Hepatitis B Core Antigen: Immunology and Electron Microscopy

LEWELLYS F. BARKER, JUNE D. ALMEIDA, JAY H. HOOFNAGLE, ROBERT J. GERETY, DANIEL R. JACKSON, AND PHILIP P. McGRATH

Division of Blood and Blood Products, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland, and the Wellcome Research Laboratories, Beckenham, Kent, England

Received for publication 20 August 1974

Two distinct viral antigens are associated with the hepatitis B virus: the hepatitis B surface antigen (HB_sAg, Australia antigen) and the hepatitis B core antigen (HB_cAg). HB_sAg, purified from the serum of asymptomatic human HB_sAg carriers, and HB_cAg, 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 HB_sAg reacted with the outer, surface component of the Dane particle and with the 20-nm spherical and tubular particles present in HB_sAg-positive serum, but not with the internal component of the Dane particle or with purified HB_cAg particles. Antisera raised against purified HB_cAg particles reacted with the internal component of the Dane particle and with HB_cAg, but not with the surface of the Dane particle or with the 20-nm spherical and tubular particles associated with HB_sAg. Purified HB_cAg 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 (HB_cAg) surrounded by an antigenically and morphologically distinct lipoprotein surface component (HB_sAg).

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 (HB_sAg, Australia antigen) and the hepatitis B core antigen (HB_cAg) (1, 3, 6, 10). HB_sAg, 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). HB_cAg, 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 HB_sAg-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). HB_sAg 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). HB_cAg is associated with the inner, 27-nm, electron-dense core component of the Dane

particle seen in HB_sAg-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 (HB_cAg) but antigenically distinct from the outer, surface component (HB_sAg) of the Dane particle.

MATERIALS AND METHODS

Serological testing. HB_sAg was detected by counterelectrophoresis (9) and by solid-phase radioimmunoassay (Ausria; Abbott Laboratories, North Chicago, Ill.) using the room temperature procedure (15). Antibody to HB_sAg (anti-HB_s) was assayed by counterelectrophoresis, passive hemagglutination (19), and solid-phase radioimmunoassay (Ausab; Abbott Laboratories [Reagents kindly provided by Abbott Laboratories.]). Selected samples were also tested for anti-HB_c by radioimmunoprecipitation (14). HB_cAg and antibody to HB_cAg (anti-HB_c) were detected by a

microtiter complement-fixation test (10) and by counterelectrophoresis (12).

Purification of HB_eAg and HB_sAg. HB_eAg 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 ($1,100 \times g$). The supernatant fluid was centrifuged at 25,000 rpm for 2 h in a no. 30 rotor in a Beckman L-2 ultracentrifuge ($75,000 \times g$). 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 ($1,100 \times g$).

The resulting supernatant was used as the antigen in serological testing for both complement fixation and counterelectrophoresis to detect anti-HB_e. 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 ($75,000 \times g$). 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_eAg by radioimmunoassay and for HB_sAg 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_eAg was purified from the plasma of human chronic HB_eAg 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_eAg or HB_sAg 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_eAg or HB_sAg) were mixed with 0.02 ml of the antisera to be tested (anti-HB_e, anti-HB_s, or normal control serum). For studies of the Dane particles, purified HB_eAg 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 $18,000 \times g$. 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_eAg (anti-HB_e) and HB_sAg (anti-HB_s, 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_eAg or HB_sAg (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_eAg and HB_sAg were prepared in rabbits and rhesus monkeys using similar immunization schedules. Naturally occurring antibodies to HB_eAg and HB_sAg were obtained from chimpanzees experimentally infected with HBV and from humans at varying stages of convalescence from type B hepatitis. Anti-HB_e was obtained from a chimpanzee (chimp 959) who developed this antibody 5 weeks after exposure to infectious serum and in whom HB_eAg was never detected (Markenson et al., *J. Infect. Dis.*, in press). Chimpanzee anti-HB_e 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_eAg and prior to the appearance of anti-HB_e). Human anti-HB_e was obtained from a hemophiliac patient, and human anti-HB_s was obtained from a patient recently convalescent from type B hepatitis (1). Control sera consisted of prebleeds from uninoculated guinea pigs, rabbits, rhesus monkeys, and one chimpanzee (chimp 23). Human control serum consisted of plasma negative for HB_eAg, anti-HB_e, and anti-HB_s 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_eAg particles and typical HB_sAg 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_eAg particles in fractions at a density from 1.20 to 1.23 g/ml. Figure 2 illustrates the patterns of reactivity of HB_eAg (by radioimmunoassay) and HB_sAg (by complement fixation) found in each fraction from a

FIG. 1. Pellet of second high-speed ultracentrifugation of the chimpanzee liver homogenate. Both HB_sAg particles (20-nm spheres and tubules) and HB_cAg particles (27-nm cores) are seen. $\times 140,000$.

FIG. 2. Pattern of serological reactivity in $CsCl$ gradient fractions of the material shown in Fig. 1. HB_sAg , as detected by radioimmunoassay ($\square$), was seen at densities ($\blacktriangle$) of 1.20 to 1.23 g/ml, whereas HB_cAg , as detected by complement fixation ($\bullet$), was seen at densities of 1.30 to 1.33 g/ml. Electron microscopy revealed HB_sAg particles in fractions 12 and 13 and HB_cAg particles in fractions 6 and 7 of this gradient.

$CsCl$ gradient. HB_cAg reactivity was seen in fractions with densities of from 1.30 to 1.33 g/ml and HB_sAg 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_cAg 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

FIG. 3. Purified HB_cAg particles seen at a density of 1.32 in a CsCl gradient shown in Fig. 2. The purity of the preparation and morphology of the core particles is shown. The average diameter of the particles was 27 nm, with occasional particles only 20 nm in diameter. $\times 230,000$. The insert shows a single core particle at a higher magnification ($\times 350,000$). This demonstrates the distinctive characteristic of the HB_cAg particle, the subunits which comprise the particle. It was not possible to establish the exact number of subunits present.

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 HB_cAg yielded antisera that contained anti-HB_c by complement fixation and counterelectrophoresis in the absence of detectable anti-HB_s by passive hemagglutination, radioimmunoassay, and radioimmunoprecipitation. These antisera failed to agglutinate HB_sAg particles or attach to the surface component of the Dane particle (HB_sAg), 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 HB_sAg gave the opposite pattern of reactivities. Although a high titer of anti-HB_s was found in these antisera, no anti-HB_c was detectable by complement fixation or counterelectrophoresis. Furthermore, when guinea pig anti-HB_s 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. 5).

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

DISCUSSION

The immune electron microscopic and serological data presented here reinforces the accu-

FIG. 4. Malformed HB_cAg 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. $\times 238,000$.

TABLE 1. Reactivity and titers of antisera with HB_cAg and HB_sAg

Antisera		Antigens ^a						
Specificity	Species	HB_cAg			HB_sAg			
		CF	CEP	IEM	PHA	RIA	IEM	RIP
Anti- HB_c	Guinea Pig	1:64	1:16	+	—	—	—	—
	Rabbit	1:32	1:8	+	—	—	—	—
	Rhesus	1:512	1:32	NT	—	—	NT	—
	Chimpanzee 23	1:256	1:16	+	—	—	—	—
	920	1:1,024	1:128	+	—	—	—	NT
	921	1:1,024	1:128	+	—	—	—	NT
	Human (Pugh)	1:512	1:64	+	—	—	—	NT
Anti- HB_s	Guinea Pig	—	—	—	1:128,000	+	+	—
	Rabbit	—	—	NT	1:64,000	+	NT	—
	Rhesus	—	—	NT	1:8,192	+	NT	—
	Chimpanzee 959	—	—	—	1:64,000	+	+	—
	Human	—	—	NT	1:256,000	+	NT	—
Control	Guinea Pig	—	—	—	—	—	—	—
	Rabbit	—	—	NT	—	—	NT	—
	Rhesus	—	—	NT	—	—	NT	—
	Chimpanzee 23 (pre)	—	—	—	—	—	—	—
	Human	—	—	NT	—	—	NT	—

^a 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_cAg particles have been purified from the liver of a chimpanzee that died during acute viral hepatitis, type B. Immune electron mi-

croscopy using this purified HB_cAg and detergent-treated, Dane-rich HB_sAg -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_cAg (anti- HB_c) 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_cAg no agglutination was seen, but the particles appeared dispersed and uncoated as shown in Fig. 3. $\times 230,000$.

FIG. 6. Reaction seen when chimpanzee antiserum against HB_cAg (anti- HB_c) was mixed with purified HB_sAg particles. The preparation shown in Fig. 3 was reacted with chimpanzee anti- HB_c , 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_cAg . The same antisera had no effect on HB_sAg particles. $\times 158,000$.

this purified HB_cAg preparation yielded antisera which had high titers of anti-HB_c, but which were negative for anti-HB_s by the most sensitive techniques (14). Also demonstrated was the ability of purified HB_sAg to induce high titers of anti-HB_s in the absence of anti-HB_c detectable by complement fixation, counter-electrophoresis, or immune electron microscopy. Finally, the antigenic duality shown by serological techniques was confirmed visually by immune electron microscopy using both purified HB_sAg 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-HB_s and anti-HB_c 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 (HB_cAg) surrounded by a lipoprotein surface component (HB_sAg). The more numerous 20-nm spherical and tubular structures seen in HB_sAg-positive serum probably represent the lipoprotein surface antigen (HB_sAg) produced by infected hepatocytes far in excess of what is necessary to encapsulate the number of nucleocapsid forms. The exact role of HB_cAg and anti-HB_c 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

We thank Audrey J. Shawver and David E. Gilbert for technical assistance and Robert Purcell for testing selected

samples by radioimmunoprecipitation.

LITERATURE CITED

- Almeida, J. D., D. Rubenstein, and E. J. Stott. 1971. New antigen-antibody system in Australia-antigen-positive hepatitis. *Lancet* **2**:1225-1227.
- Almeida, J. D., A. P. Waterson, J. M. Trowell, and G. Neale. 1970. The finding of virus-like particles in two Australia-antigen positive human livers. *Microbios* **2**:145-153.
- Barker, L. F., F. V. Chisari, P. P. McGrath, D. W. Dalgard, R. L. Kirschstein, J. D. Almeida, T. S. Edgington, D. G. Sharp, and M. R. Peterson. 1973. Transmission of viral hepatitis, type B, to chimpanzees. *J. Infect. Dis.* **127**:648-662.
- Blumberg, B. S., A. I. Sutnick, and W. T. London. 1968. Hepatitis and leukemia. Their relation to Australia antigen. *Bull. N.Y. Acad. Med.* **44**:1566-1586.
- Bond, H. E., and W. T. Hall. 1972. Separation and purification of Hepatitis-associated antigen into morphologic types by zonal ultracentrifugation. *J. Infect. Dis.* **125**:263-268.
- Brzosko, W. J., K. Madalinski, K. Krawczynski, and A. Nowoslawski. 1973. Duality of hepatitis B antigen and its antibody. I. Immunofluorescence studies. *J. Infect. Dis.* **127**:424-428.
- Dane, D. S., C. H. Cameron, and M. Briggs. 1970. Virus-like particles in serum of patients with Australia-antigen associated hepatitis. *Lancet* **1**:695-698.
- Feinstone, S. M., A. Z. Kapikian, and R. H. Purcell. 1973. Hepatitis A: detection by immune electron microscopy of a virus-like antigen associated with acute illness. *Science* **182**:1026-1028.
- Gocke, D. J., and C. Howe. 1970. Rapid detection of Australia antigen by counterimmunoelectrophoresis. *J. Immunol.* **104**:1031-1034.
- Hoofnagle, J. H., R. J. Gerety, and L. F. Barker. 1973. Antibody to hepatitis-B-virus core in man. *Lancet* **2**:869-873.
- Huang, S. N. 1972. Hepatitis-associated antigen hepatitis. An electron microscopic study of virus-like particles in liver cells. *Amer. J. Pathol.* **64**:453-470.
- Huang, S. N., and V. Groh. 1973. A study on antibodies produced with liver tissue containing Australia antigen and virus-like particles. *Lab. Invest.* **29**:743-750.
- Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson. 1973. DNA polymerase associated with human hepatitis B antigen. *J. Virol.* **12**:995-1005.
- Lander, J. J., H. J. Alter, and R. H. Purcell. 1971. Frequency of antibody to hepatitis-associated antigen as measured by a new radioimmunoassay technique. *J. Immunol.* **106**:1166-1171.
- Ling, C. M., and L. R. Overby. 1972. Prevalence of hepatitis B virus antigen as revealed by direct radioimmuno assay with I¹²⁵ antibody. *J. Immunol.* **109**:834-841.
- Lipman, M. B., W. J. Hierholzer, Jr., and A. Schluederberg. 1973. Isolation of cores from hepatitis B Dane particles. *J. Infect. Dis.* **128**:664-667.
- Prince, A. M. 1968. An antigen detected in the blood during the incubation period of serum hepatitis. *Proc. Nat. Acad. Sci. U.S.A.* **60**:814-821.
- Stannard, L. M., A. Polson, A. Kipps, and J. R. Parker. 1973. Two antigen-antibody systems in serum hepatitis: preparation of immunoglobulins and their reaction with Australia antigen and particles in liver cell homogenates. *Microbios* **8**:87-100.
- Vyas, G. N., and N. R. Shulman. 1970. Hemagglutination assay for antigen and antibody associated with viral hepatitis. *Science* **170**:332-333.