Immunological and Morphological Properties of HBeAg Subtypes (HBeAg/1 and HBeAg/2) in Hepatitis B Virus Core Particles

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SUMMARY

A polypeptide of 21500 mol. wt., structurally associated with hepatitis B virus core particles, was shown to have two kinds of HBeAg antigenicity (HBeAg/1 and HBeAg/2). This was revealed by transferring a single core peptide from polyacrylamide gels to nitrocellulose sheets (Western blotting), which reacted with anti-HBeAg/1 and anti-HBeAg/2. Selective discrimination of the two HBe antigens was achieved by radioimmunoassay (RIA). When highly purified core particles were incubated at 37 °C in a 0.1 % SDS-0.1 % 2-mercaptoethanol solution, only HBeAg/1 was released after 5 min incubation and the release of HBeAg/2 occurred only after prolonging incubation for 30 min. The course of degradation was also detected by CsCl density gradient centrifugation. These results indicate that HBeAg/1 is less closely associated with core particles than is HBeAg/2. Electron microscopy showed that the core particles from which HBeAg/1 was removed were more labile than the original preparation when incubated at 56 °C in aqueous solution, or at 37 °C in Sarkosyl solutions; when placed in 1 M-NaCl or -CsCl solution, the particles swelled to a larger diameter than untreated cores.

INTRODUCTION

The complete human hepatitis B virus (HBV) is believed to be a Dane particle, composed of a 27 nm core (HBcAg), and an antigenically different coating (HBsAg). The core particle represents the nucleoid of HBV and contains a double-stranded circular DNA molecule and a DNA polymerase. HBeAg is the third HBV-specific antigen detectable in patients' sera apart from HBV particles and HBs antigens (Magnius & Espmark, 1972). A close physical correlation between HBeAg and HBV particles, however, was later shown (Ohori et al., 1979; Takahashi et al., 1979; Yoshizawa et al., 1979) and HBeAg is now regarded as an integral component, in a cryptic form, of core particles (Yoshizawa et al., 1979; Budkowska et al., 1979; Ohori et al., 1979). There is a strong correlation between the detection in patients' sera of HBeAg and the presence of either HBV particles (Nordenfelt & Kjellen, 1975; Imai et al., 1976; Maynard et al., 1976) or the HBV particle-associated HBCag (Murphy et al., 1976; Trepo et al., 1976).

In the early days of HBeAg studies, Williams & Le Bouvier (1976) reported two subspecificities, HBeAg/1 and HBeAg/2, detected by immunodiffusion. In later studies (Yamada et al., 1979; Ohori et al., 1980b), we demonstrated that HBeAg/1 is detectable for longer periods than HBeAg/2 in patients' sera during HBV infection. HBeAg/1 can be detected in the early phase of infection and also late after recovery. On the other hand, HBeAg/2, DNA polymerase and the peak HBsAg titre were all detectable at about the same time during the course of HBV infection. Thus, HBeAg/2 is probably the original HBeAg described by Magnius & Espmark (1972), and the significance of HBeAg/1 detection is unresolved. Recent physicochemical studies of HBeAg, however, indicated that a polypeptide of mol. wt. 21000 to 21500 is the only dominant polypeptide detectable in core particles that share the antigenicities of both HBcAg and HBeAg (Ohori et al., 1980a; Mackay et al., 1981; Yamaki et al., 1982). Furthermore, we found that a polypeptide with a mol. wt. of 16000 was the degraded product...
(by-product) of a core particle with antigenicities of both HBeAg/1 and HBeAg/2, although no evidence was obtained to indicate whether a single polypeptide could share both HBeAg/1 and HBeAg/2 antigenicities (Yamada et al., 1983).

This paper describes further studies on the association of HBeAg/1 and HBeAg/2 with the HBV core particles and, in particular, the relationship between these antigens and the structure of the core particles.

METHODS

Serological testing. HBsAg titres were determined by the reversed passive haemagglutination (RPHA: Antihebscell; The Green Cross Corp., Osaka, Japan) method and anti-HBsAg was determined by the passive haemagglutination (PHA: Hebsgcencell; The Green Cross Corp.) method. HBeAg and anti-HBeAg titres were determined by the RPHA and RPHA inhibition (RPHAI) methods as described previously (Yamada et al., 1979; Ohori et al., 1980b).

Preparation of core particles from liver. An autopsy of liver (5 g) obtained from a patient who died from HBsAg-positive subacute hepatitis was the sole starting material for the experiment. Extraction and purification of core particles followed the same methods described in a previous paper (Ohori et al., 1980a).

Selective determination of total HBeAg, HBeAg/1 and HBeAg/2. (i) Immundiffusion test: HBeAg was determined by immunodiffusion (micro-Ouchterlony, MO) in agarose by the method described previously (Yamada et al., 1983). (ii) Radioimmunoassay (RIA) test: HBeAg was quantified by a modified method using a solid-phase radioimmunoassay HBeAg kit (Master Lot No. 12-545-HR; Abbott Laboratories, North Chicago, Ill., U.S.A.) as described by Mushahwar et al. (1978). Because the polystyrene beads were coated with anti-HBeAgs (anti-HBeAg/1 and anti-HBeAg/2) as well as with anti-HBeAg, titres of both HBeAg and HBCAg were assayed by the kit. Therefore, to determine total HBeAg (HBeAg/1 and HBeAg/2), it was necessary to add anti-HBCAg to the reaction mixture to obtain the exact HBeAg titre of the disintegrated core particles. The titration of total HBeAg was done as follows. Two sets of serial twofold dilutions of the test samples were prepared in phosphate buffer (0.01 M, pH 7.6) supplemented with 0.1 M-NaCl (PBS) in an acrylic plate. One set was combined with 100 μl of PBS containing anti-HBCAg (RPHAI titre, 1:215), and the other set (control) with 100 μl of PBS containing the same titre of anti-HBCAg and excess anti-HBeAg [RIA titre, 1:25 600; anti-HBeAg titre was determined by a neutralization procedure (Yamada et al., 1983)]. After incubation of the plate at 37 °C for 2 h, polystyrene beads coated with anti-HBCAg were added to each well, and the plate was further incubated at room temperature for 24 h. After washing with distilled water (15 ml), the beads were further incubated in 200 μl of 125I-labelled anti-HBeAg solution at 45 °C for 4 h. The beads were then washed with distilled water (15 ml) and counted for uptake of 125I-labelled anti-HBeAg, as described by Mushahwar et al. (1978). The first set gave the exact HBeAg titre when the counts of the second set were subtracted as the background.

Titres of HBeAg/1 and HBeAg/2 were obtained as follows. Three sets of serial twofold dilutions of the test samples were prepared in PBS containing anti-HBCAg (RPHAI titre, 1:215) and monospecific anti-HBCAg (RIA titre, 1:12800) to measure the HBeAg/1 titre, while another set was added to 100 μl of PBS containing the same titre of anti-HBCAg and excess anti-HBeAg [RIA titre, 1:25 600; anti-HBeAg titre was determined by a neutralization procedure (Yamada et al., 1983)]. After incubation of the plate at 37 °C for 2 h, polystyrene beads coated with anti-HBCAg were added to each well, and the plate was further incubated at room temperature for 24 h. After washing with distilled water (15 ml), the beads were further incubated in 200 μl of 125I-labelled anti-HBeAg solution at 45 °C for 4 h. The beads were then washed with distilled water (15 ml) and counted for uptake of 125I-labelled anti-HBeAg, as described by Mushahwar et al. (1978). The first set gave the exact HBeAg titre when the counts of the second set were subtracted as the background.

Preparation of anti-HBeAg/1, anti-HBeAg/2 and anti-HBeAg positive IgGs. Standard anti-HBeAg/1 and anti-HBeAg/2 positive sera were selected from sera of asymptomatic HBsAg carriers by determining and identifying anti-HBeAg by agar gel diffusion (MO). Anti-HBCAg was also selected from the sera which were negative for both anti-HBeAg/1 and anti-HBeAg/2. IgG was prepared by using DEAE-cellulose column chromatography (Yamada et al., 1981).

SDS–polyacrylamide gel electrophoresis. SDS–PAGE was performed by the method of Laemmlli (1970). Eighty μl of core particle suspension (RPHA titre, 1:215 or 32 768 units) was added to 20 μl of sample buffer composed of 50 mM-Tris–HCl pH 7.8, 10% SDS, 10% 2-mercaptoethanol and 8 μ-urea. The reaction mixture was incubated at 100 °C for 5 min. Each 7 μl of this sample was applied to a 15% polyacrylamide gel. Molecular weights were estimated using the following standards: rabbit muscle phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), bovine erythrocyte carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and bovine milk α-lactalbumin (14400) (all from the Pharmacia electrophoresis calibration kit). Electrophoresis was carried out at 12 mA for about 6.5 h at room temperature and the protein was detected by the silver staining method (Wray et al., 1981).
Western blotting. Electrophoretic transfer of protein from the SDS–polyacrylamide gel to nitrocellulose and detection of protein having the antigenicity of HBeAg (Western blotting) was carried out according to the method of Davis & Bennett (1982). Proteins were analysed in 15% polyacrylamide gels under the conditions described for SDS–PAGE, then the core proteins on acrylamide gels were electrophoretically transferred to a nitrocellulose sheet using the method of Towbin et al. (1979). Detection of immunoreactive polypeptides was carried out as follows: immediately following transfer, the nitrocellulose sheet was incubated in 100 ml of washing buffer containing 10 mM-Tris–HCl pH 7.4, 0.15 M-NaCl, 1 mM-EDTA, 0.2% Triton X-100 and 20 mg/ml bovine serum albumin (BSA) at room temperature for 60 min. The sheet was transferred to 1 ml of fresh washing buffer supplemented with either human serum or IgG positive or negative for anti-HBeAg/1, anti-HBeAg/2, or anti-HBcAg as the first antibody reagents and incubated at 4 °C for 12 h. The nitrocellulose sheet was then washed five times with 200 ml of washing buffer without BSA, with agitation for 15 min, and once with glycine–Triton buffer composed of 2 M-urea, 0.1 M-glycine and 1% Triton X-100 for 15 min at room temperature. Finally, the nitrocellulose sheet was washed once more with washing buffer without BSA. The sheet was then immersed in 1 ml of fresh washing buffer supplemented with 1 μl of horseradish peroxidase-conjugated rabbit IgG against human serum IgG (Miles Laboratories) as the second antibody reagent and incubated at room temperature for 2 h, then the sheet was washed as described above. The sheet was then incubated at room temperature for 10 min in 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemicals, Osaka), 0.005% H2O2, 0.01 M-Tris–HCl pH 7.0, 0.15 M-NaCl (TN buffer), and washed in distilled water. Proteins that immuno-reacted with antibody were observed as dark brown bands.

Electron microscopy. Purified core particles were scanned using the negative staining procedure. Briefly, 1 drop of sample was placed on a carbon-coated grid and was allowed to stand for approximately 2 min. Excess material was absorbed by filter paper, stained with 2% uranyl acetate for 15 s and examined with a Hitachi H-500 electron microscope.

In order to compare the morphological integrity of native cores with that of core particles from which HBeAg/1 had been removed, an electron microscopic observation using a 'sandwich' procedure with two thin carbon films was carried out according to the method reported by Boublik et al. (1977). Core particles treated during incubation at 37 °C for 13 h in TN buffer with or without 1 M-NaCl or 1 M-CsCl were adsorbed at room temperature to a very thin carbon film which had been floated completely free from a small piece of mica a few mm wide, onto the surface of a specimen solution (about 0.5 ml) in a Teflon dish. The carbon film with the adsorbed core particles (adsorption time 1 to 10 min) was transferred with the aid of the mica support into the staining solution (2% uranyl acetate in distilled water, uncorrected for pH). A reticular carbon film grid coated with an additional thin carbon film was submerged under the floating carbon film with the attached core particles. Thus, after lifting of the grid, the core particles were embedded together with the electron-dense staining solution between thin carbon films; this carbon sandwich was then air-dried. Specimens were observed with a Hitachi H-500 electron microscope.

RESULTS

Effect of physicochemical agents on the morphology of core particles, the disappearance of HBcAg and the appearance of HBeAg

The purified core particles (see Methods) were positive for HBcAg by RPHA (1:218), but negative for HBeAg by RIA. The particles displayed uniform morphology consistent with that of an icosahedron as reported by Onodera et al. (1982), and gave a single protein band on SDS–polyacrylamide gels. They are referred to as native core particles in the text. As reported previously, HBeAg was only detected when core particles were disrupted by various means, and correlated with the disappearance of HBcAg activity (Ohori et al., 1980a).

Further analysis was performed by treating core particles with various physicochemical agents, to examine the effect on morphology of core particles as well as on the disappearance of HBcAg and the appearance of HBeAg (Table 1). As seen in Table 1, the loss of HBc antigenicity clearly coincided with the morphological disintegration of core particles. For example, treatment of core particles by sonication, with a pH below 4.5 (irrespective of the presence or absence of 3 M-NH4SCN in the solution) or 8 M-urea, resulted in the complete morphological disintegration of core particles and simultaneous loss of HBcAg activity. On the other hand, when morphological disintegration was not observed, HBcAg activity remained at the same level as that before treatment (i.e. at pH 5.5 to 10.5 or heating at 37 °C or 56 °C). An exceptional finding, however, was noticed after treatment with 2.5 M-CsCl at pH 7.5, when HBeAg release occurred without disintegration of core and without loss of HBcAg. The second exception was found after treatment with 6 M-urea, which disrupted cores and released HBeAg, but without much loss of HBcAg titre.
Table 1. Effect of physicochemical agents on the morphology of hepatitis B virus cores, the disappearance of HBCAg and the appearance of HBeAg*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Morphological change†</th>
<th>Immunoreactivity of HBCAg‡</th>
<th>Immunoreactivity of HBeAg§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH 1.8</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH 2.5</td>
<td>+</td>
<td>-</td>
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<tr>
<td>pH 4.5</td>
<td>+</td>
<td>±</td>
<td>-</td>
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<tr>
<td>pH 5.5 to 10.5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH 11.5</td>
<td>±</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Denaturant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 M-NH₄SCN, pH 4.5</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3 M-NH₄SCN, pH 6.5</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 M-Urea</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>8 M-Urea</td>
<td>±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>0.18 to 2.93 M-Formaldehyde</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.5 M-CsCl, pH 7.5</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M-2-Mercaptoethanol</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.15% SDS</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.05% Sarkosyl</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heating</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>37 °C, 10 h</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>56 °C, 4 h</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* The results represent the average from data of at least three separate experiments.
† Morphology was examined by electron microscopy: +, complete disintegration of the particles; ±, morphology of the particles changed; –, no effect, particles well preserved; ND, not done.
‡ Immunoreactivity was tested by the RPHA method: +, original HBCAg titre preserved; ±, decrease in original HBCAg titre; and –, HBCAg titre was zero.
§ Immunoreactivity was tested by the immunodiffusion method: +, precipitin line observed; and –, no precipitin line.

The second finding was that HBeAg immunoreactivity was generally dependent upon the disintegration of core particles and the disappearance of HBCAg activity. However, there were exceptions. Treatments at pH 1.8 or 2.5 or with 0.18 to 2.93 M-formaldehyde, resulted in the disintegration of core particles but without loss of HBCAg in a qualitative sense and without release of detectable amounts of HBeAg. The mechanisms of such changes were left for further study.

Quantitative studies were then conducted on the release of HBeAg from the core particles by treatment with 2.5 M-CsCl at pH 7.5 and with 4, 6 or 8 M-urea. The HBCAg titre was unaltered after treatment with 2.5 M-CsCl, but was accompanied by the release of HBeAg. Disintegration of core particles did not occur.

A single protein in core particles which shares HBeAg/1 and HBeAg/2 antigenicity

Direct evidence that the protein in core particles shares the antigenic determinants of HBeAg/1 and HBeAg/2 was obtained by Western blotting. As reported previously (Ohori et al., 1980a; Yamaki et al., 1982), only one polypeptide of mol. wt. 21500 was found in core particles (Fig. 1a, lane 2). When the protein in SDS–polyacrylamide gels was transferred to the nitrocellulose sheet and subsequently reacted with anti-HBeAg/1 serum (Fig. 1b, lane 2) or anti-HBeAg/1 IgG (lane 3), the immunoreactivity to anti-HBeAg/1 was associated with a polypeptide of mol. wt. 21500. With anti-HBeAg/2 serum (lane 4) or IgG (lane 5), this protein also revealed immunoreactivity to anti-HBeAg/2. However, these reagents also contained anti-HBCag (RPHA titre of anti-HBCag was 1:212 in anti-HBeAg/1 serum, 1:211 in anti-HBeAg/1 IgG, 1:214 in anti-HBeAg/2 serum and IgG), so it was possible that the positive reaction was caused by anti-HBCag. This can be ruled out because specific anti-HBc IgG (1:217 RPHA units), which does not contain any detectable anti-HBe activities, did not stain this band at any
Fig. 1. Polyacrylamide gel analysis of core particle protein and identification of the core protein as being immunoreactive with anti-HBeAg/1 and anti-HBeAg/2 antibodies. (a) PAGE analysis of marker proteins (1) and core particles (2) (RPHA titre, 1:2^15) which were added to 20 μl of sample buffer and 7 μl was electrophoresed on a 15% polyacrylamide gel. Polypeptides were visualized by staining with silver nitrate as described by Wray et al. (1981). (b) Immunoreactive polypeptides were identified by electrophoretic transfer from polyacrylamide gels to nitrocellulose paper (see Methods), followed by exposure of the nitrocellulose to normal human serum (lane 1), anti-HBeAg/1-positive serum (lane 2), anti-HBeAg/1-positive IgG (lane 3), anti-HBeAg/2-positive serum (lane 4), anti-HBeAg/2-positive IgG (lane 5) or anti-HBcAg-positive serum (lane 6).

dilution (lane 6). These results indicate that a single polypeptide of mol. wt. 21500 released from core particles is immunoreactive to both anti-HBeAg/1 and anti-HBeAg/2 but not to anti-HBcAg.

**Sequential release of HBe antigens from disintegrating core particles as shown by CsCl centrifugation**

As is evident in Table 1, core particles treated with 2.5 m-CsCl at pH 7.5 showed no changes in morphology or in the titre of HBcAg. Nevertheless, HBeAg was apparent after this treatment. Accordingly, a suspension of core particles (HBcAg titre by RPHA, 1:2^15 or 32768 units, and total HBeAg titre by RIA, < 150 ct/min) was centrifuged on a preformed continuous CsCl density gradient. Fractions were dialysed against TN buffer and assayed for HBcAg by RPHA, total HBeAg and HBeAg/1 by RIA (Fig. 2). HBcAg titres were detected over a relatively wide range of densities between 1.29 and 1.43 g/ml, whereas morphologically intact core particles, identified by electron microscopy, banded in a narrow range between 1.32 and 1.35 g/ml. When each fraction was assayed for total HBeAg activity, a broad peak with a peak density of 1.38 g/ml was observed. These results again suggest that the activity of HBeAg can be detected in fractions of partially disintegrated cores. In addition, another HBeAg peak with a density of
Fig. 2. Sedimentation profile of core particles disrupted during CsCl density gradient centrifugation. A core particle suspension (1.0 ml, RPHA titre 1:215) which had been purified by Sepharose 4B column chromatography was layered onto a 16 ml, preformed CsCl density gradient (1.17 to 1.50 g/ml) and centrifuged at 27000 rev/min for 16 h at 4°C. Fractions were assayed for CsCl density (▲), HBcAg activity by RPHA (□), total HBeAg by RIA (○) and HBeAg/1 by RIA (●).

1.19 g/ml was detected. The latter HBeAg activity, therefore, may be free HBeAg which is released from the partially disintegrated core particles. In addition, when each fraction was further assayed for HBeAg/1 activity by adding anti-HBeAg/2 IgG to the dilution buffer in RIA, almost all of the HBeAg found in the second peak (1.19 g/ml) was HBeAg/1. On the other hand, all HBeAg found in the first peak was HBeAg/2 because no HBeAg/1 activity was detected in this fraction.

The same conclusion was drawn from results obtained under other conditions and by another assay method, when a suspension of native core particles (RPHA titre of HBcAg, 1:215 or 32768 units) was incubated at 37°C for 0, 5 or 30 min in a solution of 0.1% SDS-0.1% 2-mercaptoethanol (2-ME). As shown in Fig. 3(a) native core particles only had HBcAg activity. When they had been incubated for 5 min, the precipitin line formed between HBcAg and anti-HBcAg disappeared and a new precipitin line of HBeAg/1 and anti-HBeAg/1 appeared (between D and B in Fig. 3b). After prolonged incubation, an additional precipitin line near the antigen wells corresponding to HBeAg/2 appeared (between E and B in Fig. 3c). These results again indicated that HBeAg/1 is released more easily from core particles than is HBeAg/2.

Comparison of morphological stability of native and CsCl-treated core particles

We have demonstrated that there is a selective release of HBeAg/1 from core particles in 2.5 M-CsCl solution, during CsCl centrifugation and in 0.1% SDS solution containing 0.1% 2-ME. We then attempted to see whether core particles lacking at least a part of HBeAg/1 are physicochemically different from native core particles. CsCl-treated core particles were prepared by centrifuging native core particles (RPHA titre of HBcAg, 1:215 or 32768 units) in a CsCl density gradient (1.17 to 1.50 g/ml) at 27000 rev/min for 16 h and collecting the fractions at densities between 1.30 and 1.35 g/ml. The fractions were pooled, concentrated and dialysed against TN buffer. When the native and CsCl-treated core particles (both had RPHA titres of
HBeAg subtypes in HBV core particles

Fig. 3. Immunodiffusion of 0.1% SDS-0.1% 2-ME-treated core particles. One hundred µl of the core particle suspension (RPHA titre, 1:215) was incubated at 37 °C in the presence of 0.1% SDS-0.1% 2-ME for (a) 0, (b) 5 or (c) 30 min. A, standard antigen serum which contains both HBeAg/1 (MO titre, 1:22) and HBeAg/2 (MO titre, 1:22); B, standard anti-HBcAg (RPHA titre, 1:211), anti-HBeAg/1 (MO titre, 1:22) and anti-HBeAg/2 (MO titre, 1:22). Wells C, D and E contain core particles treated with 0.1% SDS-0.1% 2-ME for 0, 5 or 30 min.

HBcAg, 1:210 or 1024 units) were incubated at 56 °C, we found that the native core particles were stable for over 4 h, whereas cores treated with CsCl disintegrated and lost almost 88% of their HBcAg activity within 30 min (Fig. 4).

Similar results were obtained when native and CsCl-treated core particles (RPHA titres of both core particles, 1:28 or 256 units) were incubated in Sarkosyl detergent at 37 °C for 10 min and then titrated for HBcAg activity by RPHA. The CsCl-treated core particles were completely disintegrated by 0.05% Sarkosyl but about half of the native core particles remained intact at this detergent concentration (Fig. 5).

We next compared the morphological differences of native and HBeAg/1-depleted core particles by using the technique of negative staining in two thin carbon films (sandwich procedure). Native and HBeAg/1-depleted core particles (RPHA titre of HBcAg, 28 or 256 units) were incubated at 37 °C for 13 h in TN buffer with or without 1 M-NaCl or -CsCl. Virus is supposed to be deposited between two thin carbon films; however, because of the partial break of the additional carbon film, core particles were deposited in two different modes on the same grid. In Fig. 6, the dark area (b), contains particles embedded in the carbon sandwich and in the lighter area (a) particles are adsorbed to a single carbon film. No morphological differences between treated and untreated core particles were found in the lighter area (see Fig. 6a, a and b,
DISCUSSION

The results obtained here suggest that the disintegration of core particles may occur in two phases; the first phase occurs after treatment with high ionic strength salt solution at neutral pH, such as with 2.5 M-CsCl, or treatment with a low concentration of anionic detergent such as 0.05% Sarkosyl. In this phase, only HBeAg/l is released from core particles and their morphology does not change at all. A second phase reaction is caused by acidic buffer (lower than pH 4.5), heat treatment at 100 °C for 10 s (Ohori et al., 1980a), sonication or 8 M-urea treatment. In another paper (Yamaki et al., 1982), we predicted the secondary structure of the polypeptide deduced from the nucleotide sequence. The polypeptide has a high content of β-turns (48%) and one-third of the β-turn fragments are located in the COOH-terminal region. In contrast, the loci of the other two types of structure, i.e. α-helix and β-sheet, are restricted to the NH2 terminus and the internal region in the primary structure, respectively. We therefore suppose that there are hydrophobic forces between neighbouring peptides of core particles in which hydrophobic structures such as α-helix or β-sheet are located at the surface of core particles, and hence these structures may be sensitive to treatments such as high concentrations of urea.

Complete disintegration occurred below pH 4.5. In preliminary experiments (Yamaki et al., 1982), we demonstrated that a β-turn structure was induced instead of an α-helix or β-sheet by treating the core particles with 11 M-acetic acid followed by dialysis against 1 mm-acetic acid. Viewing these facts in light of the results shown in Table 1, it appears that core particles...
completely disintegrate in acidic conditions, simultaneously losing α-helix and β-sheet structures with additional release of HBeAg/2.

On the basis of the results obtained in Fig. 1, we concluded that the polypeptide of mol. wt. 21 500 is potentially reactive with antibodies directed against either HBeAg/1 or HBeAg/2. However, this does not necessarily mean that the two antigenic sites reside on the same molecule and from the results of the micro-Ouchterlony tests, we believe that there are two polypeptides of the same molecular size but with different antigenicities. Recently, we identified one major and two minor polypeptides with mol. wt. of 16000, 21000 and 14000 respectively, from HBeAg/1 and HBeAg/2 in serum (Yamada et al., 1983). These results indicate that the polypeptides of mol. wt. 16000 and 14000 are by-products of the polypeptide of mol. wt. 21000. These smaller polypeptides could be derived from the major component protein of core particles (Ohori et al., 1980a; Yamaki et al., 1982). Miyakawa & Mayumi (1982) demonstrated that the core particle-derived polypeptide of mol. wt. 19000 (P19) had two distinct antigenic determinants of HBeAg (a and b determinants in their report) and additional HBc antigenicity, by analysis of monoclonal antibodies to P19.

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