Hepatitis B virus genotypes identified by a Line Probe Assay (LiPA) among chronic carriers from Spain

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Genotypes A and D of the hepatitis B virus were found to be prevalent among 278 chronic carriers residing in Spain, and genotypes B, C, E and F were detected with significant frequency (9%). Two genotype E infections corresponded to carriers born in Spain who had never traveled to Africa. These results indicate that genotype E is beginning to circulate in the Spanish population in the same way that genotype F did in the past.

Key words: **Hepatitis B virus. Genotypes. Epidemiology. Line probe assay.**

Genotipos del virus de la hepatitis B detectados mediante hibridación reversa en tira (LIPA) en portadores crónicos residentes en España

Se ha encontrado una alta prevalencia de los genotipos A y D del virus de la hepatitis B al estudiar 278 portadores residentes en España. Además, el 9% de las infecciones se asociaron a los genotipos B, C, E y F, incluyendo 2 casos de infección por genotipo E en portadores españoles que nunca habían visitado África. Estos resultados indican que el genotipo E ha comenzado ya a circular entre la población española, como ya lo hizo el genotipo F en el pasado.

***Palabras clave*: Virus de la hepatitis B. Genotipos. Epidemiología. Hibridación reversa.**

Introduction

Molecular studies performed on hepatitis B virus (HBV) genomes have rendered the identification of six major genotypes, namely genotypes A-F, whose complete sequences have already been obtained[1]. An additional HBV genotype, the genotype G, has been found among a few chronic carriers from France and from the United States[2] and has shown to belong to an independent phylogenetic branch[3]. Recently, a novel genotype (genotype H), closely related with genotype F, has been found in Central America[4].

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On the basis of molecular clock studies, the genotype F has been identified as the closest to the putative HBV virus ancestor[5]. Since this genotype is characteristic from the human populations original from America, it is thought that HBV emerged as a human virus in that continent and was brought to other geographical regions, evolving locally to generate the remaining genotypes. Genotypes B and C are characteristic from the Far East, but a particular subset of genotype C strains, found among Australian Aborigines, seems to be genetically divergent from the Chinese strains[6]. Genotype E is prevalent in the Subsaharian Africa, whereas the genotype A prevails in the North of Europe, North America and among the Australian population of European origin. The genotype D is spread worldwide, but it is characteristic from the Mediterranean region, the Middle East and India. Introduction of exotic genotypes by immigrants coming to Western Europe has been, however, already documented[7] and such introduction may be influencing the molecular epidemiology of the HBV infection in the region.

Data regarding the distribution of HBV genotypes in Spain are still very scarce, but reveal the circulation of strains from genotypes A, D and F[8,9]. With the aim of extending such data, the genotypes present in serum samples from 278 HBV DNA-positive chronic carriers residing in Spain have been examined.

Methods

From May, 2001 to August, 2002, single serum samples taken from 722 HBV surface antigen (HBsAg) carriers were sent to our laboratory from different health care centres from Spain. Since these samples were sent for study just for diagnostic purposes and without a specific request, they are not representative of the population of HBV carriers from these regions. HBV DNA was tested by a nested, polymerase chain reaction (n-PCR) assay, targeted on the P-S region of the HBV genome[10], in all samples. Outer primers HBPr134 and HBPr135 (5'-TGC TGC TAT GCC TCA TCT TC-3' and 5'-CA(A/G) AGA CAA AAG AAA ATT GG-3', respectively) were used in the first reaction for obtaining a fragment that was amplified again in a second reaction by using nested primers HBPr75 and HBPr94 (5'-CAA GGT ATG TTG CCC GTT TGT CC-3' and 5'-GGT A(A/T)A AAG GGA CTC A(A/C)G ATG-3', respectively)[11]. A final fragment of 341 base pairs, encoding aminoacids 89 to 211 from the HBsAg molecule, was finally obtained and detected by agarose gel electrophoresis. Viral DNA was subsequently quantified by a molecular hybridisation test (Digene Hybrid Capture II, Digene Corp., Gaithersburg, MD, USA) on all the n-PCR-positive samples. Since the nested primers in the n-PCR test were biotinilated, the final amplification products from all these samples were biotin-labelled and could be directly tested for identification of HBV A-G genotypes by a reverse hybridisation test that uses a collection of genotype-specific probes adsorbed on nitrocellulose strips (Line Probe Assay, INNO-LiPA HBV Genotyping, Innogenetics

TABLE 1. **HBV genotypes found among 278 chronic carriers positive for HBV DNA in serum in regard to the HBeAg/anti-HBe status and the level of viral DNA**

HBeAg	Anti-HBe	Viral DNA (pg/ml)	Number of cases	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	NT (%)
Positive	Negative	> 1,000	57	13	2	3	32	4	1	2
		< 1,000	49	21	1	3	18	2	3	1
Total			106	34 (32.1)	3 (2.8)	6 (5.6)	50 (47.2)	6 (5.6)	4 (3.8)	3 (2.8)
Negative	Positive	> 1,000	13	1			12			
		< 1,000	159	33		1	119	5		1
Total			172	34 (19.8)		1 (0.6)	131 (76.1)	5 (2.9)		1 (0.6)
Total studied			278	68 (24.4)	3 (1.1)	7 (2.5)	181 (65.1)	11 (4.0)	4 (1.4)	4 (1.4)

NT: strains that could not be typed by the genotyping test.

N.V., Ghent, Belgium). Finally, HBV "e" antigen (HBeAg) and antibody to HBeAg (anti-HBe) were determined in all samples by an automated immunoassay test (Vitros ECi, Ortho Clinical Diagnostics, Raritan, NJ, USA).

Results

Samples from 278 carriers (38.5%) were positive in the n-PCR assay. Of them, 33 were foreigners, coming from the Far East, Africa and Eastern Europe, and 248 were Spaniards who lived in 11 different regions of Spain, namely Andalucía, Baleares, Castilla-La Mancha, Castilla-León, Ceuta, Extremadura, Galicia, Madrid, Murcia, Navarra and Valencia. Eighty-one were women and 197 men, including a two months-old infant born from a carrier mother, seven children aged six to 14 years and 270 adults (age range, 15-79 years, mean age, 45.3 years).

The results obtained from genotyping the HBV strains detected among these carriers are summarised in table 1. Four strains (1.4%) did not react with any of the probes and could not be, therefore, typed by the LiPA test. Genotypes A and D were the most commonly found (249 cases, 89.5%) and genotype D was the most prevalent (181 cases, 65.1%). However, the prevalence of genotype D was significantly lower among the HBeAg-positive carriers (47.2 vs. 76.1%; $\chi^2 = 23.01$, $p < 0.01$), especially among those showing a viral DNA level below 1000 pg/ml (18 cases, 36.7%). Genotype F strains were identified in four samples (1.4%), all of them coming from HBeAg-positive patients born in Spain.

HBV genotypes characteristic from the Far East (B and C) and from the tropics of Africa (E) were found in a total of 21 cases (7.6%). All strains belonging to genotypes B and C were detected among immigrants coming from China and residing in different areas of the country, but never among carriers born in Spain. Most of them were HBeAg-positive, as is characteristic of the HBV carriers from that region. Strains from genotype E were detected among immigrants coming from Africa, but also in samples from two Spanish carriers, residing in Navarra and Palma de Mallorca, who had never travelled to Tropical Africa.

Discussion

The results obtained in this study confirm the dominance of HBV strains from genotypes A and D in Spain, as well as the circulation of genotype F strains among the Spanish population[9], as already suggested by the prior detection of HBV strains from the antigenic subtype adw4[12]. In addition, the significantly higher prevalence of genotype D found among the anti-HBe-positive carriers agrees with prior data suggesting that strains of this genotype may show a pronounced trend to establish HBeAg-negative chronic infections due to selection of precore-defective mutants[8]. HBV genotype D strains exist in two main, separate antigenic subsets, namely ayw2 and ayw3, which present a distinct pattern of geographical distribution. Both types of strains are common in the Western world, but D/ayw3 strains are also highly prevalent in India and could have been introduced recently into Europe and North America through the intravenous drug abuse. Whether or not both antigenic groups share the same ability to establish precore-defective chronic infections is unknown and could be a matter of future investigations.

The finding of a significant proportion of HBV strains from genotypes B, C and E indicates that exotic HBV genotypes are being introduced in Spain by the immigrants and shows that, as formerly happened with genotype F, some of them are beginning to circulate among the autochthonous population. Noteworthy, no carriers of genotype F coming from Latin America were detected in this study, besides the high number of immigrants coming to Spain from Latin American countries in the last 20 years. This finding agrees with the data obtained in that region, which show a low endemicity of the HBV infection in most urban and rural areas unrelated with the Amazonian Basin[13].

Although the investigations regarding the influence of the HBV genotypes on the events of the viral persistency and the chronic liver infection are still scarce, evidence suggesting the clinical and public health relevance of these genotypes is already emerging. Most of the issues risen by these investigations are still controversial and further studies in relation with these matters should be, therefore, performed. In order to provide a better basis for interpreting the results that such studies may rise-up, an assessment of the distribution of HBV genotypes among the population of chronic HBV carriers from a given geographical area is necessary. The results obtained in this study extend the data available from Spain and evidence an epidemiological reality that seems to be more complex than previously thought.

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