Failure of Antibody to e Antigen to Precipitate Dane Particles Containing DNA Polymerase Activity and Hepatitis B Core Antigen

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SUMMARY

Serum samples of 67 asymptomatic carriers were tested for Dane particles by electron microscopy, and for e antigen by immunodiffusion, as well as for hepatitis B antigen-associated DNA polymerase activity, and four samples enriched with respect to Dane particles were selected. Hepatitis B antigen particles in them were separated from e antigen and concentrated by centrifugation, and the Dane-rich preparations were incubated with buffer, antibody to e antigen (anti-HBe) or antibody to hepatitis B surface antigen. After centrifugation, the supernatant was tested for DNA polymerase activity, and both supernatant and precipitate were tested for hepatitis B core antigen (HBcAg) by the immune adherence haemagglutination method after the coat of the Dane particles had been disrupted with NP-40 and 2-mercaptoethanol. It was found that anti-HBe did not precipitate Dane particles as measured by DNA polymerase activity and HBcAg. On the basis of the results obtained, it has been concluded that e antigen does not exist on the surface of hepatitis B virions.

INTRODUCTION

Three kinds of antigens related to hepatitis B virus (HBV) have been identified against which the host develops antibody: hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg) (Almeida, Rubenstein & Stott, 1971; Hoofnagle, Gerety & Barker, 1973), and e antigen (HBeAg) (Magnius & Espmark, 1972). Recently, increasing attention has been focused on HBeAg and its corresponding antibody, anti-HBe, owing to their apparent clinical importance. The results obtained so far indicate that HBeAg, when present in serum containing hepatitis B antigen, signals the presence of HBV; serum samples containing HBeAg were shown to be highly contagious both in vertical (Okada et al. 1976) and horizontal (Alter et al. 1976) infections.

Despite an obvious association between HBeAg and Dane particles (believed to be hepatitis B virions; Dane, Cameron & Briggs, 1970), their exact relationship has remained unclear. The present study was, therefore, designed to find out whether or not HBeAg existed on the surface of Dane particles.
Methods

Serum samples. Blood units of voluntary donors were screened for HBsAg by electrosyneresis and 67 asymptomatic carriers of HBsAg were found. These samples were further tested for the presence of Dane particles by electron microscopy, for HBeAg by immunodiffusion (Magnius & Espmark, 1972), and for HBV-associated DNA polymerase activity by the incorporation of \(^{3}H\)-thymidine-methyl-5-triphosphate into DNA (Kaplan et al. 1973). Finally, four HBeAg-positive serum samples were selected which exhibited an HBcAg titre of 2\(^{8}\) or higher by the immune adherence haemagglutination method (Tsuda et al. 1975), and a HBV-associated DNA polymerase activity of 500 ct/min or higher when tested at a 1:20 concentration. The subtype of HBsAg was determined by passive haemagglutination inhibition (Imai et al. 1974).

Preparation of Dane-rich fractions. Twenty-five ml of the serum was layered on to an equal volume of tris-HCl buffer (0.01 M, pH 7.5) containing 0.1 M NaCl and 0.5 ~ (w/v) BSA (hereafter referred to as BSA-tris) in a tube (capacity 94 ml). After centrifugation at 35000 rev/min for 2 h in a Beckman Type 35 rotor, the supernatant was discarded and the pellet was suspended in 1.25 ml of BSA-tris buffer. The samples were then centrifuged at 15000 rev/min for 20 min in a Type 35 rotor with an adaptor to accommodate a tube with a capacity of 4 ml, and the supernatant, which was rich in Dane particles and free of HBeAg contained in the original serum, was harvested.

Sedimentation of Dane particles with antibody to e antigen. One hundred \(\mu l\) of the Dane-rich fractions were delivered to each of 3 tubes designated a, b, and c. Tube a received 100 \(\mu l\) of BSA-tris buffer, Tube b received 100 \(\mu l\) of a rabbit antiserum containing antibody to HBsAg (anti-HBs; PHA titre 1:20000), and tube c received 100 \(\mu l\) of a human serum obtained from an asymptomatic HBsAg carrier and containing anti-HBe which gave a precipitin line against HBeAg up to a 1:32 dilution. Tubes were incubated successively at 37 °C for 1 h, and at 4 °C for 16 h, then centrifuged at 15000 rev/min for 20 min in a Type 35 rotor with adaptors for 4 ml tubes. The supernatant was removed and the sediment was suspended in 200 \(\mu l\) of BSA-tris buffer. DNA polymerase in the supernatant, as well as HBcAg activity in supernatant and sediment, was determined.

Determination of DNA polymerase activity. DNA polymerase activity was determined by a modification (Moritsugu et al. 1975) of the method originally described by Kaplan et al. (1973). Twenty \(\mu l\) of the supernatant was incubated with 30 \(\mu l\) of the reagent solution. The final concentration of the reagents in the reaction mixture was 0.2 mM each of dATP, dCTP, and dGTP; 0.15 mm-\(^{3}H\)-TP (0.375 \(\mu Ci/50 \mu l\)); 30 mm-MgCl\(_2\); 200 mm-KCl; 25 mm-2-mercaptoethanol; 0.2 % (v/v) Nonidet P-40 (NP-40); and 50 mm-tris (pH 8.0). After incubation at 37 °C for 3 h, the reaction mixture was spotted on a diethylaminoethyl-cellulose disc (Whatman DE81), washed 12 times with 5 % Na\(_2\)HPO\(_4\), twice with distilled water, twice with ethanol, and dried. Radioactivity retained was measured in a scintillation fluid consisting of 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 5.0 g of 2,5-diphenyloxazole per litre of toluene.

Determination of HBcAg. HBcAg in supernatant and sediment suspension was determined by the immune adherence haemagglutination (IAHA) method (Tsuda et al. 1975). One-hundred \(\mu l\) of the test sample were incubated with 100 \(\mu l\) of a rabbit antiserum to HBsAg (PHA titre 1:20000), at 37 °C for 60 min. The mixture was centrifuged at 5400 g at room temperature for 15 min, and the supernatant was discarded. The precipitate was washed twice with 5 ml of tris-HCl buffer (0.01 M, pH 7.6) containing 0.15 M-NaCl and 0.03 % Na\(_3\)N (w/v), dissolved in 50 \(\mu l\) of the same buffer supplemented with 0.1 % (v/v) NP-40,
Table 1. Precipitation of DNA polymerase activity of Dane particles by incubation with anti-HBs but not anti-HBe

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Subtype of HBsAg</th>
<th>DNA polymerase activity* in the supernatant after treatment with</th>
<th>Buffer</th>
<th>Anti-HBe</th>
<th>Anti-HBs</th>
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<td>1154</td>
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<tr>
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<td>adw</td>
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<td>1127</td>
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<tr>
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<td>adw</td>
<td>267</td>
<td>323</td>
<td>56</td>
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</table>

* Dane-rich preparations were incubated with buffer, anti-HBe or Anti-HBs and, after centrifugation, the supernatant was tested for DNA polymerase activity. The figure represents cts/min of 3H-thymidine-methyl-5-triphosphate incorporated into newly synthesized DNA.

Table 2. Precipitation of hepatitis B core antigen of Dane particles by incubation with anti-HBs but not anti-HBe

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Subtype of HBsAg</th>
<th>HBCAg activity* after treatment with</th>
<th>Buffer</th>
<th>Anti-HBe</th>
<th>Anti-HBs</th>
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<tr>
<td>4</td>
<td>adw</td>
<td>32</td>
<td>(-)</td>
<td>32</td>
<td>(-)</td>
</tr>
</tbody>
</table>

* Dane-rich preparations were incubated with buffer, anti-HBe or anti-HBs and after centrifugation, and disruption of Dane particles, supernatant and precipitate were tested for HBCAg by the immune adherence haemagglutination method. Results are expressed as reciprocal titres.

plus 0.25 mg/ml pronase E (Kaken Kagaku, Tokyo, Japan) and 0.1% (v/v) 2-mercaptoethanol, and further incubated at 37 °C for 120 min. HBCAg activity in the test sample was then titrated by the IAHA method.

The test material was serially diluted in a complement buffer (GVB++; Mayer, 1961) in a microtitre plate. Twenty-five μl of anti-HBc solution (8 IAHA units/ml) were added to each well, and the plate was incubated at 37 °C for 60 min. Then 25 μl of guinea pig complement (4 CHs0 units/ml) were added, and the plate was further incubated at 37 °C for 40 min. Finally, 25 μl each of human erythrocyte suspension (Group O, Rh(+), 1.2 x 10⁶/ml in EDTA-GVB; Mayer, 1961) and dithiothreitol solution (3 mg/ml in EDTA-GVB) were added. The plate was allowed to stand at room temperature for 60 min. The IAHA titre was expressed as the highest dilution at which haemagglutination was observed.

RESULTS

Table 1 summarizes the results of DNA polymerase determinations on four Dane-rich preparations after they had been incubated with buffer (negative control), anti-HBe or anti-HBs (positive control).

It is evident from the Table that the treatment of Dane-rich preparations with anti-HBe did not precipitate DNA polymerase activity. After treatment with anti-HBe, followed by centrifugation, the supernatant of all four samples contained DNA polymerase activity.
not essentially different from the activity after they had been treated with buffer, regardless of the subtype of HBsAg. In contrast, treatment of the sample with anti-HBs precipitated virtually all the DNA polymerase activity contained in Dane-rich preparations. Similar results were obtained when different dilutions of reagents were employed (data not shown). Thus, failure of anti-HBe to precipitate DNA polymerase activity could not be explained by the phenomenon of prozoning.

The results of determinations of HBcAg in supernatant and precipitate of Dane-rich preparations after incubation with anti-HBe are given in Table 2. Treatment with buffer and anti-HBs served as negative and positive controls, respectively, as in the determination of DNA polymerase activity. Again, it is clear that treatment of Dane-rich preparations with anti-HBe did not precipitate HBcAg; no difference in the HBcAg activity of the supernatant was noted after treatment with buffer or anti-HBe. Moreover, when the precipitate was tested for HBcAg, no detectable activity was found after treatment with either buffer or anti-HBe. In striking contrast, essentially all of the HBcAg activity in the supernatant was precipitated by anti-HBs.

**DISCUSSION**

Dane particles in the serum of asymptomatic carriers of HBsAg who were also positive for HBeAg were separated from HBeAg by centrifugation; it has been demonstrated that HBeAg-positive serum of asymptomatic HBsAg carriers contains high concentrations of both HBV-associated DNA polymerase (Imai et al. 1976) and HBcAg (Takahashi et al. 1976), indicating a high concentration of Dane particles. Four preparations containing Dane particles in such high concentrations were incubated with buffer, anti-HBe or anti-HBs. After sedimentation of any immune aggregates present by ultracentrifugation, the supernatant was separated from the precipitate. The supernatant was tested for DNA polymerase activity, and both supernatant and resuspended precipitate were tested for HBcAg after disruption of the envelope of Dane particles by detergent and 2-mercaptoethanol. DNA polymerase and HBcAg activities have been widely applied as chemical and immunological markers of Dane particles, which are believed to be hepatitis B virions. The supernatant of Dane-rich preparations which had been incubated with anti-HBe revealed essentially the same activity of DNA polymerase and HBcAg as that of preparations incubated with buffer alone, regardless of the subtype of HBV. The validity of these results was further reinforced by the results of similar experiments in which anti-HBs was used as the reagent. It was shown that little, if any, DNA polymerase was detectable in the supernatant of samples treated with anti-HBs. Moreover, the testing for HBcAg both in supernatant and precipitate revealed that HBcAg activity in Dane-rich preparations was almost completely brought down with the precipitate. With the method used in this study, no evidence was found for the presence of HBeAg determinants available for reaction with anti-HBe on the surface of Dane particles.

The present observation is somewhat at variance with the previous report of Neurath and co-workers (1976) who announced that Dane particles and 20 nm tubules were precipitated when they had been incubated with anti-HBe by an immune electron microscopic method. The difference between their results and ours would partly be ascribed to the heterogeneity of Dane particles. Kaplan, Purcell & Gerin (1976) have demonstrated that there are at least two subpopulations of Dane particles, one of which had a density at 1.22 g/ml and associated with DNA polymerase activity, and the other with a lower density at 1.20 g/ml and devoid of DNA polymerase activity. Both of these populations, however, contained HBcAg. Of course, there still remains the possibility that Dane particles which Neurath's group
observed were defective ones in which neither DNA polymerase nor HBcAg existed. The present results strongly indicate that anti-HBe did not react with complete Dane particles, at least as defined by the presence of both DNA polymerase activity and HBcAg and by inference, infectivity.

There have been a number of reports citing circumstantial evidence for a connection between HBeAg and Dane particles. Apart from the association of HBeAg with DNA polymerase activity (Imai et al. 1976), and HBcAg (Takahashi et al. 1976), HBeAg seems to signal the presence of HBV. Vertical transmission of HBV from asymptomatic carrier mothers to children was observed only from mothers whose serum contained HBeAg and/or DNA polymerase activity (Mayumi & Miyakawa, 1976; Okada et al. 1976). In contrast, vertical transmission did not occur from mothers whose serum contained anti-HBe (Okada et al. 1976). Alter et al. (1976) found that acute type B hepatitis of medical personnel accidentally pricked with a needle occurred when the inoculated blood contained HBeAg and DNA polymerase activity but not when it contained anti-e. Taken together with the observations of vertical transmission, their results strongly indicate that HBeAg serves as a marker of infectivity especially with a small dose of inoculum.

The association of HBeAg with infectivity, however, becomes less clear when a large dose of inoculum is considered. Berquist, Maynard & Murphy (1976) transmitted HBV infection to two of four chimpanzees which had been inoculated with as much as 5 ml of HBsAg-positive serum containing anti-HBe. Recently, Oda (1976) titrated and compared the infectivity of HBsAg-positive serum samples containing HBeAg with those containing anti-HBe in susceptible chimpanzees. He noted that serum samples containing anti-HBe were also infectious when given in a large dose, but the infectivity of the serum containing anti-HBe was at least a million times less than the serum samples containing HBeAg.

The results of experimental infections, coupled with the fact that HBeAg was originally described as an entity separate from HBsAg particles (Magnius & Espmark, 1972), favour the view that HBeAg is not necessarily present on the surface of Dane particles. The present findings that anti-HBe failed to precipitate either DNA polymerase or HBcAg of Dane particles further supports this view; HBeAg does not seem to be assembled into the surface of Dane particles, despite their close association. The exact relationship between HBeAg and HBV awaits additional studies, although the practical usefulness of HBeAg as an indicator of the presence of HBV has definitely been established.

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REFERENCES


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