JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2004, p. 5015–5021 Vol. 42, No. 11
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.11.5015–5021.2004

Strong Association between Genotype F and Hepatitis B Virus (HBV) e Antigen-Negative Variants among HBV-Infected Argentinean Blood Donors

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Received 25 March 2004/Returned for modification 22 June 2004/Accepted 21 July 2004

A number of reports have indicated an increased risk of cirrhosis and hepatocellular carcinoma in hepatitis B virus (HBV)-infected individuals carrying HBV e antigen (HBeAg)-negative variants. Although distinct core promoter and precore mutations distributed according to geographical locality and viral genotype have been reported, epidemiological data from South America are still scarce. The prevalences of HBV genotypes and core promoter and precore polymorphisms in 75 HBeAg-negative Argentinean blood donors were surveyed. The observed frequencies of HBV genotypes were 64.0% for genotype F, 17.3% each for genotypes A and D, and 1.3% for genotype C. Genotype F strains were widely distributed and significantly more prevalent in the northern region of the country ($P < 0.001$). An overall high proportion of a stop codon mutation (UAG) at precore codon 28 (66.7%) was observed. Wild-type codon 28 (UGG) was present in 29.3% of the samples, and the remaining 4.0% of samples had mixed variants. The combination of A at nucleotide (nt) 1762 and G at nt 1764 of the core promoter was found in 58.7% of the samples. The variant profiles—T at nt 1762 and A at nt 1764 or A at nt 1762 and A at nt 1764—were detected in 28.0 and 1.3% of the samples, respectively. The observed core promoter polymorphisms could not be related to the ratio of HBeAg to anti-HBeAg antibody, HBV genotype, or precore codon 28 status. Nevertheless, a clear association of genotype F and a precore stop codon mutation was found ($P < 0.05$). In conclusion, HBV genotype F and mutant codon 28 strains predominated and were strongly associated in a geographically broad Argentinean blood donor population.

Hepatitis B virus (HBV) is a blood-borne hepatotrophic virus that infects an estimated population of over 350 million people worldwide (33). Besides the manifestations associated with acute hepatitis, chronic HBV infection constitutes a serious risk for the development of cirrhosis and hepatocellular carcinoma (HCC) in patients with active liver disease (8, 36).

A genetic classification based on a comparison of complete HBV genomes with more than 8% nucleotide divergence has defined eight genomic groups, genotypes A to H (4, 40, 46, 49, 57). The HBV strains within each genomic group show a characteristic geographical distribution, which is only partially understood due to the limited number of samples from some parts of the world. Genotype A has a worldwide distribution, being more prevalent in non-Mediterranean Europe, North America, and sub-Saharan Africa. Genotypes B and C are found primarily in Asian patients. Genotype D, although widely distributed, is more prevalent in the Mediterranean area and the Middle East, and genotype E is predominant among African carriers (28, 47). Two recently described genotypes, G and H, were found in North America and France (57) and in Central America and Mexico (4), respectively. Genotype F is considered to be the indigenous HBV type from Native Americans (47). Besides the well-established association of genotype F with the aboriginal populations of Central America and South America, a number of specific clusters have been related to populations of different local geographical origins (42).

HBV e antigen (HBeAg) is present in the serum of HBV carriers during the early phase of acute infection and also during the replicative stages of chronic infection. HBeAg positivity is a good indicator of HBV viremia. During infection with wild-type virus, the transition from an HBeAg-positive to an HBeAg-negative state upon evolution of the chronic infection is usually associated with a loss of infectivity and with concomitant HBV DNA clearance and remission of liver disease (19, 36). The expression of HBeAg represents an important target for the anti-HBV immune response. The emergence of HBeAg-negative variants therefore allows the virus to survive the anti-HBeAg antibody (anti-HBe) response of the host. Patients without detectable serum HBeAg (and usually with anti-HBe positivity), while developing progressive liver disease, frequently harbor HBV mutants unable to express HBeAg but still capable of nucleocapsid synthesis (20, 43).

The most extensively characterized HBeAg-negative HBV variant contains a genetic defect in the precore region—a signal for translation interruption at codon 28 (Trp → X) due to a G → A substitution at nucleotide (nt) 1896 (11, 12). This nucleotide alteration consequently prevents both precore syn-

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TABLE 1. Geographical distribution of blood donor samples with positive amplification reactions for both HBV core promoter plus precore and surface genes

Region	Province	No. of PCR-positive samples
North	Chaco	9
	Formosa	1
	Jujuy	2
	Salta	24
Total		36
Central East	Córdoba	7
	Entre Ríos	1
	Santa Fe	1
Total		9
Metropolitan	Federal Capital	16
	Buenos Aires	6
Total		22
Central West	Mendoza	2
	San Juan	4
Total		6
South	Chubut	1
	Neuquén	1
Total		2

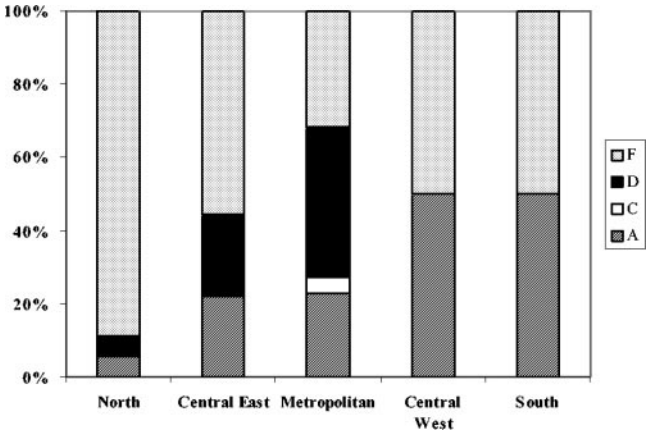

FIG. 1. HBV genotype prevalences among blood donors according to major Argentinean geographical regions.

thesis and e antigen synthesis. Other precore mutations, including single substitutions and frameshifts, have also been described but are less frequently observed and show scarce or unknown clinical associations (22, 39).

The emergence of HBeAg-negative variants (precore stop codon mutants), although first described in individuals who underwent spontaneous seroconversion and remission of chronic liver disease (51), has been associated with worsening liver pathology (3, 10). Recently, Chu et al. (15) reported that the predominance of HBeAg-negative variants before seroconversion is related to persistent viremia, liver injury, and an increased rate of cirrhosis, ascribing a time dependence to the clinical and pathological features of these mutants. Some reports do not support an association between any specific clinical status and naturally occurring precore variants (1, 59). The exact clinical relevance of precore mutants remains controversial, mainly as there have been no reports of patients who were infected initially with HBeAg variants and who progressed to chronic hepatitis (43). However, it is well recognized that these variant forms may cause more severe acute hepatitis, often with a fulminant course, in contacts (30, 61). This scenario has been observed in the majority of fulminant perinatal HBV infections occurring in infants born to HBeAg-negative–anti-HBe-positive and HBV DNA-positive mothers (54).

In some cases, the aforementioned precore stop codon mutant could not be found in HBV-infected patients with HBeAg negativity and ongoing disease. Instead, HBV basal core promoter mutants, mainly with the double substitution A1762T/G1764A, were found (34, 50). A common phenotype for these variants is decreased synthesis of precore mRNA resulting in decreased production of HBeAg and enhanced virus replication (24, 56). These mutations are almost always found together and have been identified in HBV variants derived from patients with fulminant progression, chronic active infection, and primary liver cancer (23, 32, 58). At present, despite some reported evidence, there is no final consensus as to whether these core promoter variants are indeed more virulent.

The aim of the present study was to investigate the HBV genotype distribution in a nontreated, asymptomatic, HBV-infected Argentinean blood donor population. The prevalences of precore stop codon mutation and core promoter variants in the same population were also investigated in relation to geographical localization and HBV genotypes.

MATERIALS AND METHODS

Samples. A total of 130 HBV-positive serum samples, detected by routine blood donor screening, were selected for the HBV genotype investigation. Samples were obtained from 20 Argentinean blood banks located at public hospitals distributed nationwide (15 different cities) and participating in the National Reference Laboratory External Quality Control Program in HBV and HCV Serology, coordinated by the Dr. C. G. Malbrán Institute, Buenos Aires, Argentina. Demographic and epidemiological data were collected during donation visits.

Serum samples found to be positive for antibody to HBV core antigen (anti-HBc) or HBV surface antigen (HBsAg) were additionally investigated for their HBeAg–anti-HBe profile. Serological tests were performed by commercial enzyme immunoassays for the detection of HBV infection markers (anti-HBc and HBsAg—Abbott Laboratories, North Chicago, Ill.; HBeAg and anti-HBe—Organon Teknika, Boxtel, The Netherlands).

All samples were obtained from chronically HBV-infected, asymptomatic, unrelated individuals. None of them was receiving any kind of specific antiviral therapy, reflecting prior ignorance of their infection.

HBV genotyping. HBV genotyping was performed for all PCR-positive samples by a reverse hybridization line probe assay (INNO-LiPA HBV Genotyping assay; Innogenetics NV, Ghent, Belgium [available for research use only, not for use in diagnostic procedures]) (62). One sample (1.3%) could not be genotyped. This sample was partially sequenced at Innogenetics, and the sequence was compared with published sequences to ascertain its HBV genotype.

Precore codon 28 and core promoter polymorphism testing. Serum samples were tested for precore wild-type (UGG) and mutant (UAG) codon 28 and core promoter polymorphisms (A1762/G1764, A1762/A1764, A1762/T1764, and T1762/A1764) with an INNO-LiPA HBV PreCore kit (Innogenetics [available for research use only, not for use in diagnostic procedures]) (58). The procedure was similar to that for genotyping, except for the amplified segment of the HBV genome.

Statistical analysis. Statistical analysis was performed by using the EpiInfo 2002 program. *P* values of 0.05 or less were considered significant. Differences in proportions were tested with the chi-square test.

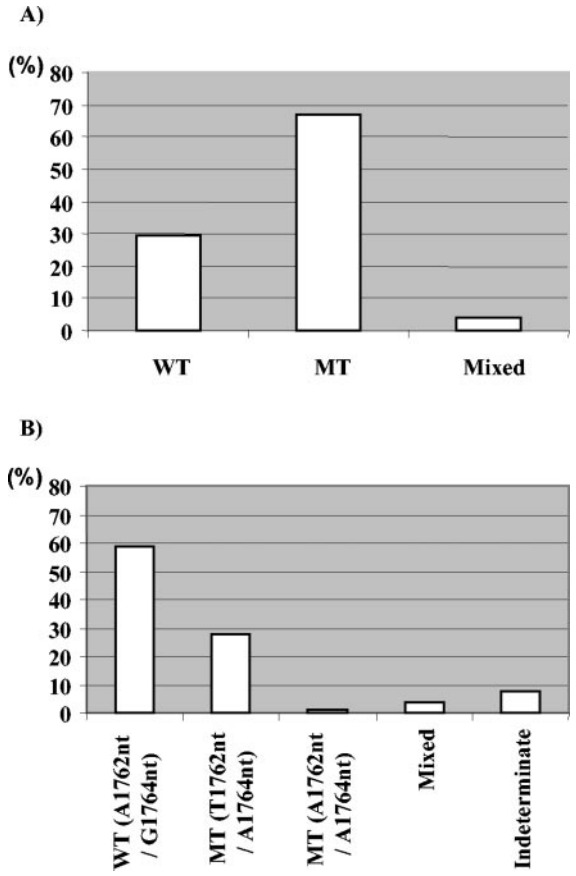

FIG. 2. Precore codon 28 (A) and core promoter (B) polymorphism prevalences among 75 Argentinean blood donors. WT, wild type; MT, mutant.

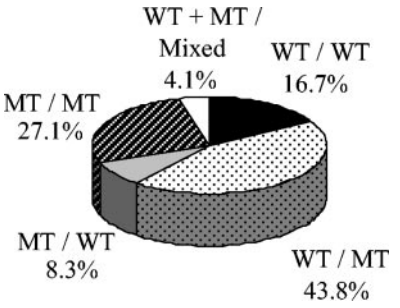

FIG. 3. Prevalences of core promoter and precore codon 28 polymorphisms in 48 HBV genotype F-infected Argentinean blood donors. WT, wild type; MT, mutant.

RESULTS

A total of 130 blood donor samples was initially screened by nested amplification of two distinct HBV genomic regions—core promoter plus precore and surface genes. Both amplification targets were detected for 75 individuals (57.7%), and the geographical distribution of these samples is shown in Table 1. Core promoter plus precore amplicons were observed in 82 of 130 samples (63.1%), while surface amplicons were obtained in 87 of 130 samples (66.9%). All of the double PCR-positive individuals were also HBeAg negative. Furthermore, 96% (72 of 75) of the individuals already showed confirmed HBe seroconversion, i.e., HBeAg negativity and anti-HBe positivity.

Prevalence of HBV genotypes. The observed prevalences of HBV genotypes in the studied population were 64.0% (48 of 75) for genotype F, 17.3% (13 of 75) each for genotypes A and D, and 1.3% (1 of 75) for genotype C. All but one sample (98.7%) could be genotyped by the INNO-LiPA HBV Genotyping assay. The remaining sample was found to be genotype D after sequencing and phylogenetic analysis. Mixed infections were not observed.

Buenos Aires and its metropolitan area were the only regions of those investigated where HBV genotype F did not predominate (31.8%). For all of the other geographical regions, the genotype F prevalence was equal to or higher than 50% (Fig. 1). Remarkably, the northern region of Argentina, which includes the provinces of Chaco, Formosa, Jujuy, and Salta, showed significantly higher genotype F prevalences than all of the other regions (88.9 versus 41.0%; $P < 0.001$).

Prevalence of precore codon 28 and core promoter polymorphisms. A high proportion of the well-known translation stop mutation (UAG) at precore codon 28 was observed in the HBV-infected blood donor population tested here (66.7%; 50 of 75 individuals). Wild-type codon 28 (UGG) was present in only 29.3% (22 of 75) of the individuals, and the remaining 4.0% (3 of 75) showed a mixed pattern (UGG and UAG in the same individual), as determined by the INNO-LiPA HBV Genotyping assay (Fig. 2A).

The combination of A1762 and G1764 at the core promoter was found in 58.7% (44 of 75) of the individuals, and the variant T1762/A1764 was observed in 28.0% (21 of 75). Only one sample had "A" at both promoter positions investigated, and the A1762/T1764 profile was not detected. Mixed core promoter polymorphisms were found in 4.0% (3 of 75) of the individuals (Fig. 2B). Indeterminate results for the core promoter region were obtained for 8.0% (6 of 75) of the individuals.

Wild-type-only polymorphisms at both surveyed regions were detected in 18.7% (14 of 75) of the individuals. A mutant core promoter containing T1762/A1764 or A1762/A1764 and mutant codon 28 containing UAG were seen in 21.3% (16 of 75) of the individuals. The relationship between polymorphism profiles at the core promoter and precore codon 28 is shown for genotype F in Fig. 3.

Prevalence of precore codon 28 and core promoter polymorphisms in relation to HBV genotypes. The distribution of core promoter or precore polymorphisms among the corresponding HBV genotypes is shown in Table 2. A clear association was observed between HBV genotype and precore region status. Genotype F was related to a high prevalence of mutant precore codon 28 ($P < 0.05$). Previously reported associations of genotype A with wild-type codon 28 and of genotype D with mutant codon 28 were also observed (14) . Although 30 of 48 samples (62.5%) with genotype F had wild-type core promoter nucleotides, this observation was not statistically significant.

The prevalence of blood donor samples containing the precore stop codon mutation in Argentina was the highest in regions where genotype F was most prevalent (Fig. 4). In the northern region of the country, 75% (27 of 36) of the samples

TABLE 2. Distribution of core promoter and precore region polymorphisms among HBV genotypes in 75 Argentinean blood donor samples

Genotype	No. of samples with the following polymorphisms:			
	Core promoter (nt 1762/nt 1764)		Precore codon 28	
	WT (A/G)	MT[a]	WT (UGG)	MT[b]
A	6	7	9	4
C	1		1	
D	7	6	1	12
F	30	18	12	36[c]

[a] MT, mutant. T/A, A/A, A/G plus T/A, and indeterminate results.
[b] UAG and UGG plus UAG.
[c] The *P* value for a comparison of genotype F against genotypes A and C was < 0.05.

contained mutant precore codon 28. Interestingly, Buenos Aires and its metropolitan area, where the highest level of HBV genotype heterogeneity was observed, also had 83.3% (five of six) of the cases in which mixed core promoter or precore region profiles were found.

DISCUSSION

The present work helps to clarify the distribution of HBV genotypes in Argentina. Studies based on blood donors are more appropriate for investigations of genotype prevalence than are patient-based surveys, as there is less bias in this set of subjects than that imposed by the selection of patients needing or under treatment (27). Nevertheless, one cannot establish the observed genotype prevalence as a trustworthy representation for the whole country, since the distribution of samples does not represent the general Argentinean population distribution or even the blood donor subgroup. For example, the central regions of Buenos Aires and surrounding provinces are known to have a low prevalence of HBV infections (41). They represent more than 45% of the Argentinean population (Censo Nacional de Población, Hogares, y Viviendas, 2001 [http://www.indec.gov.ar/webcenso/provincias_2/pais/pais.asp]) and contributed 29.3% of the genotyped samples. On the other hand, the northern provinces of Chaco, Jujuy, Formosa, and Salta constitute areas with a high prevalence of HBV infections (Epidemiological Bulletin 2003 [http://www.hepatitisviral.com.ar/ppnhv.htm]). Although they represent only 8.7% of the Argentinean population (Censo Nacional de Población, Hogares, y Viviendas, 2001), they contributed 48.0% of the analyzed samples.

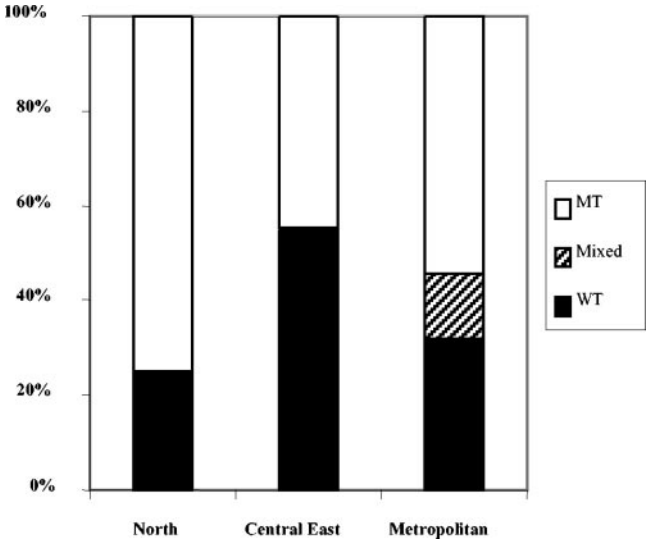

FIG. 4. Prevalence of precore codon 28 polymorphisms in Buenos Aires and the northern and central eastern regions of Argentina. WT, wild type; MT, mutant.

Samples from 74 (98.7%) of the 75 PCR-positive subjects were successfully genotyped by the INNO-LiPA HBV Genotyping assay. The only sample testing negative in the assay was found to be genotype D after partial S gene amplification and sequencing and subsequent phylogenetic analysis. This was possible because the clustering pattern of S gene sequences agrees with the HBV genotype classification based on the whole-genome sequence (5, 47, 49).

The most prevalent HBV genotype observed in Argentinean blood donors is genotype F (64%), similar to previous findings in Venezuela (9, 44) and Central America (5). Genotype F has also been sporadically detected in samples from Brazil (17, 45), North America (14, 47), Europe (21, 48), and Polynesia (16). However, none of these reports focused on blood donors. Genotype F has already been shown to be frequent in Argentina, although previous published works dealt with limited numbers of samples and were regionally restricted (Table 3).

Noteworthy was the finding that genotype F samples were significantly more prevalent among blood donors in the northern region of the country (88.9%) relative to the global Argentinean genotype F prevalence (64.0%) ($P < 0.001$). This region has a high prevalence of serological markers of HBV infection (Epidemiological Bulletin 2003 [http://www.hepatitisviral.com.ar/ppnhv.htm]). The calculated prevalence (17.3%) for genotypes A and D in Argentina, which were more prevalent in Buenos Aires and the Federal Capital (22.7 and 40.9%, respectively), may reflect the massive European immigration, especially from genotype D-enriched Mediterranean areas (25).

Precore mutant HBV strains have long been detected in chronically infected Argentinean patients (38, 39). In the present work, mutations in the precore region and in the core promoter were found to be very frequent among blood donors. Most (81.3%) of the samples had at least one variant nucleotide located at the investigated sites—precore codon 28 and core promoter nt 1762 and 1764. These mutations, which are most frequently detected in association with interrupted or reduced HBeAg expression (43), could help to explain the HBeAg–anti-HBe status. All PCR-positive samples showed anti-HBe positivity, and the majority of them (96.0%, or samples from 72 of 75 subjects) showed complete seroconversion (HBeAg negative and anti-HBe positive).

A remarkably high proportion (66.7%) of the UAG precore codon 28 mutation was detected among blood donors. The presence of this mutation was also confirmed in three other samples (4.0%) which contained both wild-type codon 28 and mutant codon 28. Similarly, López et al. (39) reported that 50.0% (13 of 26) of samples had evidence of the stop codon 28 mutation among HBeAg-negative Argentinean patients with chronic infections. In vitro studies demonstrated that the UAG

TABLE 3. Genotype F prevalence in distinct populations in Argentina

Reference or source	No. of samples	Genotype F prevalence (%)	Method	Population	Origin
60	12	41.7	Sequencing[a] and phylogenetic analysis	Adult chronic carriers	Buenos Aires
41	11	54.5	Sequencing[a] and phylogenetic analysis	Infants under treatment	Buenos Aires
39	42	45.2	Sequencing[a] and phylogenetic analysis	Patients under treatment	Not specified
This work	75	64.0	INNO-LiPA	Blood donors	Nationwide

[a] Partial S gene sequencing.

stop codon 28 mutation completely abolishes HBeAg synthesis (7). Although HBeAg is not required for viral replication, HBV strains unable to express HBeAg can be considered slow replicative variants. Therefore, the serum viral load detected in subjects infected with mutant codon 28 variants is usually lower than the load detected in patients infected with wild-type virus before seroconversion (43).

Precore mutations are restricted by the secondary structure of the HBV pregenomic encapsidation signal, depending primarily on the nucleotide at position 1858. This specific hairpin structure is essential for the packaging of viral pregenomic RNA (29). In strains with the wild-type precore, nt 1858 shows complementarity with opposing nt 1896 (which is directly involved in the nonsilent mutation G1896A) and is found in close association with the HBV genotype (48, 55). The presence of "C" at nt 1858 seems to be unfavorable to the development of a stop mutation due to its destabilizing effect on the stem of the encapsidation signal. HBV strains belonging to genotypes A and H and some strains belonging to genotype C usually harbor C1858; therefore, the transition G1896A is consistently rare in these genomic groups (2, 4, 35). On the other hand, most of the genotype B to E strains harbor T1858, which is known to allow the G1896A mutation frequently observed in precore variants (13, 55). In this work, apart from the study of nt 1858, we found the previously established associations between genotype A and wild-type codon 28 and between genotype D and mutant codon 28 for Argentinean carriers.

A clear association between genotype F and the precore stop codon mutation (34 of 48; 70.8%) was found during the investigation of Argentinean blood donor samples ($P < 0.05$). This association was previously described for HBV-infected patients of Hispanic origin in Central America (6), where most genotype F strains contain T1858. However, in other Latin American populations, in which C1858 predominates in circulating strains of HBV, the association between the precore stop codon mutation and genotype F is not clear or is uncertain (28, 48). Therefore, Argentinean strains of HBV genotype F were closer to those observed in Central America with respect to precore codon 28 status and may harbor T1858.

Important nucleotide sequence differences among HBV genotype F strains have been detected, even when the strains have been isolated from neighboring locations. Genotype F, while being the most divergent HBV genotype (46), has also shown high intragroup variability and can be subdivided into different clades (41, 42, 48). When complete genome sequences were used for phylogenetic analyses, genotype F samples from Venezuela and Colombia clustered separately from those from Central America, a pattern resembling viral genetic subgrouping in these geographical regions (4). Other phylogenetic analyses based on partial (41) or complete (42) S gene sequence information have indicated different clades for HBV strains derived from Argentina and Central America. Consequently, genotype F isolates from South America and Central America seem to fit into distinct clusters, despite the observed precore codon 28 profile similarities between Argentinean and Central American strains.

Samples from a total of 92.0% (69 of 75) of the subjects were successfully typed for core promoter nucleotides (1762 of 1764) by the INNO-LiPA HBV PreCore assay. The possible reasons for the failure of promoter analysis for samples from 8.0% (6 of 75) of the subjects were not investigated further, but less frequent single-nucleotide polymorphisms or insertions or deletions not included in this version of the assay are possibilities. Core promoter T1762/A1764 and A1762/A1764 variants were found isolated (29.3%; 22 of 75) or mixed (4.0%; 3 of 75) with wild-type strains in samples from 33.3% (25 of 75) of the subjects. This observed prevalence of HBV basal core promoter mutants was consistently lower than that observed for codon 28 (precore region) mutants (66.7%). Various investigators have reported an inverse association between the prevalences of precore coding region and core promoter mutants (13, 14). HBV strains carrying T1858 (most genotype D strains) display more UGG→UAG (codon 28) mutations than core promoter mutations during HBeAg–anti-HBe seroconversion, due to the higher stability of the encapsidation signal. The reverse has been reported for strains displaying C1858 (mostly genotype A strains), in which a UGG→UAG change would lead to a loss of hairpin stability. In this situation, core promoter mutations and/or precore changes distinct from G1896A supply the strain with the necessary mechanism for reducing or interrupting HBeAg synthesis, respectively (28, 31, 37).

In the present work, 61.1% (22 of 36) of the genotype F samples showing mutant codon 28 also showed wild-type core promoter polymorphisms. Nevertheless, only 33.3% (4 of 12) of the genotype F samples (all anti-HBe positive) showing wild-type codon 28 had confirmed mutations in the core promoter. It is therefore conceivable that the remaining non-HBeAg-expressing genotype F strains utilize other known or alternative strategies during the seroconversion phase. In Argentinean genotype F samples, despite the observed high prevalence of codon 28 mutations and the HBeAg–anti-HBe status, the investigated association between the core promoter (nt 1762 and 1764) and the precore region (codon 28) did not reach statistical significance. Core promoter status was also not related to any particular geographical region or HBV genotype. The distribution of core promoter or precore codon 28 polymorphisms in genotype F samples was very different from

recently published findings for genotypes A to D from infected patients in the United States (14).

For individuals who have HBeAg-negative chronic hepatitis B and who meet the criteria for treatment, a special regimen during and after treatment follow-up should be considered. For instance, current data suggest longer treatment schedules with alpha interferon for HBeAg-negative patients than for patients with HBeAg-positive chronic hepatitis (at least 48 weeks and 16 to 24 weeks, respectively) (36). Another major problem with alpha interferon therapy for HBeAg-negative patients is the high incidence of relapsing—approximately half of the responders relapse when therapy is discontinued (53). Lamivudine and other nucleoside analogs appear to be equally efficacious against wild-type and HBeAg-negative variants (43). However, a recent study showed that HBeAg-negative patients frequently can develop virological and biochemical breakthrough with disease progression while on lamivudine therapy (52).

The investigation of a possible correlation between HBV genotypes and severity of liver disease, therapy outcome, and other important clinical issues is in its infancy. Unfortunately, despite increasing relevance, most studies have compared genotype B with genotype C or genotype A with genotype D due to the geographical pattern of genotype distribution. For instance, several investigators have reported different prognoses attributed to genotypes B and C in Asian cohorts. Genotype C is more frequently associated with a worsening chronic clinical presentation, more severe cirrhosis, increased HCC prevalence, and a weaker response to alpha interferon-based treatment (18, 26). Recently, genotype F strains were significantly associated with HCC prevalence and lower age at diagnosis of HCC among natives of Alaska (S. Livingston, J. Simonetti, B. McMahon, L. Bulkow, K. Hurlburt, C. Homan, M. Snowball, V. Chulanov, O. Nainan, H. Margolis, and J. Williams, Abstr. 54th Annu. Meet. Am. Assoc. Study Liver Dis., abstr. 202, 2003). This new finding combined with the presumed worst outcome of HBeAg-negative chronic hepatitis should alert clinicians, especially in regions where genotype F predominates (e.g., northern Argentinean provinces) and a genotype F-stop codon mutation association has been revealed.

ACKNOWLEDGMENTS

This work was supported in part by grants from CAPES and Instituto de Diagnóstico Molecular Theranostica.

We thank Henrique Sérgio M. Coelho and Jorge A. Segadas for continuing support and advice. Special thanks are due to centers participating in the External Quality Control Program in HBV and HCV Serology for their assistance in data and sample collection and to the coordinators of the Sentinel Units of the Argentinean National Program for the Control of Viral Hepatitis.

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