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Association of e antigen with Dane particle DNA in sera from asymptomatic carriers of hepatitis B surface antigen

(hepatitis B virus/infectivity/annealing to Dane particle DNA)

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ABSTRACT **Sera containing hepatitis B surface antigen from 30 asymptomatic blood donors were assayed for e antigen (HBeAg) and antibody to e antigen (anti-HBe) by rheophoresis. Fourteen samples (47%) had detectable HBeAg, ten (33%) had anti-HBe, and six (20%) had neither. DNA was extracted from 26 of these sera and assayed for its ability to anneal to a [^{32}P]-DNA probe that is a copy of Dane particle DNA. All 10 HBeAg-positive samples tested contained DNA that formed specific hybrids with the DNA probe, as did one of 10 anti-HBe-positive samples. Hybridization was not detected in nine sera containing anti-HBe and six sera without HBeAg or anti-HBe. Because the Dane particle is thought to be the hepatitis B virus, this association between HBeAg positivity and Dane particle DNA strongly supports the hypothesis that e antigen is a marker of the presence of the virus and, consequently, potential infectivity.**

The e antigen (HBeAg, eAg) is associated with hepatitis B virus (HBV) infection but is antigenically distinct from hepatitis B surface and core antigens (HBsAg and HBcAg) (1–3). It occurs as a soluble serum protein, perhaps an immunoglobulin G (4). In the early reports of the e antigen system, Magnius and Espmark (1, 2) suggested that the presence of e antigen might be correlated with contagiousness. A number of studies have since linked the detection of HBeAg in serum with the presence of Dane particles (thought to be the HBV) in serum (3, 5–8), with elevated DNA polymerase or HBsAg-associated DNA polymerase in serum (5, 7, 9–12), and with HBcAg in serum (3) and in liver (3, 13–15). Previous reports have also indicated that the persistence of HBeAg is associated with chronic active liver disease in HBsAg carriers and the presence of antibody to e antigen (anti-HBe) with asymptomatic carriage of HBsAg and less frequently with chronic liver disease (16–20). Asymptomatic HBsAg carriers generally have a low frequency of HBeAg (21).

In this study we have ascertained the prevalence of HBeAg in a group of U.S. blood donors who were asymptomatic carriers of HBsAg and, using a specific test for viral DNA, we have tested the hypothesis that HBeAg is a marker of potential hepatitis B virus infectivity. For the specific test, we have assayed sera for DNA hybridizable to a ^{32}P-labeled probe prepared from Dane particle DNA.

MATERIALS AND METHODS

Sera. Sera from U.S. blood donors who were asymptomatic carriers of HBsAg with no history of clinical hepatitis were obtained from the American Red Cross Blood Research Laboratory, Bethesda, MD, through the courtesy of S. Mazzur.

HBeAg and Anti-HBe Identification. Sera were assayed for HBeAg and anti-HBe by rheophoresis in 0.8% agarose plates (Abbott Laboratories, North Chicago, IL). The first and fourth wells of the six peripheral wells were filled with a known HBeAg(+) serum. The center well was filled twice with serum containing anti-HBe. The plates were incubated in a moist chamber at room temperature and were read daily for up to 2 weeks. Additional buffer was added to the trough at 4 days if longer incubation was necessary to determine reactivity. The HBeAg and anti-HBe reagents used were sera from asymptomatic carriers of HBsAg; they were tested for identity with standard sera containing HBeAg/1 and HBeAg/2 specificities kindly provided by B. L. Murphy (Phoenix Laboratories, Center for Disease Control, Phoenix, AZ).

Preparation of Dane Particle [^{32}P]DNA Probe. Dane particle [^{32}P]DNA probe was prepared by *in vitro* replication of Dane particle DNA as previously described (22). Approximately 5 ng of Dane particle DNA was denatured in 0.2 M NaOH 5 min at 4° and incubated at 15° in a 10-μl reaction mixture containing 1 μmol of Tris·HCl (pH 7.5); 0.2 μmol of NaCl; 100 pmol each dGTP, dCTP, and TTP; 50 pmol of [α-^{32}P]dATP (100 Ci/mmol, New England Nuclear Corp.); 100 nmol of $MgCl_2$; 1 μg of oligonucleotide primers (22); and 0.1 μg of *Escherichia coli* DNA polymerase I. The reaction was diluted into 0.1 ml of 0.5% sodium dodecyl sulfate and 10 mM EDTA when the incorporation was complete (40 min), and the double-stranded [^{32}P]DNA product was precipitated by the addition of two volumes of ethanol. For use as a hybridization probe, the [^{32}P]DNA was denatured in 0.3 M NaOH at 98° for 20 min.

Extraction of DNA. Ten milliliters of serum was centrifuged at 40,000 rpm for 4 hr in a Beckman 65 rotor. The pellet from each sample was banded on a discontinuous 20% over 65% (wt/vol) sucrose density gradient (23). The interphase was dispersed in 5 ml of water and repelleted in a 50.1 rotor for 2½ hr at 50,000 rpm. The pellet from each was incubated with 1 ml of a solution containing 10 mM Tris·HCl (pH 7.4), 10 mM EDTA, 0.5% sodium dodecyl sulfate, and Pronase at 500 μg/ml (Calbiochem., San Diego, CA) for 1 hr at 37°. The resultant pellet was extracted once with an equal volume of phenol, adjusted to 0.1 M NaCl, and precipitated with two volumes 95% ethanol.

DNA Hybridization. The ethanol precipitate was dissolved in 1.0 ml of water and 0.5 ml was used for the hybridization. Calf thymus DNA (100 μg) was added as carrier and the DNA was denatured by heating to 100° in the presence of 0.3 M NaOH. Denatured DNA was then annealed in 25 μl of 1 M NaCl, 68°, with a 400 cpm Dane particle [^{32}P]DNA probe (10^8 cpm/μg) for 10 or 30 hr. HBV sequences in the denatured DNA were detected by their ability to accelerate annealing of the ^{32}P probe. Annealing was assayed by resistance to digestion with S-1 nuclease.

Electron Microscopic Examination for Dane Particles. Pellets of sera were prepared as for the extraction of DNA. In this case, they were resuspended in 10 mM Tris·HCl buffer (pH 7.4) with 10 mM EDTA for examination by electron microscopy. The sample was applied to Formvar-coated copper grids

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, e antigen; HBcAg, core antigen; anti-HBe, antibody to e antigen.

Table 1. Specificity of Dane particle [^{32}P]DNA probe

Sample	% hybridization at 27 hr
Calf thymus DNA, 5 mg/ml	8
Human DNA, 5 mg/ml	8
Dane particle DNA, 1.5 ng/ml + human DNA, 5 mg/ml	27
Dane particle DNA, 4.5 ng/ml + human DNA, 5 mg/ml	40
Dane particle DNA, 15.0 ng/ml + human DNA, 5 mg/ml	49

and, after the excess liquid had been removed, was washed with distilled water and stained with uranyl acetate (saturated in water at room temperature).

RESULTS

The specificity of the [^{32}P]DNA probe prepared from Dane particle DNA was tested by annealing with denatured human or calf thymus DNA at 5 mg/ml, or with DNA from Dane particles (Table 1). Only the DNA extracted from Dane particles accelerated the annealing of the probe. This assay specifically detects Dane particle DNA at concentrations as low as 1.5 ng/ml, equivalent to 7.5 pg/ml (approximately 2×10^6 particles per ml) in the original serum samples.

Of 30 sera tested by rheophoresis, 14 had detectable HBeAg, 10 had anti-HBe, and in 6 samples, neither could be detected. None of the sera had both HBeAg and anti-HBe by this test. Twenty-six of these samples were assayed for Dane particle DNA, including 10 containing HBeAg, all 10 with anti-HBe, and the 6 that had neither HBeAg nor anti-HBe (Table 2). A strong association was found between HBeAg positivity and hybridization with a [^{32}P]DNA probe. A serum was considered positive for viral DNA if the percent hybridization was twice that of the controls after both 10 and 30 hr annealing. All 10 samples with HBeAg and one of 10 with anti-HBe (sample 6) were scored as positive. The single serum with anti-HBe and viral DNA had a lower percent hybridization than any of the sera with HBeAg. None of the 6 sera in which neither HBeAg nor anti-HBe could be detected showed hybridization.

By electron microscopy, the serum containing anti-HBe and Dane particle DNA was found to have many tubular and 20 nm particles (excess viral coat with HBsAg specificity, but devoid of DNA) but only a few Dane particles. In addition, some particles similar to Dane particles were observed which had an ill-defined low contrast surface. Sera containing anti-HBe (samples 1, 3, and 4) which had values slightly higher than the controls in Table 2 also had only a few Dane particles. Again their surfaces looked unusual; some particles also appeared to be aggregated. In pellets of serum from 3 of the 10 HBeAg samples (sera 4, 6, and 7) many intact Dane particles were observed.

Table 2. Percent Dane particle [^{32}P]DNA probe annealed to sample DNA

Sample	% hybridization 10 hr	% hybridization 30 hr
HBsAg(+) sera		
HBeAg(+)		
1	31	43
2	36	66
3	18	61
4	43	61
5	31	55
6	33	61
7	31	56
8	30	45
9	33	68
10	48	50
Anti-HBe(+)		
1	8	15
2	5	13
3	5	24
4	7	18
5	2	12
6	8	33
7	4	9
8	3	10
9	3	n.d.
10	5	13
HBeAg(−), anti-HBe(−)		
1	4	n.d.
2	3	15
3	5	9
4	4	11
5	4	9
6	4	12
Controls		
HBsAg(−) sera		
1	3	9
2	5	13
3	4	7
4	4	6
No sera*		
1	4	n.d.
2	n.d.	16
3	n.d.	12
4	n.d.	12

n.d. = not determined.

* Only labeled DNA is present in the reaction mixture; the hybridization observed is due to self-annealing of the probe.

DISCUSSION

The Dane particle is generally accepted as the hepatitis B virion. These particles contain a unique DNA and an endogenous DNA polymerase (22, 24, 25). Free serum DNA having homology to the DNA of the Dane particles has also been reported in HBsAg carrier sera (26). The serum antigen HBeAg has been associated with the detection of Dane particles in serum (3, 5–8) and also with infectivity (see below).

In addition to the association between HBeAg and HBV markers revealed by microscopy and histochemical and immunological tests (3, 5, 7–15), the relation of e antigen to infectivity has been supported by transmission data. Okada and coworkers showed in a group of mother–child pairs that all 10 infants born to HBsAg- and HBeAg-positive mothers developed HBsAg; conversely, all 7 HBsAg- and anti-HBe-positive mothers had infants who did not develop HBsAg (8). Beasley *et al.* have also recently reported that maternal e antigenemia is a good predictor of vertical transmission (27). In addition, a cooperative study of accidentally inoculated medical personnel also revealed a correlation between HBeAg and HBV infectivity (28).

There is not total agreement, however, about the infectivity of HBsAg-positive sera that contain anti-HBe. Magnius (17) has reported that 10 of 12 healthy blood donor carriers with anti-HBe donated a total of 95 units of blood with no reported cases of post-transfusion hepatitis; and some investigators report that anti-HBe indicates the absence of Dane particles (11). We and others (5, 6), however, find Dane particles in certain sera positive for e antibody; and anti-HBe is apparently not always

associated with the absence of HBV infectivity. In an experimental study, Berquist *et al.* (29) inoculated four chimpanzees with HBsAg(+) sera containing anti-HBe but no detectable Dane particles. They found that a human serum with anti-HBe was infectious for one chimpanzee and that a chimpanzee serum with anti-HBe may have been infectious for a second animal. In addition, Schweitzer *et al.* (30) reported HBsAg in the newborn offspring of a mother whose serum contained HBsAg and anti-HBe, although the infant was only transiently positive and eventually made antibody to HBsAg (anti-HBs). Gerety (31) observed HBsAg in babies of two HBsAg- and anti-HBe-positive mothers; one of these infants also cleared the antigen and later developed anti-HBs. Therefore, one cannot rule out the possibility that some anti-HBe-positive sera may be infective.

The prevalence of HBeAg found in our sample was higher than that reported in other studies of asymptomatic blood donors and other healthy carriers of HBsAg (1–3, 5, 6, 9, 17–20, 32). In contrast to previous reports, HBeAg was not rare in sera from apparently asymptomatic HBsAg chronic carriers found among volunteer U.S. blood donors. This may be due in part to the longer incubation of the rheophoresis plates, which allowed ascertainment of HBeAg or anti-HBe positivity in 80% of the samples tested, as compared to an average of 50% in other reports. It should be noted also that these sera were all positive for HBsAg by immunodiffusion as well as radioimmunoassay, suggesting high titers of HBsAg. Because an association has been found between the presence of the e antigen and high titers of HBsAg in sera (8, 11, 15, 27, 33), this provides a likely explanation for the large number of HBeAg-positive specimens in our sample.

The single serum containing both anti-HBe and Dane particle DNA is of interest with regard to infectivity. This donor presumably had had e antigen and was sampled at a time when the infection was abating. This possibility is supported by the fact that seroconversion from HBeAg to anti-HBe has been shown in some HBsAg-positive individuals (ref. 9; B. G. Werner, unpublished) and that this specimen had the lowest percent hybridization among the DNA-positive samples. The presence of less viral DNA would suggest that this serum is less infectious than the e-antigen-positive samples. In addition, one might also speculate that the Dane-like particles with ill-defined surface structure are unstable and in the process of becoming less organized (disintegrating?). Similarly, this may also be the case for the few irregular Dane particles seen in the other anti-HBe-positive sera examined by electron microscopy. It is important to examine sequential serum samples from hepatitis-B-infected individuals to study the presence of e antigen and its antibody, viral DNA, and Dane particles in relation to the host response to HBV.

Our findings are consistent with the infectivity data on sera with HBeAg or anti-HBe. We have shown that e antigen is a good marker of the presence of Dane particle DNA in sera, but that Dane particle DNA may also be detected in some sera with anti-HBe. These results are consistent with the hypothesis that e antigen is a reliable marker of increased infectivity. Sera with anti-HBe, on the other hand, are much less likely to be infective because the probability of finding viral DNA in these sera is greatly reduced, and when DNA is present, the levels are lower.

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