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Screening for Hepatitis B Virus in Healthy Blood Donors by Molecular DNA Hybridization Analysis

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A DNA molecular hybridization technique employing a purified *adw* subtype hepatitis B virus (HBV) cloned DNA of 3.2 kilobase pairs as a probe was used to screen for the presence of HBV DNA in blood samples collected from 486 apparently healthy blood donors. Eighteen of 104 (17.3%) hepatitis B surface antigen (HBsAg) carriers and 7 of 382 (1.8%) HBsAg-negative individuals had circulating HBV DNA in their sera. Among the seven individuals who were positive for HBV DNA but negative for HBsAg, three had antibodies against both HBsAg (anti-HBsAg) and hepatitis B core antigen, one had only anti-HBsAg, one had both anti-hepatitis B core antigen and anti-hepatitis B e antigen and two were negative for all the above HBV markers. The results suggest that the absence of HBsAg in otherwise apparently healthy individuals may not be enough to ensure lack of circulating HBV.

Despite the screening of blood donors with third-generation hepatitis B surface antigen (HBsAg) tests and an all-volunteer donor system, hepatitis B virus (HBV) still causes approximately 7 to 13% of the cases of posttransfusion hepatitis (1, 2). This is probably related in part to the reported observations that some individuals with persistent HBV infection may not always have detectable HBsAg in their sera (5, 6, 10–13, 17, 21, 22; J. Rakela, J. W. Mosley, R. D. Aach, G. L. Gitnick, F. B. Hollinger, C. E. Stevens, and W. Szmuness, Gastroenterology **78**:1318, 1980). Therefore, the establishment of a sensitive and direct laboratory test to identify these potentially infectious units of blood would be of considerable value in controlling the horizontal transmission of HBV.

The presence of hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg), and HBV-specific DNA polymerase are all considered indices of active HBV replication and infectivity (3, 15, 18, 23). However, these markers are less than satisfactory in clinical practice owing to their relatively low sensitivity or their being indirect markers. While anti-HBcAg or anti-HBsAg or both may be of some value in determining the infectivity among blood donors (10, 11, 13; Rakela et al., Gastroenterology **78**:1318, 1980), the high prevalence of anti-HBcAg (more than 90%) and anti-HBsAg (more than 65%) in certain endemic areas would make it neither acceptable nor suitable to use these markers for the purpose of screening blood donors (26).

Because of their generally higher sensitivity and specificity and closer correlation with viral infectivity than the serological tests, which depend on the detection of virus-encoded proteins, DNA hybridization techniques could potentially be the method of choice for examining blood samples for HBV (4, 5, 7, 16, 21, 25). The simplified spot techniques described by Scotto et al. (21) and Lieberman et al. (16) allow simultaneous direct measurement of HBV DNA sequences in many sera in a relatively short time and are easy and inexpensive to perform.

In this article, we report the use of a simplified DNA hybridization technique in assessing the presence of HBV DNA sequences and thus HBV infectivity in the sera of apparently healthy blood donors.

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MATERIALS AND METHODS

A total of 486 serum samples were randomly selected from more than 10,000 blood samples collected from apparently healthy blood donor candidates between November 1984 and March 1985 in the Department of Clinical Pathology of Chang Gung Memorial Hospital. These donors, 292 males and 194 females, were between 18 and 58 years of age. None of them had signs or symptoms of hepatitis at the time of sampling. All serum specimens were stored at −80°C before analyses.

Serum samples were analyzed for HBV DNA by a modified DNA-DNA hybridization technique with direct trapping of DNA on a nitrocellulose filter, which was based on a method initially described by Berninger et al. (4), Leiberman et al. (16), and Scotto et al. (21). All samples positive for HBV DNA were then analyzed by the Southern blot technique to ensure the presence of 3.2 kilobase pairs of HBV genome. HBsAg was tested in all samples, and tests for other serological markers, including HBeAg, anti-HBeAg, anti-HBcAg, and anti-HBsAg, were performed in those samples positive for HBV DNA.

Serological tests. The presence of HBV serological markers HBsAg, HBeAg, anti-HBeAg, anti-HBcAg (immunoglobulin G and immunoglobulin M) and anti-HBsAg was determined with solid-phase radioimmunoassay kits obtained commercially from Abbott Laboratories (North Chicago, Ill.) and from General Biologicals Corp. (Hsin Chu, Taiwan, Republic of China). The tests were performed according to the specifications of the manufacturer.

DNA hybridization technique. A purified *adw* subtype HBV cloned DNA of 3.2 kilobase pairs consisting of the entire HBV genome was used as a probe. This HBV DNA was originally cloned by Williams S. Robinson and was kindly made available to us by S. C. Lee of the National Taiwan University School of Medicine. The DNA probe was labeled with [α-^{32}P]dCTP by nick translation of only the cloned HBV genome DNA to between 5×10^7 and 1×10^8 cpm/μg of DNA.

Serum samples (20 μl) were incubated at 56°C for 2 h with proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) at 250 μg/ml in 24 μl of 0.25% sodium dodecyl sulfate–5 mM EDTA–10 mM Tris hydrochloride buffer, pH 8.0. After denaturation with an equal volume of 2 M NaOH–2 M NaCl for 10 min at room temperature, 75 μl of 1 M Tris hydrochloride (pH 7.4)–4 M NaCl was added to neutralize the reaction mixture. Each specimen was then deposited on a nitrocellulose membrane filter sheet (0.45-μm pore size) through wells of a microsample filtration manifold under vacuum (BA85 and Minifold II; Schleicher & Schuell, Inc., Keene, N.H.). Positive and negative control sera as well as graded dilutions of cloned HBV DNA standards of known concentrations were all included in each assay. After being loaded, the nitrocellulose filter was gently removed from the manifold under continued vacuum, blotted, air dried, and baked at 70°C for 2 h in vacuo. Following prehybridization treatment, hybridization was performed at 65°C with between 5×10^6 and 2×10^7 cpm of the labeled DNA as a probe for each filter of 63 by 228 mm. The filters were washed sequentially in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 30 min twice at room temperature; 0.1× SSC–0.1% sodium dodecyl sulfate for 30 min twice at room temperature; and 0.1× SSC–0.5% sodium dodecyl sulfate for 30 min twice at 65°C. The filters were then air dried and autoradiographed for 24 to 48 h. After autoradiography, the filters were cut out and the radioactivity was counted in a liquid scintillation counter. The amount of HBV DNA in the serum samples was calculated according to the ^{32}P counts obtained from a series of dilutions of purified HBV DNA prepared from a stock of known concentration and hybridized to the probe DNA on the same filters.

The level of detection of our method was determined to be approximately 0.5 pg of HBV DNA. However, an HBV DNA concentration of 1 pg or more per 20 μl of serum was interpreted as a positive result in this study.

For Southern blot hybridization, DNA was extracted with phenol from 100 μl of serum, digested with restriction endonuclease *Eco*RI, and electrophoretically separated on 0.8% agarose gels. After the DNA was transferred to a nitrocellulose membrane filter, it was probed with radioactively labeled HBV genome DNA. DNA with a known size as well as purified HBV DNA of 3.2 kilobase pairs were included as size standards.

RESULTS

Figure 1 represents autoradiographs of an HBV DNA slot-blot hybridization test of 136 serum samples which showed seven HBV DNA-positive sera, and Table 1 summarizes the results of the analysis of the 486 serum specimens. The HBsAg carrier rate among these apparently healthy blood donor was 21.4%. HBV DNA was found in 17.3% or 18 of the 104 HBsAg carriers and in 1.8% or 7 of the 382 HBsAg-negative blood donors. The concentrations of HBV DNA in HBsAg-positive and -negative individuals were 405 (standard deviation, 310) and 301 (standard deviation, 149) pg/ml of serum, respectively. There is no statistically significant difference in the HBV DNA concentrations between these two groups ($P > 0.2$).

Table 2 shows the results of the HBV DNA concentrations and the HBV markers from the HBV DNA-positive sera. All 18 HBsAg- and HBV DNA-positive individuals (group A) were also positive for anti-HBcAg but lacked anti-HBsAg.

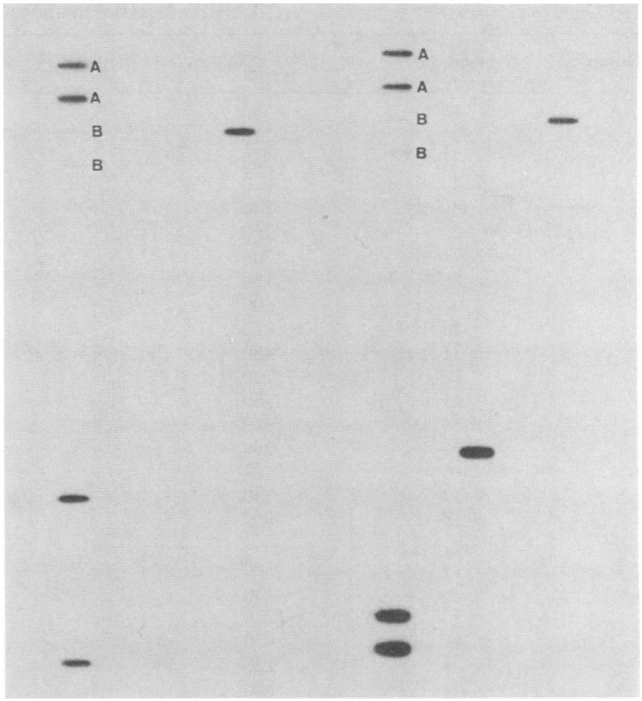

FIG. 1. Autoradiographs of HBV DNA slot-blot hybridization test of serum samples from healthy blood donors. A total of 68 each of HBsAg-positive and -negative blood samples are shown here. A total of seven serum specimens in these autoradiographs gave positive HBV DNA results. Serum samples of 20 μl were treated and handled by the procedures outlined in Materials and Methods. This particular autoradiograph represented a 48-h exposure. The actual size of each slot was 0.75 by 8 mm, or 6 mm^2. Slots A and B were positive controls of 10 and 1 pg of purified HBV DNA, respectively. The 10-pg HBV control slots (slots A) were between 500 and 700 cpm in a typical experiment.

Eleven serum specimens were HBeAg positive, and six had neither HBeAg nor anti-HBeAg.

Among HBsAg-negative but HBV DNA-positive individuals (Table 2, group B), one individual (donor 4) was positive for anti-HBcAg but lacked anti-HBsAg. Three donors (1, 2, and 3) were positive for anti-HBcAg and anti-HBsAg (one also had anti-HBeAg). Another one in group B (donor 5) showed only anti-HBsAg. Two individuals (donors 6 and 7) were seronegative for all HBV markers tested.

All sera positive for HBV DNA were further analyzed by Southern blotting, and the results indicated the presence of 3.2 kilobase pairs of full-length HBV genome whenever slot-blot hybridization tests were positive (Fig. 2). The serum HBV DNA was also found to be DNase resistant (data not shown).

TABLE 1. Prevalence of serum HBsAg and HBV DNA in healthy blood donors

Serum HBsAg		Serum HBV DNA positive		
Presence	No.	No. (%)	pg/ml	SD
+	104	18 (17.3)*	405**	310
−	382	7 (1.8)*	301**	149

* Chi-square distribution, $P < 0.005$.
** Student's t test, $P > 0.2$.

TABLE 2. HBV marker of HBV DNA-positive individuals

Group	Donor no.	HBV DNA (pg/ml)	HBsAg	Anti-HBsAg	HBeAg	Anti-HBeAg	Anti-HBcAg
A	1	360	+	−	−	−	+
	2	210	+	−	−	−	+
	3	250	+	−	−	−	+
	4	70	+	−	−	−	+
	5	240	+	−	−	−	+
	6	290	+	−	−	−	+
	7	240	+	−	−	ND[a]	+
	8	150	+	−	±	−	±
	9	90	+	−	+	−	+
	10	1,210	+	−	+	−	+
	11	480	+	−	+	−	+
	12	700	+	−	+	−	+
	13	500	+	−	+	−	+
	14	340	+	−	+	−	+
	15	290	+	−	+	−	+
	16	180	+	−	+	−	+
	17	740	+	−	+	−	+
	18	950	+	−	+	−	+
B	1	340	−	+	−	−	+
	2	150	−	+	−	−	+
	3	210	−	+	−	+	+
	4	470	−	−	−	+	+
	5	120	−	+	−	−	−
	6	500	−	−	−	−	−
	7	320	−	−	−	−	−

[a] ND, Not determined.

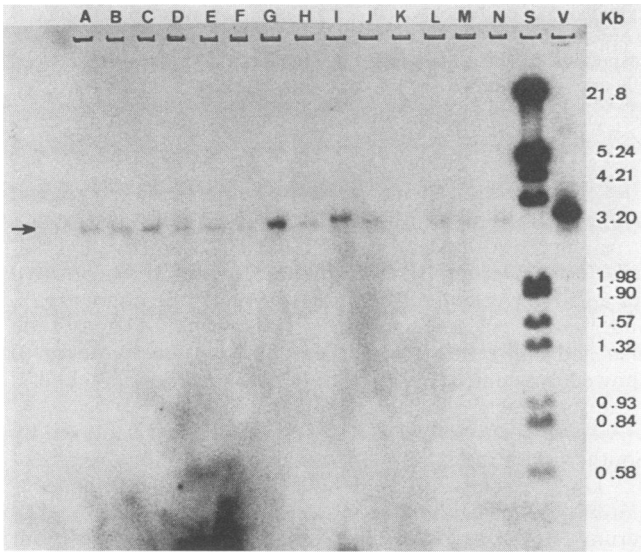

FIG. 2. Autoradiographs of Southern blot hybridization analysis of HBV DNA from sera of healthy donors with positive slot hybridization tests. In each case, DNA was extracted by phenol from 100 μl of serum, digested with *Eco*RI, and electrophoretically separated on 0.8% agarose gels. After the DNA was transferred to a nitrocellulose membrane filter, it was probed with radioactively labeled HBV genome DNA. The origin was at the top of the figure, and DNA of known size as well as purified HBV DNA of 3.2 kilobase pairs (kb) were included in lanes S and V, respectively, as standards. Lanes K, L, M, and N were the DNA from serum HBV DNA-negative individuals, while the remaining lanes were the DNA from HBV DNA-positive individuals. The arrow indicates the position of purified HBV DNA standard.

DISCUSSION

Detection of HBV DNA in sera by the DNA hybridization method has been shown to be more sensitive and is a more direct in vitro test for assessing HBV infectivity, when compared with other HBV serological markers (4, 5, 7, 16, 21–25). It thus would be a potentially valuable method to monitor the infectivity of blood units for HBV that may otherwise escape detection by HBsAg screening.

The presence of HBV DNA in HBsAg-negative subjects has been reported in patients with acute or chronic liver disease (5, 6, 12, 17, 21, 22), in chronic alcoholics (17, 22), and in healthy individuals (8, 22). We have also reported recently the finding of HBV DNA in HBsAg-negative immunocompromised cancer patients (20) and in patients with end-stage renal failure who are receiving hemodialysis (19). Our detection of HBV DNA in 7 of 382 (1.8%) serum samples that were negative for HBsAg pointed to an interesting epidemiological and public health issue which is particularly relevant to the blood banks. It does not seem that this group of people represented merely borderline cases since their HBV DNA concentrations did not differ significantly from those of people who were HBsAg positive (Table 1). These cases thus exemplify the value of the higher sensitivity of DNA hybridization tests.

The HBsAg-negative, HBV DNA-positive individuals (Table 2, group B) consisted a heterogeneous group of donors who were either low-level carriers or in the convalescent phase of recent infection (9), or others. Several possibilities might explain the presence of HBV DNA in sera of HBsAg-negative individuals. HBsAg may be present in low levels or masked by HBsAg-anti-HBsAg complexes and thus undetectable by regular immunoassays. Katchaki et al. (13) and Hopkins et al. (11) reported that after concentration of the sera from HBsAg-negative, anti-HBcAg-positive do-

nors who were implicated as transmitting posttransfusion hepatitis, tests for HBsAg became positive in these specimens. Wands et al. (24) were able to demonstrate the presence of HBsAg in serum samples using monoclonal anti-HBsAg antibody, whereas previous tests with conventional radioimmunoassay kits had given false-negative results. Shafritz et al. (22) reported that 13 of 36 samples of such sera contained HBV DNA and six of these HBV DNA-positive samples also contained anti-HBsAg, with HBsAg being demonstrable with monoclonal anti-HBsAg but not with conventional antibodies. The fact that five of the seven HBsAg-negative but HBV DNA-positive individuals were also positive for either anti-HBsAg or anti-HBcAg or both in their sera was compatible with the notion that molecular DNA hybridization was more sensitive than conventional HBsAg immunoassay. Partial or no expression of the HBV genes, absence of humoral responses, or infection by a different subtype of HBV can also be reflected in the absence of detectable HBsAg and other serological markers in the remaining cases.

HBsAg was detected in 104 of 486 (21.4%) otherwise healthy blood donors. This figure agrees with the HBsAg-positive rate for the general population of Taiwan (26) and indicates the randomness of the selection of the specimens in this study. The 17.3% prevalence rate for serum HBV DNA in HBsAg-positive individuals is also in close agreement with that of Krogsgaard et al. (14) and Harrison et al. (8), who found that 19.2 and 20.6%, respectively, of their blood donors who were positive for HBsAg were also positive for HBV DNA.

Even though there is a great variation in the degree of infectiousness of HBsAg carriers that does not correlate with amount or titer of HBsAg, blood from all HBsAg-positive persons is still considered potentially infectious (9). HBV DNA hybridization could potentially be offered as an adjunct test for pretransfusion screening, making blood and its products even safer, particularly for those who have acquired immune suppression and are at higher risk for hepatitis.

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