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Clinical and Serological Variation between Patients Infected with Different Hepatitis B Virus Genotypes

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Hepatitis B virus (HBV) has eight genotypes which have distinct geographical distributions. Studies comparing differences in the clinical outcomes of infections caused by strains with genotype-related variations in the HBV genome have largely compared genotypes B and C and genotypes A and D but not all four genotypes. The present study included 196 HBV-infected patients attending an infectious diseases outpatient clinic in Sweden. The age and geographic origin, liver function, HBeAg and anti-HBe status, and the presence or absence of HBV DNA were analyzed for each patient. HBV DNA was detected in 144 patients, and the HBV genotype and the core promoter and precore sequences were determined for the isolates from 101 of these patients. Among the patients who might be considered most likely to be nonviremic, namely, anti-HBe-positive HBV carriers with normal alanine aminotransferase (ALT) levels, 65% had detectable HBV DNA and were thus viremic. Among the viremic patients, HBeAg-positive patients were more likely to have elevated ALT levels than anti-HBe-positive patients. HBV genotypes A to F were represented in the study, and their distributions coincided accurately with the origin of the patient. A significantly higher number of genotype D-infected patients were anti-HBe positive and had elevated ALT levels (42% of genotype D-infected patients but 0% of patients infected with genotypes B and C). Genotype D strains with mutations in the core promoter and precore regions were significantly correlated with elevated ALT levels in the patients. The differences were not age related. Therefore, in this large-scale cross-sectional study, genotype D appears to be associated with more active disease.

Infection with hepatitis B virus (HBV) can lead to chronic carriage of the virus and progressive liver disease, including liver cirrhosis and hepatocellular carcinoma (HCC). The association between HBV and HCC has been proven both epidemiologically (2) and experimentally (8). Perinatal infection, in which viral transmission is vertical (i.e., from mother to child), leads to a high frequency (up to 90%) of chronic carriage. Infection with HBV is preventable by vaccines, but, as yet, there is no long-standing efficient treatment for chronic carriers of the virus. HBV infection constitutes a major global health problem, with an estimated 350 million chronic carriers worldwide and approximately 1 million HBV-associated deaths from HCC every year.

HBV belongs to the family *Hepadnaviridae* and has some unique properties. It is highly species specific, infecting only humans, chimpanzees, and some other primates. Attempts to grow HBV in standard cell lines have not been successful, although a recent study with a new hepatoma cell line has shown some promise (13). Other members of the hepadnavirus family infect animals, such as the woodchuck hepatitis virus (28) and the duck hepatitis B virus (22), but no far-reaching conclusions about HBV and its interactions with the host have been able to be extrapolated from the animal models. The replication of HBV is unusual for DNA viruses, in that it replicates through an intermediate reverse transcription step. The effect of the increased mutation rate that this leads to is partly counteracted by the extreme compactness of the HBV genome (12). There are eight genotypes of HBV (genotypes A to H); and there is some evidence that the long-term prognosis, the initial clinical picture, and the response to treatment may differ depending on which genotype has infected the patient (16). The vast majority of studies comparing the outcomes of patients infected with different genotypes have not compared all four main genotypes, however, and only genotype A has been compared with genotype D and only genotype B has been compared with genotype C (15, 23, 27).

Since HBV cannot be grown in cell culture and its species specificity precludes studies with large animals, the evidence used to measure infectivity in the individual patient has largely been circumstantial. The presence of HBsAg, the main surface protein of HBV, in serum indicates infection. After the initial characterization of HBeAg (21), a truncated form of the core (nucleocapsid) protein, all patients with both HBsAg and HBeAg are considered highly infectious. Conversely, it was believed for several years that patients who had cleared HBeAg and seroconverted to anti-HBe positivity were noninfectious. Clinical studies in which HBV DNA was detected in HBsAg-positive patients with anti-HBe, initially by hybridization and in subsequent studies by PCR, demonstrated that a proportion of HBsAg-positive patients who clear HBeAg still show signs of replicative infection (14). Carman et al. (9) put forward a molecular explanation for this.

In this study, we wanted to analyze the relationship between the presence of HBV DNA, liver function (as assessed by alanine aminotransferase [ALT] levels), and anti-HBe status in

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a large groups of patients who were of various geographical origins and who thereby harbored HBV strains of different genotypes. We also wanted to assess whether any of the genotypes appeared to be more prone to the development of mutations in the core promoter and precore regions of the genome, which are essential for replication.

MATERIALS AND METHODS

Patients. Patients with HBV infection attending the outpatient clinic at the Department of Infectious Diseases, University Hospital, Lund, Sweden, between 1992 and 2001 were included in the study. The inclusion criteria comprised a serologically proven HBV infection (HBsAg positivity) with concomitant tests for HBeAg, anti-HBe, and ALT. One hundred ninety-six patients (102 females and 94 males) were included. Clinical data and sampling of serum for tests for liver function and HBV DNA were part of the standard clinical management of the patients. The mean age of the patients was 29 years, and there was no gender difference. The patients originated from Sweden ($n = 34$), northern Europe ($n = 15$), southeastern Europe ($n = 54$), the Middle East ($n = 32$), India and Central Asia ($n = 10$), Southeast Asia ($n = 38$), Africa ($n = 12$), and Central America ($n = 1$). The origin of the HBV infection was assessed to coincide with the geographical origin of the patient.

Liver function and serological tests. ALT levels were measured by standard methods. The upper normal level in our clinical chemistry laboratory is 0.7 μkat/liter. HBsAg, HBeAg, and anti-HBe were detected with commercial test kits from Abbott Laboratories (North Chicago, Ill.).

PCR. Serum was separated and stored at −20°C. Throughout the process of sampling, extraction, and PCR, strict measures were taken to prevent the carryover of DNA; and the suggestions of Kwok (18) were carefully followed. DNA was extracted from serum by the phenol-chloroform method with subsequent ethanol precipitation. After resuspension in sterile, double-distilled water, the DNA was amplified with HBV DNA-specific primers by a previously described protocol (19), with slight modifications in cycling temperatures: the annealing temperature was 45°C in both PCRs, and the extension time was increased to 4 min when primer pair KL12-KL14 was used. The oligonucleotide primer pair used to amplify the core promoter and precore regions was KL28 (5′-1611-GAG ACC ACC GTG AAC GCC) and KL6 (5′-1974-GGA AAG AAG TCA GAA GGC A). Primer pair KL12 (5′-2814-GGG TCA CCA TAT TCT TGG G) and KL14 (5′-990-ACA TAC TTT CCA ATC AAT AG) was used to amplify the pre-S and S regions. The nucleotide numbering of Okamoto et al. (26) was used. Negative controls (sterile, double-distilled water) and positive controls (serum from highly viremic chronic carriers and DNA extracted from highly viremic chronic carriers diluted $1:10^2$, $1:10^4$, and $1:10^6$) were included in every extraction and PCR. There was never any sign of contamination or carryover of DNA. The sensitivity of this PCR was originally described to be 1 to 10 genome copies when whole purified HBV DNA is used (19), and we have recently confirmed this (data not shown). The sensitivities of the extraction and PCR protocols do not seem to be affected by long-term storage of serum samples, as demonstrated in a recent study in which HBV DNA in 30-year-old samples was successfully amplified (4).

Sequencing and genotyping. Amplified DNA was sequenced by the method of Kretz et al. (17). The sequencing primers used were described previously (5). Genotyping of HBV was performed by sequencing the core promoter, precore, and S regions and comparing key nucleotide positions with those from genotypically well defined strains after alignment of the sequences.

Statistical methods. Fisher's exact test (two tailed) and Pearson chi-square statistics (JMP, version 5; SAS Institute Inc., Cary, N.C.) were used to analyze the relationship between positive PCR results, raised ALT levels, and the geographical origin of the patient.

RESULTS

Retrospective analysis of the 196 patients with HBV infection who were included in the study demonstrated that the proportion of males and females and the mean ages of the patients from different countries were fairly uniform. The patients originating from Sweden were slightly older, but the difference was not significant.

HBV DNA was detected by PCR in serum from 144 of the 196 patients (73%). The proportions of PCR-positive patients from the different geographical regions were similar (Table 1). Two-thirds (66%) of the patients whose sera had detectable HBV DNA had normal ALT levels. In the whole study, 69% of patients had normal ALT levels, irrespective of their HBV DNA status. When the presence of HBV DNA was correlated with the serological status of the patient, 97 of the 144 PCR-positive patients (67%) had anti-HBe, and in total, 66% of the anti-HBe-positive patients were PCR positive.

TABLE 1. Proportion of patients from different geographical regions with detectable HBV DNA, elevated ALT level, and anti-HBe-positive phenotype

Geographical region (no. of patients)	% of patients		
	HBV DNA positive	With elevated ALT levels	Anti-HBe positive
Sweden (34)	73	45	88
Northern Europe (15)	67	7	93
Southeastern Europe (54)	68	39	60
Middle East (32)	68	42	71
India (10)	90	0	80
Southeast Asia (38)	79	21	56
Africa (12)	83	17	92
Central America (1)	100	100	100

Among the patients who would be considered nonviremic and noninfectious by some clinicians, i.e., anti-HBe-positive patients with normal ALT levels, 65% were PCR positive and thus demonstrated signs of viremia.

The genotype and full core promoter and precore sequences were determined for the viruses in 101 samples (70% of all PCR-positive samples). In all but two cases, the genotype corresponded to the expected genotype distribution for the geographical area from which the patient originated (Fig. 1). The two exceptions were a Swedish patient who had been nosocomially infected through a blood transfusion with HBV genotype C and a patient originating from India who carried a genotype C strain. In total, 13% of strains belonged to genotype A, 11% belonged to genotype B, 15% belonged to genotype C, 58% belonged to genotype D, 2% belonged to genotype E, and 1% belonged to genotype F.

When the correlation between the patient's serological status and the HBV genotype in the patient's sample was analyzed, the results for genotype B and C strains were similar. In contrast, the results for genotype D strains were significantly different, in that 64% of the samples containing genotype D were anti-HBe positive, whereas only 35% of the samples containing genotypes B and C were anti-HBe positive ($P < 0.05$). This difference was not age related, as the mean age of the patients from the different regions was similar. The ALT levels detected in samples infected with strains of different genotypes varied. The ALT levels in samples from patients infected with genotypes B and C were similar and differed from those in samples from patients infected with genotype D: 31% of samples from patients infected with genotypes B and C had elevated ALT levels, whereas 51% of samples from patients infected with genotype D had elevated ALT levels. This difference between ALT levels by the infecting genotype was significant when anti-HBe-positive samples were compared. All samples containing genotypes B and C with an anti-HBe phenotype but only 58% of samples containing genotype D

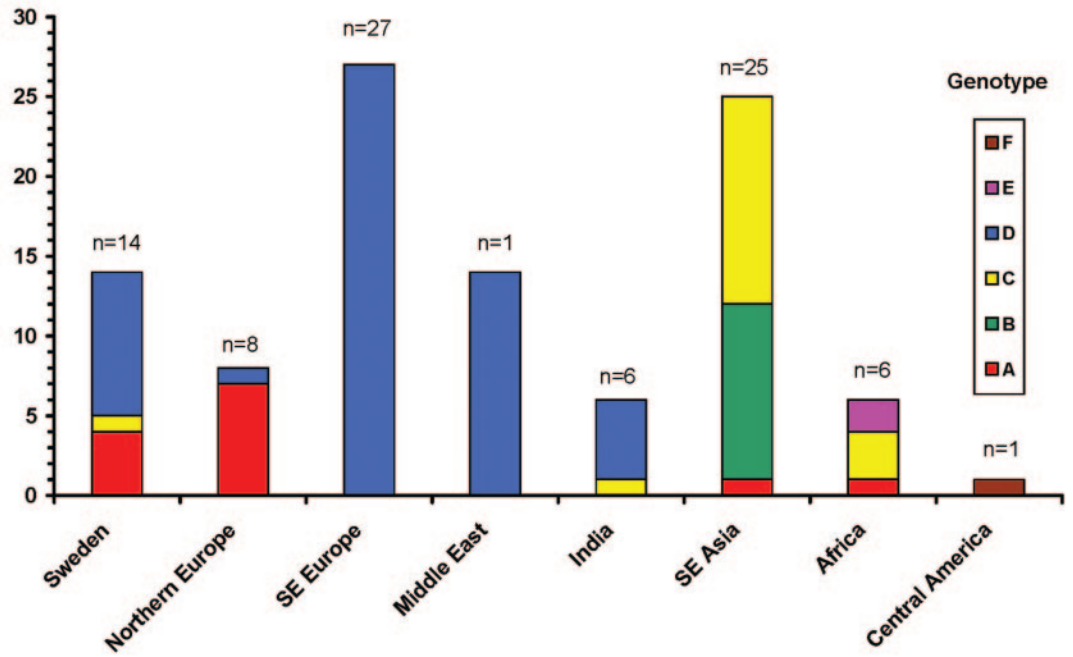

FIG. 1. Distribution of HBV genotypes in patients originating from different geographical regions.

with an anti-HBe phenotype had normal ALT levels ($P < 0.02$).

In addition to the genotype differences described above, the sequences of the strains showed considerable variability over the regions sequenced. In all, 51 of the 101 strains had mutations at nucleotide positions 1762, 1764, 1896, and/or 1899 (Table 2). There was no difference in the proportion of mutations in the core promoter region (A to T at nucleotide 1762 and G to A at nucleotide 1764) by genotype. In total, 27% of the strains carried mutations in the sequence for the core promoter region. The G-to-A mutation at nucleotide 1899 was found in 16% of the strains and was found only in strains of genotypes C and D. The G-to-A mutation at nucleotide 1896, which, together with the core promoter region-specific mutations, has been associated with an anti-HBe phenotype, was found in 38% of the sequenced strains. There was no significant difference in the proportions of strains of different genotypes displaying this mutation.

To study the clinical importance of the specific mutations in the core promoter and precore regions, the mutations were correlated to the ALT levels in the sample. Thirty-four percent of the mutated strains were isolated from serum samples with elevated ALT levels, which is similar to the proportion of samples with elevated ALT levels in the whole study. When the mutated strains were analyzed by genotype and by correlation of mutations with ALT levels, a difference between genotypes came to light. Significantly more samples containing mutated strains of genotype D than samples containing mutated strains of the other main three genotypes had elevated ALT levels ($P < 0.03$).

TABLE 2. Number of strains of different genotypes with mutations in the precore region and the core promoter region[a]

Genotype	No. of strains with mutation at nucleotide(s):		
	1896 (G→A)	1899 (G→A)	1762–1764 AGG→AGA/TGA
A	0	0	5
B	5	0	1
C	4	1	5
D	29	15	15
E	0	0	1
F	0	0	0

[a] Mutations in the precore region at nucleotide 1896 and 1899 and mutations in the core promoter region from nucleotides 1762 to 1764, according to the nucleotide numbering of Okamoto et al. (26).

DISCUSSION

The patient samples included in this study represent unselected material from a northern European infectious diseases outpatient clinic. The distribution of the geographic origins of the patients reflects both international migratory patterns and the varied prevalence of HBV in the world (6). It is likely that the effects of increased migration of HBV-carrying individuals from regions of high endemicity to regions of low endemicity will be counteracted by the efficient programs of vaccination against HBV that are being implemented in an increasing number of countries (1).

In this context, it is interesting that although the patients included in this study were all living in Sweden, the genotypes of the HBV strains infecting the patients coincided very accurately with the HBV genotypes expected from the patients' countries of origin. The genotype which is typically expected in northern Scandinavia, genotype A, was found in less than half of the Swedish strains that were genotyped. The remaining Swedish strains were of genotype D, supporting the findings of another study (3), which also found an overrepresentation of genotype D. The predominance of genotype D in southeasterm

Europe and the Middle East (25) was confirmed in this study, and genotypes B and C predominated entirely in Southeast Asia, as reported previously (6). In this cross-sectional study, anamnestic data other than the origin of the patient were not obtained. It is very likely that the duration of infection was longer in the patients who were infected with genotype B and C strains and who originated from the Far East than in genotype D-infected northern Europeans. The main route of transmission in Southeast Asia remains vertical, while a large number of genotype D infections in northwestern Europe are transmitted by intravenous drug use.

In the absence of more generally available in vitro systems that can be used to grow HBV, it can only be assumed that positive HBV DNA PCR results reflect infectivity. Ulrich et al. (30) have been the only investigators to perform an experimental study to analyze this question. Those workers tested serum that had been used to inoculate chimpanzees. There was a strict parallel between the minimal chimpanzee infectious titer and the levels of HBV DNA detected by PCR, thus providing experimental evidence that a positive HBV DNA PCR result reflects infectivity. Before these results can be generally applied, it is essential to confirm the sensitivity of the PCR being used. A number of quantitative PCRs have been described, both commercially available and in-house versions. A drawback with some of them is their rather low sensitivity level. In this study, we chose instead to use a well-validated qualitative PCR with a high sensitivity.

Before PCR became a readily available technique for the detection of HBV DNA, it was suggested that the patient's HBe and anti-HBe status and ALT levels could be used to assess the infectivity of the individual patient (20). The high cost of PCR often still prevents its routine use for the regular clinical assessment of HBV carriers. In this large study, normal ALT levels were found more often than elevated ALT levels in anti-HBe-positive patients. In 65% of the anti-HBe-positive patients with normal ALT levels, however, HBV DNA was detectable by PCR. This represents more than one-third (38%) of the total number of patients who were included in this study. We have therefore demonstrated that normal ALT levels cannot be used as a substitute for PCR to assess the infectivities of anti-HBe-positive patients.

When the patients were analyzed for signs of viral replication, i.e., positive HBV DNA results, it was seen that HBeAg-positive patients were more likely than anti-HBe-positive patients to have elevated ALT levels (55 and 24%, respectively). This is an important finding, as most earlier studies have compared all anti-HBe-positive patients with HBeAg-positive patients and thereby included nonreplicating strains. This finding is also interesting in the context of the immunotolerance that is thought to exist during the HBeAg-positive phase (7), as elevated ALT levels suggest the reaction of rather than tolerance by the immune system.

Some of the differences in the proportion of anti-HBe-positive patients seen were found to be genotype related. Genotype D-infected patients were found to be anti-HBe positive significantly more often, and among the anti-HBe-positive patients, those infected with genotype D had significantly higher ALT levels. Although the age differences between the groups might account for these differences, this was not found to be the case. Thus, genotype D may lead to more severe disease in some patients. This was suggested by McMillan et al. (24) as a result of a study in which they analyzed the outcomes for 15 liver transplant patients. However, in that study most of the isolates from genotype D-infected patients had single or double mutations at the downstream end of the gene for the precore region. These mutations have been found in other studies in which strains leading to fulminant hepatitis were analyzed. Two reports from India have provided conflicting results in this context. In a study of chronic carriers in which, surprisingly, 50% of the patients were infected with genotype A strains and 50% were infected with genotype D strains, Thakur et al. (29) found that patients infected with genotype D strains had more severe liver disease. In contrast, Gandhe et al. (11) could not find that genotype D influenced the outcome of chronic HBV infection in Indian patients. In a recent study by Chu et al. (10), patients infected with genotype B stood out as having significantly lower ALT levels, whereas in their study it was not possible to assess any difference between genotypes in anti-HBe-positive patients with elevated ALT levels.

In order to try to differentiate between the clinical importance of the infecting genotype and mutations in the core promoter and precore regions, we analyzed mutated strains of different genotypes. There was a clear and significant difference between genotype D and genotypes A, B, and C, in that patients infected with mutated genotype D strains had higher ALT levels than those infected with mutated strains of other genotypes. No differences in ALT levels were found when mutated and nonmutated strains were compared, irrespective of genotype. Therefore, genotype D appears to be associated with more active disease, as assessed by ALT levels.

In summary, this study has shown that the geographical distribution of HBV genotypes depends entirely on the geographical origin of the patients and not on random sampling in a particular region of the world. We have also demonstrated that HBV DNA detection cannot be replaced by ALT testing for anti-HBe-positive patients for the clinical assessment of infectivity. Finally, by correlating the genotype of the infecting strain with HBeAg and anti-HBe status, ALT levels, and mutations in the precore and core promoter regions, we have found evidence that HBV genotype D may be associated with more active disease.

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