Demonstration of Hepatitis B e Antigen (HBeAg) in Association with Intact Dane Particles

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SUMMARY

Mild detergent treatment (0.1 % Sarkosyl-0.1 % β-mercaptoethanol) of Dane particle-rich fraction from human serum resulted in the release of core particles together with HBe antigen activity when examined by the reversed passive haemagglutination method. Furthermore, when the core particles isolated by the above procedure were exposed to stronger detergent (1 % Sarkosyl-0.1 % β-mercaptoethanol), additional HBe antigen activity was released only from intact core particles with DNA polymerase activity and not from empty core particles.

The hepatitis B e antigen (HBeAg) discovered by Magnius & Espmark (1972) is known to be intimately associated with hepatitis B virus (HBV) infection but to be distinct from the other known particle associated antigens, i.e. hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg). The fact that HBeAg is most commonly detected in the sera in the presence of circulating Dane particles, HBV-specific deoxyribonucleic acid (DNA) polymerase activity (Nordenfelt & Kjellen, 1975) and virus DNA (Werner et al. 1977) may indicate the possibility that HBeAg is a component of the HB virion as has been suggested by Lam et al. (1977).

Another explanation for the nature and significance of the HBe antigen was proposed by Neurath & Strick (1977) who suggested that it shared the properties of immunoglobulin. An extended explanation of Neurath's hypothesis was made by Takahashi et al. (1978a), who described the duality of HBeAg, one in the free form and the other in a form bound to immunoglobulin.

These contradictory hypotheses stimulated us to examine the possible extraction of HBeAg from Dane particles using different detergent treatments. So far, the most clear-cut result has been obtained by using Sarkosyl (sodium N-lauroylsarkosinate, Wako Pure Chemicals Co. Ltd., Tokyo) as the detergent.

For the preparation of Dane particles, sera from 350 chronic carriers were screened by Ouchterlony's double immunodiffusion method for the presence of HBeAg (Magnius & Espmark, 1972); those specimens with relatively high HBeAg titres, almost 20 %, were pooled. The serum pellets were obtained from centrifugation of 7 l of serum in a Hitachi Type RP-42 rotor at 40000 rev/min for 3 h at 4 °C. The pellets were suspended in phosphate buffered saline (PBS), pH 7.4, at 1/50 of the original volume and recentrifuged as described above. After the two repeated sedimentations, the precipitates were resuspended in 20 % (w/v) sucrose in PBS. Thirty ml samples were then layered on top of discontinuous 40 %-over-60 % (w/v) sucrose density gradients containing 0.01 M tris-HCl, pH 7.4, 0.15 M-NaCl (TN buffer). Centrifugation was carried out in a Hitachi Type RPS-27-2 rotor at 24000 rev/min for 13 h at 4 °C and 2 ml fractions were assayed for HBcAg. The fraction positive for HBcAg, between 35 and 50 % sucrose, was collected, concentrated and dialysed overnight against TN buffer (the Dane particle-rich fraction). At this step, none of the free HBeAg present in the serum was detected in the Dane particle-rich fraction since it did not contain...
Fig. 1. Sedimentation profile of the Dane particle-rich fraction in a sucrose density gradient and the results of Sarkosyl treatment. Two ml of Dane particle-rich fraction (RPHA titre of HBcAg, 1:4096) were layered on a 5 to 20% sucrose gradient solution containing 0.01 M-tris-HCl, pH 7.4, and 0.15 M-NaCl and centrifuged at 24,000 rev/min for 75 min at 4 °C. Each fraction was assayed for HBsAg (O--O) by the RPHA method. After this, to 80 μl of each fraction, 20 μl of 0.5% Sarkosyl-0.5% β-mercaptoethanol was added to make a final concentration of 0.1% Sarkosyl and this was then incubated at 37 °C for 60 min and assayed for HBcAg (●●) and HBeAg (▲▲) by the RPHA method. □□, Density.

HBeAg activity when determined by reversed passive haemagglutination (RPHA) as described by Takahashi et al. (1977). For the quantitation of HBc antigen (H. Ohori et al. unpublished data) and HBs antigen (Schurs & Kacaki, 1974), the RPHA method was employed. DNA polymerase activity was examined by the modified method of Robinson (1975).

Preliminary experiments showed that HBeAg was released when 200 μl of Dane particle-rich fraction were treated with 0.1% Sarkosyl-0.1% β-mercaptoethanol at 37 °C for 60 min. The released HBeAg was first monitored by double immunodiffusion and the precipitin line formed between anti-HBe and detergent treated material was identified as being identical to that formed between standard HBeAg and anti-HBe. After this, the quantitative titration of HBeAg was carried out by RPHA.

The release of HBe antigen from the Dane particle-rich fraction was further tested by sucrose density gradient centrifugation (Fig. 1). Two ml of Dane particle-rich fraction were layered on top of a continuous 5 to 20% (w/v) linear sucrose density gradient (30 ml) and centrifuged in a Hitachi Type RPS-27-2 rotor at 24,000 rev/min for 75 min at 4 °C. Eighty μl from each 1.2 ml fraction were collected and each received detergent treatment as described above. In Fig. 1, three HBsAg peaks can be identified. The fraction having the smallest sedimentation coefficient was shown by electron microscopy to contain almost exclusively small spherical particles. After Sarkosyl treatment of each fraction in this gradient, three HBe antigen peaks appeared in the 12 to 18% sucrose fractions whereas detectable activity was not seen in any fraction before treatment. Two peaks (Peaks 1 and 2, Fig. 1) corresponded to two peaks of HBs antigen before Sarkosyl treatment. The third peak of HBeAg which had the highest HBeAg titre appeared in fraction 16 (12% sucrose). Concordant with the appearance of HBc antigen in these three peaks was HBeAg.
Fig. 2. The sedimentation profile of core particles by sucrose density gradient centrifugation. Detergent (0.1% Sarkosyl–0.1% β-mercaptoethanol) treated Dane particle-rich fraction was layered on a 5 to 20% sucrose gradient containing 0.01 M-tris-HCl, pH 7.4, and 0.15 M-NaCl and centrifuged at 24000 rev/min for 150 min at 4°C. Each fraction was assayed for HBsAg (○—○) by RPHA and for DNA polymerase activity (▲—▲) as described in the text. After this, to 80 µl of each fraction, 20 µl of 5% Sarkosyl–0.5% β-mercaptoethanol was added and incubated at 37°C for 60 min. Each detergent treated fraction was dialysed against the same buffer overnight at 4°C and titrated for HBcAg (●—●) and HBeAg (△—△). ■—■, Density.

activity which also was not detectable before Sarkosyl treatment. A relatively higher titre of HBeAg when compared to that of HBcAg was liberated from the two peaks of larger sedimentation coefficient (Peaks 1 and 2 in Fig. 1); the titres of HBcAg and HBeAg being 1:16 and 1:8 (Peak 1) and 1:32 and 1:32 (Peak 2), respectively. In the fraction with the smallest sedimentation coefficient (Peak 3, fraction 16), the titres of the two antigens were 1:128 and 1:16, respectively. These results may suggest that HBeAg can be released from Dane particles after Sarkosyl treatment as each of the three fractions before treatment did not reveal any HBeAg activity. Compared with the HBcAg activity, a relatively higher titre of HBeAg was recovered from the heavier Dane particles.

The next experiment was conducted to examine whether or not HBeAg was still associated with core particles after detergent treatment. Before conducting the experiment, the disruption of Dane particles by various concentrations of detergent was examined. For this, Dane particle-rich fractions were treated by exposure to Sarkosyl concentrations of 0.1%, 0.25%, 0.5% and 1% in the presence of 0.1% β-mercaptoethanol at 37°C for 60 min. This was followed by centrifugation in 5 to 20% (w/v) sucrose density gradients at 24000 rev/min for 150 min. When each fraction from this centrifugation was assayed for HBcAg and the core particles examined by electron microscopy, 1% Sarkosyl–0.1% β-mercaptoethanol was found to be the best disruption buffer for the Dane particles; more than 90% of HBcAg was solubilized and detected in the top fractions and in addition, HBC particles could not be visualized by electron microscopy in any fraction. Treatment with 0.5% Sarkosyl disrupted Dane particles by 50% while 0.25% Sarkosyl had no effect.

Based upon these results, the possible release of HBeAg from core particles was examined. Two ml of the 0.1% Sarkosyl–0.1% β-mercaptoethanol treated Dane particle-rich fraction (RPHA titre of HBcAg; 1:4096) was layered on top of a continuous 5 to 20% (w/v)
Sucrose density gradient and centrifuged in a Hitachi RPS-27-2 rotor as described above (Fig. 2). The first and second peaks of HBcAg positive fractions had DNA polymerase activity but the third peak in which empty core particles were found by electron microscopy did not have any detectable DNA polymerase activity. When each fraction was further treated with 1 % Sarkosyl–0.1 % β-mercaptoethanol to disrupt the core structure and dialysed against TN buffer overnight at 4 °C, the highest titre of HBeAg was found in the first peak (HBcAg and DNA polymerase positive) but a lower titre of HBeAg was also found in the second peak. These results suggest that HBeAg is a component of the Dane particles and that this antigen may be closely associated with the core of the HB virion.

Other reports describe two populations of core particles in the heterogeneous Dane particle population (Neurath et al. 1978), one with a buoyant density of 1.36 (in CsCl) and an $s_{20, w}$ of 110 and another with a buoyant density of 1.28 to 1.30 and an $s_{20, w}$ of 70. The pioneer work of Kaplan et al. (1976) is in accord with these results in the sense that there are two core particles, one with DNA polymerase activity and a high density ($\rho = 1.36$ in CsCl) and another without DNA polymerase activity and a low density ($\rho = 1.30$). The three peaks of HBcAg and core (visualised by electron microscopy, data not shown) obtained in this study by sucrose density gradient centrifugation may suggest further heterogeneity of the core particles when the presence of HBeAg is taken into consideration; the first peak associated with DNA polymerase, HBcAg and HBeAg, the second peak with DNA polymerase, HBcAg and a small amount of HBeAg and the third peak (empty particle) not associated with DNA polymerase or HBeAg.

In the past, Neurath et al. (1976) claimed that HBeAg was present on the surfaces of Dane particles and tubular forms but not on the small spherical particles. The work of Gerin et al. (1978) and Takahashi et al. (1978b), however, denied the presence of HBeAg on the Dane particles. Our data shows that there was no detectable HBeAg activity on the untreated Dane particle-rich fraction which is in accord with the results of the latter two papers. In our study, by treating such Dane particles and core particles with different concentrations of Sarkosyl, measurable activity of HBeAg was recovered.

The detergent used was very useful in that it separated the core from the Dane particles without any loss of HBsAg titre (Fig. 1). Neither HBsAg nor HBcAg titre was affected by prior 0.1 % Sarkosyl treatment (data not shown). About 40 % of the HBeAg associated with the Dane particles was released by 0.1 % Sarkosyl treatment. Furthermore, when the Sarkosyl concentration was increased to 1 %, the remaining 60 % of HBeAg activity associated with the core particles was liberated without any loss of HBcAg titre.

The results obtained may indicate the presence of significant amounts of HBeAg inside the Dane particles and the use of the RPHA assay for HBeAg made this analysis possible. The antigen may be closely associated with core surfaces as evidenced by further release after stronger detergent treatment of the core particles. However, whether HBeAg is situated on the core surfaces or inside the core was left for further studies. The fact that the second liberation of HBeAg was obtained only with core particles of high density may suggest the attribution of this antigen to the HB virion assembly and that it may function as a maturation protein of the virus core.

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