Antigens of Hepatitis B Virus: Failure to Detect HBeAg on the Surfaces of HBsAg Forms

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SUMMARY

The particulate forms of HBsAg were analysed for the presence of HBeAg on their surfaces. By immunodiffusion analysis, anti-HBe did not form precipitin bands with the purified forms of HBsAg and hyperimmune guinea pig antisera to these forms did not react with HBeAg. Lines of non-identity were observed between the HBeAg determinants (e1 and e2) and the Dane particles and filaments isolated from an HBeAg-positive serum. Finally, anti-HBe failed to precipitate the polymerase-positive subpopulation of Dane particles, indicating that anti-HBe has no direct role in virus neutralization.

The virion of Type B hepatitis appears to be a 43 nm spherical particle (Dane particle; Dane, Cameron & Briggs, 1970) with a complex morphology consisting of an outer lipoprotein coat and an internal 27 nm core. Two antigen systems appear to be specified by the genome of hepatitis B virus (HBV) and are associated with structural components of the Dane particle. The determinants of hepatitis B surface antigen (HBsAg) are expressed on the lipoprotein coat of the Dane particle and other subviral forms found in the sera of chronically-infected individuals. The hepatitis B core antigen (HBcAg) is distinct from HBsAg and is associated with the particulate 27 nm cores of the Dane particle (Almeida, Rubenstein & Stott, 1971), some of which contain an endogenous DNA polymerase activity (Kaplan et al. 1973, 1976) and circular double-stranded DNA (Robinson, Clayton & Greenman, 1974).

The hepatitis B e-antigen (HBeAg) and its antibody (anti-HBe) represent yet another antigen system in association with Type B hepatitis and not other liver diseases (Magnius & Espmark, 1972) including Type A hepatitis (Maynard et al. 1976). The HBeAg complex appears to consist of more than one specificity (Williams & LeBouvier, 1976) and physical characterization (Magnius, 1975) indicated that it is composed of soluble proteins of approx. 300000 mol. wt. A number of studies demonstrated that the presence of HBeAg correlates well with Dane particles (Nielsen, Dietrichson & Juhl, 1974) and HBV-specific DNA polymerase activity (Nordenfelt & Sandberg, 1976; Takahashi et al. 1976) in serum and that these markers are important indicators of HBV infectivity (Alter et al. 1976; Okada et al. 1976). Despite the specific association of HBeAg with Type B hepatitis, most authors have cautioned that the evidence is not yet conclusive that it represents a HBV gene product; HBeAg might also represent a specific response of the host to HBV replication (El Sheikh et al. 1975; Magnius et al. 1975). However, in a recent report, Neurath et al. (1976) concluded that HBeAg was exposed on the surface of Dane particles and tubular forms of HBsAg and was not present on the surface of the approx. 20 nm form. This demonstration of HBeAg as a protein associated with the structure of the presumptive hepatitis B virus supports the possibility that it represents a virus gene product and has significant implications for approaches to prophylaxis. Thus, it predicts that anti-HBe might have a direct or additive role in virus neutralization and might provide a basis for the use of anti-HBe in passive immunization as suggested by Okada et al. (1976). Current vaccine programs (Purcell &
Short communications

Gerin, I975; Buynak et al. I976), which are based on the use of the 20 nm subviral form of HBsAg, would have to be evaluated on the basis that the immunogen lacks a potentially important surface antigen of the HBV which is involved in virus neutralization. Because of these important implications, we attempted to confirm the findings of Neurath et al. (I976) using other methods of analysis but failed to find evidence for the presence of HBeAg on the Dane particle surface.

Two types of analysis were used in this evaluation: immunodiffusion in agarose gels and double-antibody immunoprecipitation of Dane particles as measured by the endogenous DNA polymerase activity. The plasma of a chronic HBsAg/ayw carrier (NCB), positive for HBsAg-specific DNA polymerase activity and HBeAg, was used as a source of ‘heavy’ Dane particles (Kaplan et al. 1976). The Dane particles were isolated by centrifuging the plasma for 4 h at 20000 rev/min in the Spinco 21 rotor, resuspending the pellet in phosphate buffered saline (PBS; 0.85 % NaCl, 0.01 M-phosphate, pH 7.4) to 1/20 volume, followed by re-centrifuging and resuspending the pellet in 1/50 of the original volume. Electron microscopic examination of the preparation, designated NCB P-2, revealed Dane particles and filaments as the predominant particulate forms. HBsAg was also purified from the plasma of a chronic HBsAg/ayw carrier (Ytr) by zonal centrifugation procedures as previously described (Gerin, Holland & Purcell, 1971; Gerin, 1973). The rate sedimentation profile (see Gerin, 1973, Fig. 15) of twice CsCl-banded HBsAg revealed the usual 20 nm forms as well as filamentous forms which sedimented further in the sucrose gradient. Three pools were made from the gradient fractions: pool one contained 20 nm forms of HBsAg; pool two, the long filamentous forms plus the low-density (1.20 g/ml) Dane particles (Gerin, Ford & Purcell, 1975); and pool three, short filamentous forms found in gradient fractions intermediate between those which made up pools one and two. Each pool was re-banded in CsCl and HBsAg recovered at 1.20 g/ml. After dialysis against 0.01 M-tris-HCl buffer, pH 7.4, the individual pools were used to hyperimmunize guinea pigs as previously described (Purcell et al. 1970). Each guinea pig was inoculated in the hind footpad at time zero with a dose of 20 μg of HBsAg, 1:1 in Freund’s complete adjuvant. At 4 and 6 weeks, each animal was again inoculated intraperitoneally with 20 μg of aqueous HBsAg and exsanguinated by heart puncture 10 days after the 6-week boost. The sera of individual guinea pigs were assayed for anti-HBs by passive haemagglutination (PHA; Vyas & Schulman, 1970) using commercial reagents (Electronucleonics, Bethesda, Maryland). When tested against red blood cells coated with an ayw antigen, the hyperimmune sera to pools one, two and three titred 1:3200000, 1:640000 and 1:512000, respectively. The standard HBeAg reagent was obtained by plasmapheresis of a chronic HBsAg/adw carrier chimpanzee and contained both the e1 and e2 specificities (Williams & LeBouvier, 1976). The standard anti-HBe was obtained from a human chronic carrier, specified both anti-HBe1 and HBe2, and also contained anti-HBc activity. The anti-HBe reagent (795) was from a chronic HBsAg carrier and has been previously characterized (Moritsugu et al. 1975; Budkowska, Shih & Gerin, 1977); it contained HBeAg and therefore lacked anti-HBe. The human anti-HBs serum was obtained from a donor convalescent from Type B hepatitis and was predominantly anti-HBs/a.

Immunodiffusion analyses were performed by the procedure of Williams & LeBouvier (1976) with 0.4 % agarose (L’Industrie Biologique Francaise, Gennevilliers, France) in 0.15 M-NaCl and tris-HCl, pH 7.6. Wells, 3 mm in diam., were cut in the agarose and filled with approx. 20 μl of sample; wells loaded with the standard HBeAg and anti-HBe reagents were refilled twice within the first few hours. All other wells were filled once. Guinea pig antisera to the HBsAg forms were absorbed with normal human serum before analysis. The
Fig. 1. Non-identity is demonstrated between the HBeAg antigenic determinants (e₁ closer to the antibody well, e₃ closer to the antigen well) and the Dane particle-anti-Dane precipitin band. The line of precipitation between wells 3 and 4 represents an HBsAg-anti-HBsAg reaction. In test patterns (not presented here) in which the positioning of wells was changed, this line shows partial identity with the Dane-anti-Dane precipitin band. The spurring reaction in this case results from the subtype difference between the HBsAg in the HBeAg positive serum (subtype \textit{adw}) and the Dane particle preparation (subtype \textit{ayw}). Well 1, Dane particle preparation (subtype \textit{ayw}); well 2, anti-HBeAg (anti-e₁ and anti-e₃) serum; well 3, guinea pig anti-Dane and long filament serum; well 4, HBeAg (e₁ and e₃), HBsAg subtype \textit{adw} positive serum.

Fig. 2. Electron micrograph of the precipitin band formed by the reaction of anti-HBs with the NCB P-2 preparation. The precipitin band was excised from the agarose gel, extracted with PBS and examined in an Hitachi HU 11E after negative staining with 1% phosphotungstic acid.
Table 1. Failure of antibody to HBeAg to precipitate DNA polymerase-positive Dane particles

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>DNA polymerase activity remaining in supernatant*</th>
<th>(^{3}H, \text{ct/min} )</th>
<th>P/N†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal serum</td>
<td>1591</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>138</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>1742</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>1567</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td><strong>Guinea pig</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preserum</td>
<td>1752</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>Anti-pool one (20 nm)</td>
<td>82</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Anti-pool two (Dane particles &amp; filaments)</td>
<td>91</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Anti-pool three (short filaments)</td>
<td>90</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

* Dane particle concentrate (NCB P-2; 25 \( \mu l \)) was incubated with 20 \( \mu l \) of 1:5 dilution of antiserum for 18 h at 4 °C at which time 75 \( \mu l \) of an optimum concentration of second antibody (goat anti-human IgG or rabbit anti-guinea pig IgG) was added. After 2.5 h at room temperature the mixture was centrifuged at 3000 g for 30 min. The supernatant (75 \( \mu l \)) was solubilized by the addition of 10 \( \mu l \) of 1% Nonidet P-40 and 1% \( \beta \)-mercaptoethanol. Polymerase reaction mixture (40 \( \mu l \)) was added to yield a final concentration of 0.128 M-tris-HCl, pH 7.5; MgCl\(_2\), 32 mm; NH\(_4\)Cl, 96 mm; 0.4 mm each of dATP, dCTP and dGTP, and 1.25 \( \mu Ci \) of \( ^{3}H\)-TTP, sp. act. 45Ci/mmol (New England Nuclear). A portion (40 \( \mu l \)) of the mixture was withdrawn at zero time and after 3 h at 37 °C and processed for counting as described by Kaplan et al. (1973). The zero time values ranged from 30 to 40 ct/min.

† P/N means positive/negative and represents the \(^{3}H \text{ct/min} \) value of the experimental sample divided by that obtained using either normal human serum (134 ct/min) or guinea pig serum (99 ct/min) in place of the NCB P-2 concentrate.

plates were stored in a humid chamber at room temperature and examined daily for up to 4 days. Precipitin lines were observed between the three HBsAg pools and the respective guinea pig antisera. Each individual antiserum showed a line of identity with all three HBsAg pools and a reference HBsAg/\( \text{ayw} \) serum, and partial identity with the HBsAg/\( \text{adw} \) in the standard HBeAg serum. The reaction between the standard HBeAg serum and the anti-HBe reagent revealed lines (\( e_1 \) and \( e_2 \)) of non-identity with the HBsAg-anti-HBs line; no anti-HBe\(_1\) or anti-HBe\(_2\) activity was detected in any of the guinea pig antisera to the various HBsAg forms. Likewise, the anti-HBe reagent failed to react with the three pools of purified HBsAg. Finally, reactions of complete non-identity were observed between the HBeAg (\( e_1 \) and \( e_2 \)) determinants and the HBsAg precipitin line formed from the reaction of the Dane particle (NCB P-2) preparation with the guinea pig antiserum to pool two (Dane particles and filaments; Fig. 1). In order to be certain that the HBsAg precipitin line was not due to the presence of residual 20 nm forms, the precipitin line was excised from the gel, extracted in PBS buffer with mechanical disruption, and examined by electron microscopy after negative-staining with 1% phosphotungstic acid. The morphological forms (Fig. 2) participating in the HBsAg reaction consisted of Dane particles and filaments. The reaction of non-identity between these particles and the \( e_1 \) and \( e_2 \) specificities of HBeAg clearly indicated that these specificities were not present on the surfaces of these particulate forms.

The NCB P-2 preparation contained endogenous DNA polymerase activity which was precipitated by anti-HBs and not by anti-HBe or normal serum (Table 1). These criteria establish the activity as a marker of the subpopulation of intact Dane particles considered to be the infectious hepatitis B virus. The standard anti-HBe reagent failed to precipitate the
polymerase activity indicating that the HBe1Ag and HBe2Ag specificities were not exposed on the surface of the presumed HBV. The guinea pig antisera to the various HBsAg forms precipitated the polymerase activity as expected from their high anti-HBs titres.

In summary, the conclusion that the determinants of HBeAg are not expressed on the surfaces of the HBsAg particles is based on the following lines of evidence: anti-HBe did not react with any of the purified particulate forms and hyperimmune guinea pig antisera to these forms failed to react with the reference HBeAg; clear lines of non-identity were observed between the HBeAg determinants and the Dane particles and filaments isolated from an HBeAg-positive serum; and the failure of anti-HBe to precipitate the polymerase-positive Dane particles using a sensitive double antibody radioimmunoprecipitation assay. Whether the HBeAg complex is coded for by the HBV genome or represents a specific response of the host to HBV infection still remains an important question. Lam, Tong & Rakela (1977) reported that the treatment of Dane particle-rich preparations with 0.5% Tween 80 released HBeAg suggesting that HBeAg represents an internal structural protein of the HBV. On the other hand, Neurath & Strick (1977) have found that HBeAg has properties of an immunoglobulin and suggested it represents an idiotypic antibody; clearly a host response to HBV infection. The earlier results (Neurath et al. 1976) were explained by the possibility that a rheumatoid factor might have been present in their anti-HBe reagent. However, another report from the same laboratory (Trepo et al. 1976) localized HBeAg to the cytoplasm of infected hepatocytes. In any case, we report here that the determinants of HBeAg are not expressed on the surfaces of Dane particles, filaments or 20 nm HBsAg forms. Significantly, HBeAg was not found on the surface of the population of Dane particles that contains DNA polymerase and correlates with HBV infectivity indicating that anti-HBe has no direct role in virus neutralization. Therefore, the correlation between anti-HBe and lack of infectivity is probably an indirect one; if so, there appears to be no basis for active immunization for anti-HBe or the passive administration of anti-HBe as prophylactic measures for Type B hepatitis.

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