Hepatitis B Core Antigen: Immunology and Electron Microscopy

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Two distinct viral antigens are associated with the hepatitis B virus: the hepatitis B surface antigen (HBsAg, Australia antigen) and the hepatitis B core antigen (HBcAg). HBsAg, purified from the serum of asymptomatic human HBsAg carriers, and HBcAg, purified from the liver of a chimpanzee acutely infected with hepatitis B virus, were examined by serological and immune electron microscopic methods. Antisera raised against HBsAg reacted with the outer, surface component of the Dane particle and with the 20-nm spherical and tubular particles present in HBsAg-positive serum, but not with the internal component of the Dane particle or with purified HBcAg particles. Antisera raised against purified HBcAg particles reacted with the internal component of the Dane particle and with HBsAg, but not with the surface of the Dane particle or with the 20-nm spherical and tubular particles associated with HBcAg. Purified HBsAg particles, 27 nm in diameter, demonstrated distinct subunits. The infectious form of hepatitis B virus appears to be represented by the 42-nm Dane particle composed of a 27-nm nucleocapsid core component (HBcAg) surrounded by an antigenically and morphologically distinct lipoprotein surface component (HBsAg).

Evidence from several laboratories has demonstrated that the hepatitis B virus (HBV) possesses at least two separate and distinct antigens: the hepatitis B surface antigen (HBsAg, Australia antigen) and the hepatitis B core antigen (HBcAg) (1, 3, 6, 10). HBsAg, the lipoprotein surface component of HBV, can be readily detected by a variety of immunological techniques in the sera of patients acutely or chronically infected with this virus (4, 17). HBcAg, the nucleocapsid component of HBV, is not found free in serum but is found in the nuclei of hepatocytes from patients with type B hepatitis and can also be extracted from HBsAg-reactive sera containing Dane particles by detergent treatment (1, 11–13, 16, 18; R. H. Purcell, J. L. Gerin, J. D. Almeida, and P. V. Holland, Intervirology, in press). Both antigens are associated with distinct virus-like structures visible on electron microscopy (1, 2, 11, 12, 18). HBsAg in serum is associated with 20-nm spherical particles, elongated tubular particles (20-nm in width and several hundred nanometers in length), and with the outer, surface component of the complex, 42-nm Dane particles (1, 7). HBcAg is associated with the inner, 27-nm, electron-dense core component of the Dane particle seen in HBsAg-positive serum and with the 27-nm nucleocapsid found in the nuclei of hepatocytes of patients infected with HBV (1–3, 11, 12, 18).

This report describes the purification and characterization of nucleocapsid core particles from the liver of an experimentally infected chimpanzee (3; J. A. Markenson, R. J. Gerety, J. H. Hoofnagle, and L. F. Barker, J. Infect. Dis., in press), and the demonstration by immune electron microscopy that these particles are antigenically identical to the inner, core component (HBcAg) but antigenically distinct from the outer, surface component (HBsAg) of the Dane particle.

MATERIALS AND METHODS

Serological testing. HBsAg was detected by counter electrophoresis (9) and by solid-phase radioimmunoassay (Austria; Abbott Laboratories, North Chicago, Ill.) using the room temperature procedure (15). Antibody to HBsAg (anti-HBs) was assayed by counter electrophoresis, passive hemagglutination (19), and solid-phase radioimmunoassay (Austria; Abbott Laboratories, Reagents kindly provided by Abbott Laboratories.) Selected samples were also tested for anti-HBc by radioimmunoprecipitation (14). HBsAg and antibody to HBsAg (anti-HBs) were detected by a
Purification of HB,Ag and HB,Ag. HB,Ag was purified from the liver of a chimpanzee who died of pneumonia during the acute phase of an experimentally induced HBV infection (subtype ayw) and while being "immunosuppressed" with cyclophosphamide (Markenson et al., J. Infect. Dis., in press). The liver was removed at necropsy and stored at -20°C until further processing. Electron microscopy of liver sections revealed numerous 27-nm intranuclear core particles. Because of this, attempts were made to purify core particles from this liver by differential centrifugation. Liver (80 g) was suspended in 320 ml of hypotonic (0.45%) saline and homogenized in a Waring blender. The 20% (wt/vol) homogenate was clarified by centrifugation at 2,500 rpm for 30 min in an International PR-2 centrifuge (1,100 × g). The supernatant fluid was centrifuged at 25,000 rpm for 2 h in a no. 30 rotor in a Beckman L-2 ultracentrifuge (75,000 × g). The resulting pellet was resuspended in 35 ml of distilled water, and the high-speed centrifugation was repeated. The pellet was then resuspended in 35 ml of distilled water and clarified again by centrifugation at 2,500 rpm for 30 min (1,100 × g).

The resulting supernatant was used as the antigen in serological testing for both complement fixation and counter electrophoresis to detect anti-HB, To further purify this supernatant, it was layered onto a continuous CsCl gradient, density 1.2 to 1.5 g/ml. The gradient mixture was centrifuged at 22,000 rpm for 16 h in an SW25.2 rotor in a Beckman L-2 ultracentrifuge (75,000 × g). Fractions were collected dropwise from the bottom of the tube in approximately 1-ml amounts. The specific gravity of the resultant fractions was determined by using an American Optical refractometer. Each fraction was assayed for HB,Ag by radioimmunoassay and for HB,Ag by complement fixation and examined for virus-like particles under the electron microscope. Fractions containing typical 27-nm core particles were dialyzed for 1 to 3 days against phosphate-buffered saline and used to immunize animals and for immune electron microscopic studies.

HB,Ag was purified from the plasma of humans with chronic HB,Ag carriers by a series of isopycnic bandings in CsCl and rate zonal ultracentrifugations in sucrose (5). Samples were dialyzed against phosphate-buffered saline prior to use.

Immune electron microscopy. Specimens for electron microscopy were prepared by dialyzing either purified HB,Ag or HB,Ag against phosphate-buffered saline. Specimens were then diluted with an equal quantity of phosphate-buffered saline, and 0.5-ml portions of either antigen (HB,Ag or HB,Ag) were mixed with 0.02 ml of the antisera to be tested (anti-HB, anti-HB, or normal control serum). For studies of the Dane particles, purified HB,Ag was treated with Tween 80 prior to mixing with antisera (1). Antisera were tested under code, identified by species only. The antigen-antibody mixtures were allowed to react overnight at 4°C and then centrifuged for 1 h at 18,000 × g. The supernatant was discarded, and the pellet was resuspended in distilled water. One drop of this suspension was mixed with one drop of 3% phosphotungstic acid previously adjusted to pH 6 with potassium hydroxide. Mixtures were then placed on a 400-mesh Formvar-carbon-coated grid and examined under a Philip's 300 electron microscope. Electron micrographs were taken at a plate magnification of 57,000.

Antisera. Antisera to both HB,Ag (anti-HB,) and HB,Ag (anti-HB, anti-core) were prepared in guinea pigs (10; R. J. Gerety, J. H. Hoofnagle, and L. F. Barker, J. Immunol., in press). Hartley guinea pigs weighing 200 to 400 g were inoculated subcutaneously with 0.2 ml of HB,Ag or HB,Ag (diluted 1:3 to 1:10 in normal saline), emulsified in an equal volume of complete Freund adjuvant, and were boosted with a similar amount of antigen emulsified in incomplete Freund adjuvant at 14 days. The animals were bled by cardiac puncture at 21 days and at weekly intervals thereafter. In addition, antisera to both HB,Ag and HB,Ag were prepared in rabbits and rhesus monkeys using similar immunization schedules. Naturally occurring antibodies to HB,Ag and HB,Ag were obtained from chimpanzees experimentally infected with HBV and from humans at varying stages of convalescence from type B hepatitis. Anti-HB, was obtained from a chimpanzee (chimp 959) who developed this antibody 5 weeks after exposure to infectious serum and in whom HB,Ag was never detected (Markenson et al., J. Infect. Dis., in press). Chimpanzee anti-HB, was obtained from three animals (chimps 23, 920, and 921) all in the immediate convalescent period of typical, experimentally induced type B hepatitis (after the disappearance of HB,Ag and prior to the appearance of anti-HB,). Human anti-HB, was obtained from a hemophiliac patient, and human anti-HB, was obtained from a patient recently convalescent from type B hepatitis (1). Control sera consisted of prebleeds from un inoculated guinea pigs, rabbits, rhesus monkeys, and one chimpanzee (chimp 23). Human control serum consisted of plasma negative for HB,Ag, anti-HB, and anti-HB, from a person with no known history or exposure to type B hepatitis.

RESULTS

Electron microscopic examination of intact liver sections from the chimpanzee revealed many intranuclear core particles. Both the crude homogenate and pellet from the second high-speed ultracentrifugation revealed 27-nm HB,Ag particles and typical HB,Ag particles (20-nm spheres and tubules) (Fig. 1). Examination of fractions from CsCl gradients revealed typical 27-nm nucleocapsid core particles in fractions at a density from 1.30 to 1.33 g/ml and the typical HB,Ag particles in fractions at a density from 1.20 to 1.23 g/ml. Figure 2 illustrates the patterns of reactivity of HB,Ag (by radioimmunoassay) and HB,Ag (by complement fixation) found in each fraction from a
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FIG. 1. Pellet of second high-speed ultracentrifugation of the chimpanzee liver homogenate. Both HB$_A$Ag particles (20-nm spheres and tubules) and HB$_B$Ag particles (27-nm cores) are seen. ×140,000.

FIG. 2. Pattern of serological reactivity in CsCl gradient fractions of the material shown in Fig. 1. HB$_A$Ag, as detected by radioimmunoassay (O), was seen at densities (▲) of 1.20 to 1.23 g/ml, whereas HB$_B$Ag, as detected by complement fixation (●), was seen at densities of 1.30 to 1.33 g/ml. Electron microscopy revealed HB$_B$Ag particles in fractions 12 and 13 and HB$_A$Ag particles in fractions 6 and 7 of this gradient.

CsCl gradient. HB$_B$Ag reactivity was seen in fractions with densities of from 1.30 to 1.33 g/ml and HB$_A$Ag reactivity at densities of from 1.20 to 1.23 g/ml. This pattern was found repeatedly on subsequent isopycnic bandings in CsCl.

Because of the high degree of purity of the HB$_B$Ag particles, it was possible to obtain better morphological characterization than has been possible with previous serum-derived preparations. The great majority of particles were 27 nm in diameter. From the viewpoint of substructure, the core particles displayed distinct subunits (Fig. 3). Although subunits could be resolved, it was not possible to determine the exact number of capsomeres forming the capsid. In some areas, particles showed breakdown, and
in these it appeared that partially disrupted core particles coalesced to form highly aberrant forms (Fig. 4).

Results of serological testing and immune electron microscopy on antisera from guinea pigs, rabbits, rhesus monkeys, chimpanzees, and humans are summarized in Table 1. Immunization of guinea pigs with HBcAg yielded antisera that contained anti-HBc by complement fixation and counterelectrophoresis in the absence of detectable anti-HBs by passive hemagglutination, radioimmunoassay, and radioimmunoprecipitation. These antisera failed to agglutinate HBcAg particles or attach to the surface component of the Dane particle (HBsAg), but distinct antibody attachment was seen to the internal core component of the Dane particle previously released by Tween 80 treatment of the serum (1). Antisera raised in guinea pigs against purified HBcAg gave the opposite pattern of reactivities. Although a high titer of anti-HBc was found in these antisera, no anti-HBc was detectable by complement fixation or counterelectrophoresis. Furthermore, when guinea pig anti-HBc was reacted with purified, disrupted Dane particles, a large amount of antibody was seen attached to the outer surface component of the Dane particle as well as to the 20-nm spherical and tubular particles, but none was seen attached to the inner, core component of the Dane particle (Fig. 4).

Immune electron microscopy using purified core particles and purified HBcAg particles confirmed these findings. Guinea pig, rabbit, chimpanzee, and human sera positive for anti-HBc by serological tests demonstrated both agglutination and antibody attachment to purified core particles (Fig. 6). However, when these anti-HBc reactive sera were reacted with HBcAg purified from serum, no agglutination or antibody attachment was seen. Conversely, guinea pig, chimpanzee, and human anti-HBc did not react with the purified HBcAg preparations, but demonstrated agglutination and antibody attachment to HBcAg particles (Fig. 5). Control sera (prebleeds and human serum negative for antibodies to HBV antigens) showed no reactivity with either HBcAg or HBcAg particles.

DISCUSSION

The immune electron microscopic and serological data presented here reinforces the accu-
FIG. 4. Malformed HB$_A$g particles. Occasionally groups of malformed and seemingly coalesced particles were seen. These particles may have been altered and disrupted by the purification procedure. Alternatively, such particles may represent incompletely formed virus. ×298,000.

Table 1. Reactivity and titers of antisera with HB$_A$g and HB$_B$g

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Specificity</th>
<th>Species</th>
<th>HB$_A$g CF</th>
<th>HB$_A$g CEP</th>
<th>HB$_A$g IEM</th>
<th>HB$_A$g PHA</th>
<th>HB$_A$g RIA</th>
<th>HB$_A$g IEM</th>
<th>HB$_A$g RIP</th>
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<tr>
<td>Anti-HB$_C$</td>
<td>Guinea Pig</td>
<td>1:64</td>
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<td></td>
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<td>1:32</td>
<td>1:8</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Rhesus</td>
<td>1:512</td>
<td>1:32</td>
<td>NT</td>
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<td>-</td>
<td>NT</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>Chimpanzee 23</td>
<td>1:256</td>
<td>1:16</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>920</td>
<td>1:1,024</td>
<td>1:128</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
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<tr>
<td></td>
<td>921</td>
<td>1:1,024</td>
<td>1:128</td>
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<td>-</td>
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<td>Human (Pugh)</td>
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<td>+</td>
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<td>Rabbit</td>
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<td>Rhesus</td>
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<td>NT</td>
<td>-</td>
<td>-</td>
<td>NT</td>
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<td></td>
<td>Chimpanzee 23 (pre)</td>
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<td>-</td>
<td>-</td>
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<td></td>
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<td>-</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>NT</td>
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</table>

*Abbreviations: CF, complement fixation; CEP, counterelectrophoresis; IEM, immune electron microscopy; PHA, passive hemagglutination; RIA, radioimmunoassay; RIP, radioimmunoprecipitation; NT, not tested.

mulating evidence regarding the existence of two independent antigen-antibody systems associated with type B hepatitis (1, 3, 6, 10, 12). HB$_B$g particles have been purified from the liver of a chimpanzee that died during acute viral hepatitis, type B. Immune electron microscopy using this purified HB$_B$g and detergent-treated, Dane-rich HB$_B$g-positive preparations have revealed the antigenic identity of the 27-nm intranuclear core particle with the internal component of the Dane particle. Immunization of a variety of laboratory animals with
FIG. 5. Reaction seen when guinea pig antiserum against HB,Ag (anti-HB,) was mixed with a preparation of Dane particles disrupted by detergent treatment. The antibody agglutinated and attached to the surface component of the Dane particle and to the 20-nm spherical and tubular particles. Antibody was not seen attached to the internal component of the disrupted Dane particles, and when this antiserum was mixed with purified HB,Ag no agglutination was seen, but the particles appeared dispersed and uncoated as shown in Fig. 3. ×230,000.

FIG. 6. Reaction seen when chimpanzee antiserum against HB,Ag (anti-HB,) was mixed with purified HB,Ag particles. The preparation shown in Fig. 3 was reacted with chimpanzee anti-HB, naturally acquired from an HBV infection. The particles are agglutinated and are obscured by a halo of attached antibody. Similar results were obtained using antiserum from animals immunized with HB,Ag. The same antisera had no effect on HB,Ag particles. ×158,000.
this purified HBsAg preparation yielded antisera which had high titers of anti-HBc, but which were negative for anti-HBx by the most sensitive techniques (14). Also demonstrated was the ability of purified HBsAg to induce high titers of anti-HBc in the absence of anti-HBe detectable by complement fixation, counterimmunoelectrophoresis, or immune electron microscopy. Finally, the antigenic duality shown by serological techniques was confirmed visually by immune electron microscopy using both purified HBsAg and purified, disrupted serum-derived Dane particles.

The purity of the core preparation described here has allowed for a better morphological description of the core particle. Distinct subunits were visualized, although the exact number of capsomeres forming the nucleocapsid could not be determined. The presence of subunits differentiates this agent from enteroviruses and rhinoviruses which also fall in the 27-nm range in diameter. Indeed, the structure, size, and characteristics of HBV appear to place it in a virus category of its own. Failure of anti-HBc and anti-HBe to react with the recently discovered hepatitis A antigen in stools of patients acutely ill with viral hepatitis, type A, makes it unlikely that the hepatitis A and B viruses are immunologically related (8). The recent demonstration of a specific DNA-dependent DNA polymerase in the heavy core fractions from detergent-disrupted Dane particles is strong evidence that HBV is a DNA virus (13).

Immunological and electron microscope data now allows the description of the physical and antigenic made up of the hepatitis B virus. The 42-nm Dane particle is most likely the intact, infectious form of the virus and is made up of a 27-nm nucleocapsid core (HBcAg) surrounded by a lipoprotein surface component (HBsAg). The more numerous 20-nm spherical and tubular structures seen in HBcAg-positive serum probably represent the lipoprotein surface antigen (HBsAg) produced by infected hepatocytes far in excess of what is necessary to encapsulate the number of nucleocapsid forms. The exact role of HBcAg and anti-HBc in the course of type B hepatitis is not known. Hopefully, the discovery and characterization of this second antigen-antibody system in type B hepatitis will permit a clearer understanding of this still uncontrolled and sometimes fatal viral disease.

ACKNOWLEDGMENTS

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LITERATURE CITED