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# Distribution of Hepatitis B Virus Genotypes among American Blood Donors Determined with a PreS2 Epitope Enzyme-Linked Immunosorbent Assay Kit

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**We genotyped 418 sera from volunteer blood donors from two large, regional blood centers in the United States who were HBsAg positive by an enzyme-linked immunosorbent assay (ELISA). The HBV genotypes were determined by a serological method using a preS2 epitope ELISA kit (Institute of Immunology, Tokyo, Japan) with monoclonal antibodies. Of the 418 samples, the genotypes of 320 could be determined (76.6%). One hundred forty-three (34.2%) were genotyped as A (preS2 subtype su), 31 (7.4%) were genotyped as B (subtype m), 59 (14.1%) were genotyped as C (subtype ks), 83 (19.9%) were genotyped as D or E (subtype ksu), and 4 (1.0%) were genotyped as F (subtype k). This kit cannot differentiate genotypes D and E. For 98 (23.4%) of the 418 samples, the genotype could not be determined; 11 of these 98 samples were positive for at least one of the preS2 genotype-specific epitopes (m, k, s, and u), but the combinations of positive epitopes were different from those of samples that could be genotyped; 45 had only the common epitope (b). In the group with a high signal-to-cutoff (S/C) ratio, the HBV genotype could be determined for 199 (84%) of 237 samples; in contrast, in the low-S/C-ratio group, only 10 (20%) of 51 samples could be genotyped ($P < 0.001$). These findings may indicate the limitation of genotyping samples with low S/C ratios for HBsAg by ELISA or the existence of genotype G or other new HBV genotypes in HBsAg-positive blood donors in the United States. Of the genotyped samples, 201 were assayed for HBeAg; only 9 (4.5%) were positive for HBeAg. The frequency of genotype C in HBeAg-positive donor samples (5 of 9 or 56%) was higher than that in HBeAg-negative donor samples (33 of 192, or 17%) ($P = 0.022$).**

Hepatitis B virus (HBV)-positive samples can be classified into one of six genotypes, A to F (8, 10). Previous studies have suggested that the natural histories of HBV carriers, patients' responses to interferon therapy, and the development of chronic hepatitis and/or liver cirrhosis are associated with specific HBV genotypes (4, 5, 9). Since genotyping previously required labor-intensive methods like HBV DNA sequencing or PCR plus restriction fragment length polymorphism analysis, little HBV genotyping of HBsAg-positive American blood donors has been performed. With the advent of an enzyme-linked immunosorbent assay (ELISA) kit with monoclonal antibodies against the preS2 region (16), it is possible to genotype large numbers of blood donors' samples with HBsAg more easily. The correlation between the results of genotyping with this ELISA kit and those of nucleic acid-based technologies has been excellent (16).

Initially, HBsAg subtypes (adr, adw, ayw, and ayr) were used for studies of the geographic distribution of HBV (2, 3, 13). However, the HBsAg subtype does not reflect true genotypic variation. The HBV subtype classification was based on a limited number of amino acid substitutions; sometimes the HBsAg subtype can be changed by a nucleotide point mutation of the S gene (12). Since the HBV genotype is due to the entire nucleotide sequence, the HBV genotype is more appropriate for investigation of geographic distribution and epidemiologic connections.

Previous studies reported the distribution of HBV genotypes by area. In the Far East, genotype B and genotype C predominate (8). In Western European countries, genotype A is predominant (11, 16). In Central America, genotype F is predominant (1). Little information is available from the United States. The genotype frequency may also be impacted by the ethnic backgrounds of HBsAg-reactive individuals in different geographic areas (1). We elected to genotype samples from blood donors identified as HBsAg positive at two large regional blood centers with ethnically diverse populations and individuals from many parts of the United States. The areas of California and Texas that are served by the regional blood centers whose samples were used in this study cover large geographic regions which are representative of each of these states as a whole, as well as include individuals from the remaining 48 states because of the moderate weather and employment and educational opportunities, which prompt extensive movement of people to these two states.

## MATERIALS AND METHODS

A total of 418 sera from blood donors confirmed to be positive for HBsAg by ELISA were tested with a monoclonal genotyping ELISA kit (16). Three hundred seventeen samples were from the Gulf Coast Blood Center in Texas, and 101 were from the Sacramento Medical Foundation Blood Centers in California. Both centers serve large parts of their respective states. Each draws donors from its major universities and a diverse work force from many regions of the United

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TABLE 1. Distribution of HBV genotypes among 418 HBsAg-positive American blood donors

| Genotype | PreS2 serotype | No. of samples | % of samples |
|---|---|---|---|
| A | su | 143 | 34.2 |
| B | m | 31 | 7.4 |
| C | ks | 59 | 14.1 |
| D or E[a] | ksu | 83 | 19.9 |
| F | k | 4 | 1.0 |
| UD[b] | | 98 | 23.4 |

[a] Genotypes D and E could not be differentiated with the HBV genotype ELISA kit used.

[b] UD, undetermined. Eleven of 98 samples were positive for at least one of the preS2 type-specific epitopes (m, k, s, and u), but the epitope pattern of positive samples was different from that of samples with defined genotypes.

States; in addition, the population served by each is ethnically varied. In California, Caucasians now make up less than 50% of the population and there are sizable numbers of Asians, Hispanics, and African-Americans. In Texas, Caucasians make up greater than 50% of the population but there are sizable numbers of Hispanics and African-Americans. Since all samples were from asymptomatic, volunteer blood donors, none were from patients under treatment with antiviral therapy (e.g., interferon or lamivudine). Samples were initially tested for HBsAg with an HBsAg ELISA kit (HBsAg ELISA 2.0; Ortho Diagnostics, Raritan, N.J.; or Auszyme Monoclonal; Abbott Laboratories, North Chicago, Ill.). Samples tested for HBsAg by Auszyme were selected based on optical density (OD) values higher than 2.0, except with two samples. Two hundred one of the samples were tested for HBeAg with an HBeAg ELISA kit (AusE; Abbott Laboratories).

HBV genotypes were determined using a preS2 epitope ELISA kit (HBV Genotype; Institute of Immunology, Tokyo, Japan) by following the manufacturer's package insert procedure (16). Briefly, at the first step, HBsAg in the serum was captured on a 96-well microplate coated with monoclonal antibody to the common determinant "a" of HBsAg. In the second step, each of four wells received enzyme-labeled monoclonal antibodies to a genotype-specific preS2 epitope (m, k, s, or u). Genotypes were determined by the combination of preS2 epitopes: su for genotype A, m for genotype B, ks for genotype C, ksu for genotypes D and E, and k for genotype F. In this assay, genotypes D and E have the same reactions with the defining monoclonal antibodies (17). When the sera reactive for HBsAg had no genotype-specific epitopes, antibody to epitope b, expressed on all HBV genotypes, was used in the second step to determine the presence of a preS2 product in the sample. Statistical analysis was performed by the chi-square test. This study was approved by the Sacramento Medical Foundation Institutional Review Board.

## RESULTS

Of 418 samples, 320 (76.6%) could be genotyped. One hundred forty-three (34.2%) were genotyped as type A (preS2 subtype su), 31 (7.4%) were genotyped as type B (subtype m), 59 (14.1%) were genotyped as type C (subtype ks), 83 (19.9%) were genotyped as type D or E (subtype ksu), and 4 (1.0%) were genotyped as type F (subtype k) (Table 1). Two hundred one of the samples were tested for HBeAg. Nine (4.5%) of these 201 samples were positive for HBeAg. The frequency of genotype C for HBeAg-positive samples was 56% (5 of 9 samples), while that for HBeAg-negative samples was 17% (33 of 192 samples) ($P = 0.022$) (Table 2).

The genotype could not be determined for 98 (23.4%) of the samples that were reactive for HBsAg by ELISA. Eleven of these samples were positive for one or more of the preS2 genotype-specific epitopes; however, the combinations of detected epitopes (seven of subtype s, three of subtype msu, and one of subtype mk) were different from the combinations for known genotypes with this assay. Of the 87 samples negative for all preS2 genotype-specific epitopes (m, k, s, and u), 45 were positive for the preS2 epitope b (the common epitope of the preS2 region). Thirty-nine of the 42 samples that were negative for any epitope, including b, had low levels of HBsAg by ELISA as detailed below.

TABLE 2. Association of HBeAg with HBV genotypes

| Genotype | Total no. of samples | No. of samples that were HBeAg | |
|---|---|---|---|
| | | Positive | Negative |
| A | 83 | 2 | 81 |
| B | 17 | 1 | 16 |
| C | 38 | 5 | 33 |
| D or E | 59 | 1 | 58 |
| F | 4 | 0 | 4 |
| Total | 201 | 9 | 192 |

According to the level of HBsAg determined by the Ortho ELISA kit, 288 samples could be divided into two groups: samples with a signal/cutoff (S/C) ratio greater than 50 (high) and those with an S/C ratio from 1.0 to 50 (low). In the high-S/C-ratio group, the HBV genotype could be determined for 199 of 237 samples (84%). By contrast, in the low-S/C-ratio group, only 10 of 51 samples (20%) could be genotyped ($P < 0.001$). Of 79 untypeable samples, of which 38 had a high level of HBsAg and 41 had a low level of HBsAg, 35 samples (92%) of the high-level group had one or more detectable preS2 epitopes while only 2 (4.9%) of the low-level samples had any detectable preS2 epitope. Because of this strong relationship between a high HBsAg S/C ratio and the ability to determine the HBV genotype, for samples initially tested by Abbott's Auszyme ELISA kit, only samples with an HBsAg OD greater than 2.000 were used in the study, except with two samples (for these the OD readings were 1.728 and 1.892). For 129 of these 130, one or more preS2 epitopes were detected and 111 were fully genotyped.

## DISCUSSION

Recently, an ELISA kit for genotyping samples reactive for HBsAg containing monoclonal antibodies against preS2 region epitopes became available (16). The results of the ELISA method for genotyping agreed closely with those of the more laborious nucleic acid-based techniques originally used to genotype HBsAg-positive samples (16). Therefore, we genotyped a large number of samples from asymptomatic, American blood donors with HBsAg detected by licensed ELISA screening tests. Genotype A was the most frequent genotype (34.1% of samples), with the frequencies of other genotypes being as follows: 19.8% of samples were of genotypes D and E, 14.1% were of genotype C, 7.4% were of genotype B, and 1.0% were of genotype F. The diversity of HBV genotypes identified reflects the diversity of the HBsAg-positive individuals in large geographic areas from two populous states, California and Texas (although most residents of these two states have migrated from other parts of the United States).

In our study, we did not know the ethnic backgrounds of the HBsAg-positive individuals because most of the blood donor samples were tested after being unlinked from the donor. From other studies, it is known that the HBV genotype distribution is closely related to the ethnic backgrounds of HBV-

infected persons. For example, genotype A is predominant in whites, while genotypes B and C are predominant in Asians (1, 6, 8). There is a question of which factor, viral genotype or ethnicity of the host, plays a more important role in the determination of the clinical course of HBV carriers. Further investigations are required to clarify this. In the region of northern California served by the Sacramento Medical Foundation Blood Centers, Caucasians comprise less than 50% of the population; the next most common ethnic group is Hispanics, followed by Asians and African-Americans. In the area of Texas covered by Gulf Coast Blood Centers, Caucasians predominate in the donor population but there is a large representation of Hispanics and African-Americans.

Using the same ELISA preS2 kit, Usuda and colleagues found only 7 (1.4%) genotype-indeterminable samples among 514 HBsAg-positive sera initially detected by reverse passive hemagglutination in Japan (16). The population they studied was quite homogenous. By contrast, in our study, 98 (23.4%) samples from 418 healthy ELISA HBsAg-positive American blood donors could not be genotyped. ELISAs for HBsAg are generally considered more sensitive than the reverse-passive-hemagglutination assay (RPHA). In a recent study of transfusion-associated cases of hepatitis B from blood that tested negative for HBsAg by the RPHA used in Japan, a number of blood donors were subsequently found to be reactive for HBsAg by an ELISA technique applied to the stored serum samples of these implicated donors; these originally RPHA-negative samples were also reactive for HBV DNA by PCR (7). We believe that the substantial rate of indeterminable samples found among our sera is explainable primarily by their low levels of HBsAg (as determined by a low S/C ratio on ELISA). We found a strong correlation between the ability to define the HBV genotype and HBsAg levels; samples with low S/C ratios were much more likely to have undetectable preS2 epitopes by the genotyping ELISA that we used. We believe that the distribution of genotypes based on the samples which could be genotyped by this method nonetheless provides a valid representation of the genotypes in the entire sample.

Another reason for our inability to define HBV genotypes by the monoclonal antibody ELISA kit used is the possibility of the occurrence of preS2 variants in HBsAg carriers in the United States different from those in Japan. We found 56 ungenotypeable samples where one or more preS2 epitopes were detected. For 11 of these samples, the combinations of detected epitopes were different from the patterns of genotypes known for this assay. In the remaining 45, only the common epitope (b) was detected. For these 56 samples, which were not genotypeable but which had detectable preS2 epitopes, all but 2 had high levels of HBsAg. These samples may thus indicate the existence of a new genotype(s) among U.S. samples and/or the existence of mutations in the preS2 region. Recently, the existence of genotype G was described by Stuyver et al. (15). With the assay we used, we were unable to define genotype G, even though it, like other HBV genotypes, has the common epitope (b) (15). As HBsAg samples of genotype G have not been evaluated with the preS2 ELISA kit used in our study, we do not know if any samples of this genotype would more likely be only among those with epitope b or also among those with additional determinants.

Differences in the natural histories of HBV carriers and in the responses to interferon therapy of HBV-infected patients have been described previously (13). Some recent studies have implied that this difference may be due to genotype distribution (4, 5,9). Kao et al. have suggested that HBV genotype C is associated with more severe liver disease and with a lower rate of response to interferon therapy (4, 5). Mayerat et al. (9) reported an association between genotype A and chronic hepatitis. In our study, genotype C was more frequently associated with HBeAg-positive samples (5 of 9) than with HBeAg-negative samples (33 of 192). While our numbers are small and the samples were only from asymptomatic blood donors, this result may indicate that HBV carriers with genotype C will seroconvert less often from HBeAg to anti-HBe during the natural course of their HBV infection than patients with other genotypes or that the apparent association is due to the genetic or ethnic backgrounds of these carriers or even the geographic regions from which they may have emigrated. This finding is consistent with reports that HBV carriers infected with the subtype adr, most of whom are of genotype C, are more frequently positive for HBeAg (14).

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