significantly lower in those with normal ALT levels than in those with elevated ALT levels. There was no difference in response according to ALT levels among patients with HCV-RNA levels of 1 Meq/ml or less. Our data suggest that IFN therapy for patients with normal ALT levels before treatment and HCV-RNA of 1 Meq/ml or less was effective. Moreover, ALT levels flared up after IFN therapy in some patients who had had persistently normal ALT levels before treatment. Our results indicate that, in patients with HCV-RNA levels of more than 1 Meq/ml, IFN therapy should be commenced only after there is an increase in the ALT level.

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Low rate of YMDD motif mutations in polymerase gene of hepatitis B virus in chronically infected patients not treated with lamivudine

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Background. Lamivudine is used for the treatment of chronic hepatitis B (CH-B), and exhibits excellent antiviral activity. However, longterm administration increases the likelihood of the emergence of resistant viruses, with an accompanying relapse of hepatitis. However, recent studies have reported lamivudine-resistant viruses in patients with CH-B before such treatment. The aim of this study was to investigate whether YMDD mutants occur in nature. ***Methods.*** The existence of lamivudine-resistant viruses was examined in 20 asymptomatic carriers of hepatitis B virus (ASC), 10 patients who lost hepatitis B surface antigen (HBsAg) during follow-up and in 20 lamivudine-treated patients with and without breakthrough hepatitis. Both polymerase chain reaction (PCR) restriction fragment length polymorphism and SMITEST hepatitis B virus (HBV)-YMDD mutation detection methods were used to detect resistant viruses. ***Results.*** No YMDD mutants were detected in the sera of the 20 ASC at the initial and final medical examinations, nor were YMDD mutants detected in sera collected at the initial medical examination, about 6 months before, or immediately after the loss of HBsAg in the 10 patients. In the 20 patients treated with lamivudine, YMDD mutants were not detected in any of them before treatment, whereas mutants were detected in the sera of 10 patients during treatment. ***Conclusions.*** Our results suggest that lamivudine-resistant YMDD mutant viruses were present in a few patients with HBV infection who before they have been treated with lamivudine.

Key words: hepatitis B virus, lamivudine, YMDD mutant

Introduction

Lamivudine is used for the treatment of chronic hepatitis B, for which it exhibits excellent antiviral activity, and is clinically useful.[1–4] One of the problems with lamivudine therapy, however, is that longterm administration increases the likelihood of the emergence of resistant viruses.[5–10] It has been reported that lamivudine-resistant viruses emerge after more than 6 months' treatment, with accompanying relapse of hepatitis. However, it was reported recently that lamivudine-resistant viruses were present in patients with chronic hepatitis B before such patients were treated with lamivudine.[11] The method for detecting YMDD motif mutation combines polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA) and the minisequence method (PCR-enzyme-linked mini-sequence assay [ELMA] method).[12] Of interest subjects found to be infected with mutant viruses before lamivudine therapy were asymptomatic hepatitis B virus carriers (ASC) who were positive for anti-hepatitis B e (HBe) antibody.[11]

The aim of the present study was to determine whether YMDD mutants exist spontaneously in nature. For this purpose, we investigated the existence of lamivudine-resistant viruses in ASC with normal transaminase levels for 10 years or more, irrespective of treatment with lamivudine, and in patients with hepatitis B who had lost hepatitis B surface antigen (HBsAg) and were treated with lamivudine. Studies were conducted using two methods for the detection of YMDD motif mutation; the above PCR-ELMA method and a PCR restriction fragment length polymorphism (PCR-RFLP) method, which was previously reported by Chayama et al.[5]

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Subjects

The study subjects were 50 patients with hepatitis B who were being followed-up at Toranomon Hospital. Three groups were investigated: (1) group A, 20 ASC who showed normal transaminase levels for 10 or more years (aged 25 to 77 years; median, 47.5 years), comprising 8 men and 12 women. Hepatitis B e antigen (HBeAg) was positive in 1 patient and negative in 19 patients, as determined by radioimmunoassay (RIA) or enzyme immunoassay (EIA). None of these patients had been treated with antiviral agents. (2) Group B, 10 patients with hepatitis B who had lost HBsAg during the follow-up (aged 31 to 51 years; median, 43 years), comprising 8 men and 2 women. At the initial medical examination at Toranomon Hospital, HBeAg was positive in 1 patient and negative in 9 patients. The HBeAg-positive patient became antigen-negative during the follow-up. (3) Group C, 20 patients who were treated with lamivudine at Toranomon Hospital, 10 of whom had breakthrough hepatitis and the other 10 who did not. They were aged 26 to 56 years (median, 43 years) and comprised 18 men and 2 women. The patients were treated with lamivudine for 25 to 290 weeks (median, 108.5 weeks), and 7 of the 10 patients who had breakthrough hepatitis were concomitantly treated with interferon (IFN). In patients in group A, serum samples were collected at the initial medical examination and at the final examination during follow-up. In patients in group B, the samples were collected at the initial medical examination, and about 6 months before, and immediately after the loss of HBsAg. In patients in group C who had breakthrough, the samples were collected before lamivudine treatment and at breakthrough (before IFN treatment in IFN-treated patients) and in patients in group C who did not have breakthrough, the samples were collected before lamivudine treatment and at the final examination at the end of lamivudine treatment.

Methods

Detection of YMDD mutant viruses

HBV-DNA was extracted from 100 µl of serum by using SMITEST EX-R&D (Genome Science, Tokyo, Japan). YMDD mutant viruses were detected by a combination of PCR-ELISA and a minisequence method (PCR-ELMA method; SMITEST HBV-YMDD mutation detection kit; Genome Science)[11,12] and by a PCR-RFLP method.[5]

HBV DNA levels were measured by transcription-mediated amplification and hybridization protection assay (TMA-HPA) (Chugai Diagnostics Science, Tokyo, Japan)[13] in patients treated with lamivudine, at baseline and 3 months after commencement of the therapy. The lower and higher limits of detection of this assay are 3.7 and 8.7 log genome equivalents per milliliter (LGE/ml), respectively.

Results

Serum samples obtained from ASC at the initial medical examination were HBV-DNA negative in 2 of the 20 patients and positive (YMDD) in the remaining 18 patients by PCR-RFLP, whereas the serum samples were HBV-DNA-positive (YMDD) in all patients by PCR-ELMA. Serum samples obtained from ASC at the final examination were HBV-DNA-negative in 7 patients and positive (YMDD) in the remaining 13 patients by PCR-RFLP, whereas they were HBV-DNA-negative in 6 patients and positive (YMDD) in 14 patients by PCR-ELMA. No YMDD mutant virus was detected by either method (Table 1).

Sera obtained at the initial medical examination from the patients who had lost HBsAg were HBV DNA-positive (YMDD) in all patients by PCR-RFLP, but were HBV DNA-positive (YMDD) in five of the ten patients and negative in the remaining five patients by PCR-ELMA. Serum samples obtained approximately 6 months before the loss of HBsAg were HBV DNA-negative in four patients and positive (YMDD) in six patients by both PCR-RFLP and PCR-ELMA. The serum samples obtained immediately after the loss of HBsAg were HBV-DNA-negative in four patients and positive (YMDD) in six patients by PCR-RFLP, but they were negative in nine patients and positive (YMDD) in 1 patient by PCR-ELMA. No YMDD mutant virus was detected by either method (Table 2).

Sera obtained from lamivudine-treated patients before such treatment were HBV DNA-positive in all patients by the PCR-RFLP method as well as by PCR-ELMA. Sera obtained during lamivudine treatment were HBV DNA-negative in ten patients by PCR-RFLP, whereas YIDD and/or YVDD was detected in the sera of the remaining ten patients by this method. By PCR-ELMA, the sera were HBV DNA-negative in three patients and positive (YMDD) in seven patients, whereas YIDD and/or YVDD was detected in the sera of ten patients by this method. The YMDD mutant viruses were detected in the same ten patients by both methods, and all of these patients had breakthrough (Table 3). On the other hand, HBV DNA levels were decreased after 3 months of lamivudine treatment in all patients (Fig. 1).

Table 1. Detection of mutant viruses in asymptomatic carriers

No.	Age (years)	Sex	ALT (IU/l)	HBeAg	YMDD motif at initial medical examination			YMDD motif at final examination		
					PCR-RFLP[a]	PCR-ELMA[b]	Date of measurement	PCR-RFLP[a]	PCR-ELMA[b]	Date of measurement
1	77	F	14	−	Negative	YMDD	07/10/1987	YMDD	YMDD	15/11/2000
2	62	F	20	−	YMDD	YMDD	22/06/1983	Negative	YMDD	20/03/1998
3	56	F	12	−	YMDD	YMDD	19/06/1986	YMDD	YMDD	16/02/1999
4	55	F	18	−	YMDD	YMDD	22/06/1983	Negative	Negative	29/05/1998
5	55	M	28	−	YMDD	YMDD	08/03/1984	YMDD	Negative	15/10/1998
6	54	F	24	−	YMDD	YMDD	07/09/1989	Negative	YMDD	24/01/2001
7	53	M	18	−	YMDD	YMDD	23/05/1988	Negative	YMDD	06/12/2000
8	52	F	26	−	YMDD	YMDD	02/07/1986	YMDD	YMDD	21/05/1996
9	51	M	16	−	YMDD	YMDD	30/05/1988	YMDD	YMDD	11/06/1996
10	51	M	16	−	YMDD	YMDD	14/05/1987	Negative	Negative	03/06/1996
11	44	F	18	−	YMDD	YMDD	25/09/1985	YMDD	Negative	01/11/2000
12	37	F	16	−	YMDD	YMDD	08/06/1983	Negative	Negative	13/07/1999
13	37	M	20	−	YMDD	YMDD	25/09/1984	YMDD	Negative	22/12/2000
14	36	M	38	−	YMDD	YMDD	26/06/1980	YMDD	YMDD	29/07/1996
15	30	F	18	+	YMDD	YMDD	13/03/1985	YMDD	YMDD	19/07/2000
16	29	M	26	−	YMDD	YMDD	06/06/1979	YMDD	YMDD	03/07/1996
17	29	F	14	−	YMDD	YMDD	19/12/1984	YMDD	YMDD	21/02/2001
18	29	F	66	−	YMDD	YMDD	12/03/1981	YMDD	YMDD	24/01/1996
19	26	M	16	−	YMDD	YMDD	21/09/1983	YMDD	YMDD	28/02/1996
20	25	F	18	−	Negative	YMDD	06/06/1985	Negative	YMDD	18/10/2000
			Sum[c]		0/20 (0%)	0/20 (0%)		0/20 (0%)	0/20 (0%)	

PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism; ELMA, enzyme-linked mini-sequence assay

[a] By the method of Chayama et al.[5]

[b] By SMITEST HBV-YMDD mutation detection kit

[c] Numbers of YMDD mutations/all samples

Table 2. Detection of mutant viruses in patients who showed clearance of HBsAg during treatment

No.	Age (years)	Sex	ALT (IU/l)	HBeAg	YMDD motif at initial medical examination			YMDD motif 6 months before loss of HBsAg			YMDD motif immediately after loss of HBsAg		
					PCR-RFLP[a]	PCR-ELMA[b]	Date of measurement	PCR-RFLP[a]	PCR-ELMA[b]	Date of measurement	PCR-RFLP[a]	PCR-ELMA[b]	Date of measurement
1	31	M	286	+	YMDD	Negative	26/08/1982	Negative	Negative	10/04/1996	YMDD	Negative	21/10/1998
2	43	M	28	–	YMDD	YMDD	15/12/1976	YMDD	YMDD	15/03/1990	YMDD	Negative	28/02/1991
3	43	M	22	–	YMDD	Negative	29/09/1982	Negative	YMDD	20/12/1995	Negative	Negative	28/08/1996
4	38	M	28	–	YMDD	YMDD	25/05/1981	YMDD	YMDD	24/01/1994	Negative	Negative	19/10/1994
5	38	M	66	–	YMDD	Negative	05/02/1987	YMDD	YMDD	30/10/1996	YMDD	Negative	03/03/1999
6	41	M	44	–	YMDD	YMDD	04/11/1988	YMDD	YMDD	29/11/1995	Negative	Negative	24/06/1998
7	51	F	60	–	YMDD	Negative	29/09/1982	Negative	Negative	12/12/1990	YMDD	YMDD	07/08/1991
8	46	F	14	–	YMDD	YMDD	14/10/1991	YMDD	YMDD	28/02/1996	YMDD	Negative	24/06/1998
9	45	M	22	–	YMDD	YMDD	21/01/1987	Negative	Negative	18/01/1994	Negative	Negative	23/08/1994
10	50	M	30	–	YMDD	Negative	14/04/1992	YMDD	Negative	10/11/1998	YMDD	Negative	31/08/1999
					Sum[c]	0/10 (0%)		0/10 (0%)	0/10 (0%)		0/10 (0%)	0/10 (0%)	

[a] By the method of Chayama et al.[5]
[b] By SMITEST HBV-YMDD mutation detection kit
[c] Numbers of YMDD mutations/all samples

Fig. 1. Hepatitis B virus (HBV) DNA levels in 20 patients at baseline and after 3 months of lamivudine therapy. HBV DNA level was measured by a transcription-mediated amplification and hybridization protection assay (TMA-PHA) method. The lower and higher limits of detection of this assay were 3.7 and 8.7 log genome equivalents per milliliter (*LGE/ml*), respectively (*dotted lines*). *Each symbol* represents an individual patient

Discussion

Lamivudine is widely used for the treatment of hepatitis B mainly in Western and Asian countries. Among the problems associated with lamivudine are the relapse of hepatitis when such treatment is discontinued, which results in the release of HBV replication suppressed by lamivudine, and the emergence of resistant viruses during the treatment.[5–10] The latter factor may cause a relapse of hepatitis, necessitating the concomitant use of other antiviral agents.[14–16] Prediction of the emergence of lamivudine-resistant viruses before treatment with lamivudine would provide clinically useful information. Lamivudine is an antiviral agent that inhibits HBV replication through the suppression of RNA-dependent DNA polymerase. What are the mechanism(s) underlying the appearance of YMDD mutant viruses? One possible mechanism is that YMDD motif mutations represent the induction of a new resistant viral strain(s) to cope with this stress. Another possible mechanism is the selection of minor preexisting resistant strains. Recently, Kobayashi et al.[11] reported that resistant viruses were present in the blood of patients who had never been treated with lamivudine. We attempted to confirm their observation by looking for YMDD mutations in patients with various stages of hepatitis B. We used two methods for the detection of the mutations; one method that was employed by Kobayashi et al.[12] and the other method that we have been using in our hospital.[5] In our present study, no YMDD mutant viruses were detected in any of the patients not treated with lamivudine or in

Table 3. Detection of mutant viruses in patients treated with lamivudine

No.	Age (years)	Sex	ALT (IU/l)	HBeAg	YMDD motif before lamivudine treatment			YMDD motif during lamivudine treatment		
					PCR-RELP[a]	PCR-ELMA[b]	Date of measurement	PCR-RELP[a]	PCR-ELMA[b]	Date of measurement
1	43	M	74	+	YMDD	YMDD	16/11/1995	Negative	Negative	22/06/2001
2	43	F	469	+	YMDD	YMDD	07/12/1995	Negative	Negative	18/06/2001
3	28	M	22	+	YMDD	YMDD	22/09/1998	Negative	YMDD	06/06/2001
4	45	M	926	+	YMDD	YMDD	12/02/1999	Negative	YMDD	06/06/2001
5	45	M	71	+	YMDD	YMDD	06/11/1998	Negative	Negative	11/05/2001
6	45	M	53	−	YMDD	YMDD	07/11/1995	Negative	YMDD	30/05/2001
7	47	M	355	−	YMDD	YMDD	22/01/1996	Negative	YMDD	16/03/2001
8	35	M	79	−	YMDD	YMDD	18/06/1997	Negative	YMDD	20/06/2001
9	38	M	68	−	YMDD	YMDD	19/01/1994	Negative	YMDD	04/04/2001
10	31	F	31	−	YMDD	YMDD	15/03/1999	Negative	YMDD	21/06/2001
11	26	M	204	+	YMDD	YMDD	28/09/1995	YVDD	YVDD	24/10/1996
12	56	M	130	+	YMDD	YMDD	14/11/1995	YIDD + YVDD	YIDD	08/12/1997
13	38	M	182	+	YMDD	YMDD	18/12/1995	YIDD	YIDD	06/01/1997
14	55	M	104	+	YMDD	YMDD	26/01/1996	YIDD + YVDD	YIDD + YVDD	04/04/1997
15	45	M	206	+	YMDD	YMDD	27/10/1999	YIDD + YVDD	YVDD	12/10/2000
16	47	M	109	+	YMDD	YMDD	09/11/1999	YIDD	YIDD	02/10/2000
17	47	M	209	+	YMDD	YMDD	29/11/1999	YIDD + YVDD	YIDD + YVDD	20/02/2001
18	29	M	416	+	YMDD	YMDD	13/01/2000	YIDD	YIDD	09/08/2000
19	42	M	64	+	YMDD	YMDD	06/03/2000	YIDD + YVDD	YIDD	01/11/2000
20	43	M	37	−	YMDD	YMDD	24/11/1995	YVDD	YVDD	16/01/2000
				Sum[c]	0/20 (0%)	0/20 (0%)		10/20 (50%)	10/20 (50%)	

[a] By the method of Chayama et al.[5]
[b] By SMITEST HBV-YMDD mutation detection kit
[c] Numbers of YMDD mutations/all samples