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Major Polypeptide of Duck Hepatitis B Surface Antigen Particles

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The 40- to 50-nm pleomorphic particles found in the sera of domestic Pekin ducks infected with duck hepatitis B virus were purified by rate zonal and isopycnic centrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic polypeptide analysis of these particles, called duck hepatitis B surface antigen particles, revealed the major component to be a single 17,500-dalton polypeptide. This result is in contrast to polypeptide analyses of the surface antigens of related mammalian viruses, including hepatitis B, in which a major doublet of polypeptides is seen with molecular weights ranging from 23,000 to 29,000. Tryptic maps of 17,500-dalton polypeptide resembled that of the major non-glycosylated polypeptide of the *adw* subtype of hepatitis B surface antigen. A serological assay for antibody to the purified duck virus particles is also described.

Duck hepatitis B virus (DHBV) was discovered in 1980 first in sera of ducks from the People's Republic of China (J. Summers, W. T. London, T. T. Sun, and B. S. Blumberg, unpublished data) and shortly thereafter in ducks of Chinese ancestry in commercial flocks in the United States (9). DHBV sufficiently resembles the known members of the hepatitis B, or hepadna, family of viruses (hepatitis B virus [HBV], woodchuck hepatitis virus [WHV] [18], and ground squirrel hepatitis virus [GSHV] [7]) to be classified with them. The DHB virion is of similar size (40 nm) and has a similar appearance in an electron microscope (9). Virion DNA is found to be partially single stranded, and a DNA polymerase in the virus can repair the single-stranded region of the DNA (9). As in HBV, a protein is covalently attached to the 5′ end of the long or complete strand of viral DNA (11). Furthermore, DHBV replicates primarily in the liver (10), as do the mammalian hepadnaviruses (4, 15, 18).

DHBV differs from the mammalian viruses in several ways. DHBV clearly replicates in the pancreas, as well as the liver (10), whereas GSHV and WHV appear to replicate in the liver only (4, 18). Recent work, however, suggests that HBV may also replicate in the pancreas (16). The DHB virion DNA is 3,000 base pairs in length compared with DNA of 3,182 to 3,300 base pairs in the mammalian viruses (17). The buoyant density of DHBV is 1.16 g/ml compared with 1.225 to 1.24 g/ml for the mammalian viruses (9). The core or nucleocapsid structure of the DHB virion appears to have spikes, whereas those of the other viruses do not (9). During infections with the mammalian viruses, the most numerous viral forms circulating in the blood are incomplete viral coat particles without viral DNA, DNA polymerase, or nucleocapsid. These forms are called surface antigen particles as they appear to bear the same antigenic determinants as complete virions in studies of HBV (reviewed in reference 15). Numerous particles thought to be analogous to the surface antigen particles of the mammalian viruses have been observed in sera of DHBV-infected ducks (9), and we will refer to these as duck hepatitis B surface antigen (DHBsAg) particles. The surface antigen particles of HBV, WHV, and GSHV have buoyant densities of 1.18 to 1.20 g/ml in CsCl and diameters of 15 to 25 nm, whereas the most numerous particles of DHBV are reported to have a significantly lower buoyant density of 1.14 g/ml and are more pleomorphic in size, ranging from 40 to 60 nm (17).

The polypeptides of the mammalian surface antigen particles have been analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), tryptic peptide mapping, and serological techniques. The characteristic polypeptide pattern of the mammalian virus surface antigen particles, as determined by SDS-PAGE,

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is a major pair of polypeptides, one appearing to be a glycosylated version of the other in the case of HBsAg (5, 13, 14). A variety of higher-molecular-weight polypeptides which cross-react serologically are also present (3). In HBsAg particles, the major polypeptides have apparent molecular weights of 25,000 and 29,000, whereas those of WHV and GSHV are ca. 23,000 and 27,000, respectively (3). There is considerable tryptic peptide map homology between surface antigen polypeptides of HBV, GSHV, and WHV (3). Although the mammalian surface antigen particles are all serologically cross-reacting, the particles associated with DHBV infection do not bind antibody to the mammalian virus antigens (17).

In this report, we describe the purification of the pleomorphic particles in sera of DHBV-infected ducks, the analysis of their polypeptides by SDS-PAGE and tryptic peptide mapping, and a comparison of the results with those obtained with the mammalian virus surface antigens. Serological assays for antibody to the purified duck virus particles are also described.

MATERIALS AND METHODS

Isolation of DHBsAg particles. The sera of yearling Pekin ducks were tested for the presence of DHBV first by assaying for virion DNA polymerase after pelleting the virus, carrying out a DNA polymerase reaction with ^{32}P-labeled nucleotide, and detecting the radioactive virion DNA by autoradiography after agarose gel electrophoresis (7). Later, the virus was detected by dot blot hybridization, using as a probe DHBV DNA cloned in Charon 16A by William Mason. For the preparation of DHBsAg particles from sera of animals with a high titer of virus, 4 ml or more of serum was layered over 4 ml of 10% (wt/vol) sucrose containing TNE (10 mM Tris-hydrochloride, 0.1 M NaCl, and 5 mM EDTA) in a 50 Ti rotor and centrifuged at 45,000 rpm and 4°C for 16 h. The pellet was suspended in 50 μl of TNE, made up to a volume of 1 ml and a density of 1.2 g/ml with a CsCl solution, and layered over a discontinuous gradient of 0.5 ml of 1.4-g/ml and 0.5 ml of 1.25-g/ml CsCl in TNE. The tube was then topped with 1.1-g/ml CsCl in TNE and centrifuged for 48 h at 45,000 rpm and 10°C in an SW60 rotor. Fractions collected from the bottom of the tube were tested for absorbance at 280 nm, solution density, and the presence of virus particles by electron microscopy. Fractions in the density range of 1.15 to 1.20 g/ml contained virus particles. These fractions were pooled and centrifuged in an SW60 rotor at 45,000 rpm and 10°C for 48 h. Fractions were tested as before and stored for further use at 4°C.

Electron microscopy. Samples from CsCl gradients were prepared by diluting 20 μl of each 200-μl fraction into 0.5 ml of TNE with 1% bovine serum albumin (BSA) and centrifuging overnight at 24,000 rpm and 4°C in a type 25 rotor. Each pellet was suspended in 10 μl of water and stained and examined as previously described (7).

Radiolabeling of DHBsAg and HBsAg. Purified preparations of DHBsAg and HBsAg particles were radiolabeled with ^{125}I, using Iodogen (Pierce Chemical Co.), by the method of Gerlich et al. (5). Free ^{125}I was removed from ^{125}I-labeled HBsAg preparations by chromatography in Sepharose CL-6B with 1% BSA. ^{125}I was removed from ^{125}I-labeled DHBsAg by dialysis against TNE, as heavy losses of ^{125}I-labeled DHBsAg occurred when chromatography was used. Labeling with the Bolton-Hunter reagent was as described by Gerlich et al. (5).

SDS-PAGE and polypeptide mapping. Antigen preparations were analyzed by SDS-PAGE after heating at 100°C for 3 min in 0.07 M Tris-hydrochloride (pH 6.8)–11% glycerol–0.0015% bromophenol blue–3% SDS–10% 2-mercaptoethanol as previously described (8). Gels with non-radiolabeled proteins were stained with a commercial silver stain (Bio-Rad Laboratories), following the recommendations of the supplier, or with Coomassie brilliant blue as previously described (8).

Tryptic peptide mapping, described by Gerlich et al. (5), was done on cellulose thin-layer plates (Brinkmann Instruments, Inc.) and employed electrophoresis in the first dimension and ascending chromatography in the second dimension. Tryptic peptides containing ^{125}I were detected by autoradiography.

Serological assays for anti-DHBsAg. Serological testing for antibody to DHBsAg was done by solid-phase radioimmunoassay, using Falcon flexible microtiter plates (Falcon Plastics). Wells were coated with 50 μl of purified DHBsAg at 1 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Plates were washed with 0.1% BSA in PBS. A total of 25 or 50 μl of serum to be tested was incubated in the coated wells overnight at 4°C, the plates were washed as before, and 60,000 cpm of ^{125}I-labeled monoclonal mouse anti-mouse immunoglobulin G2a (IgG2a) heavy chain (12) was incubated in the wells overnight at 4°C. Individual wells were cut from the plates and counted in a Beckman Gamma 4000 (Beckman Instruments, Inc.). In blocking experiments, 25 μl of antiserum was mixed with 25 μl of test serum before addition to the microtiter wells.

Antisera. Rabbit no. 1 was injected intramuscularly with DHBsAg particles purified from duck serum by the method described above. Boosts of 1.6 μg of purified DHBsAg in TNE were given at 2 and 4 weeks. The anti-DHBsAg serum from rabbit no. 2 was made and generously given to us by William Mason.

Four BALB/c mice were each injected intraperitoneally with particles centrifuged from 0.1 ml of virus-bearing duck serum as above and boosted at 3 and 8 weeks with 1.6 μg of purified DHBsAg.

RESULTS

Purification of DHBsAg particles. DHBsAg particles were purified by pelleting through 10% sucrose, followed by centrifugation to equilibrium in two or more CsCl gradients as described above. The two CsCl centrifugation steps of DHBsAg particle purification from the serum of animal Y26 are shown in Fig. 1. In the first gradient (Fig. 1A), particles detected by electron microscopy were found at buoyant densities ranging from 1.15 to 1.25 g/ml, with the maximum concentration found at 1.17 g/ml. A peak of

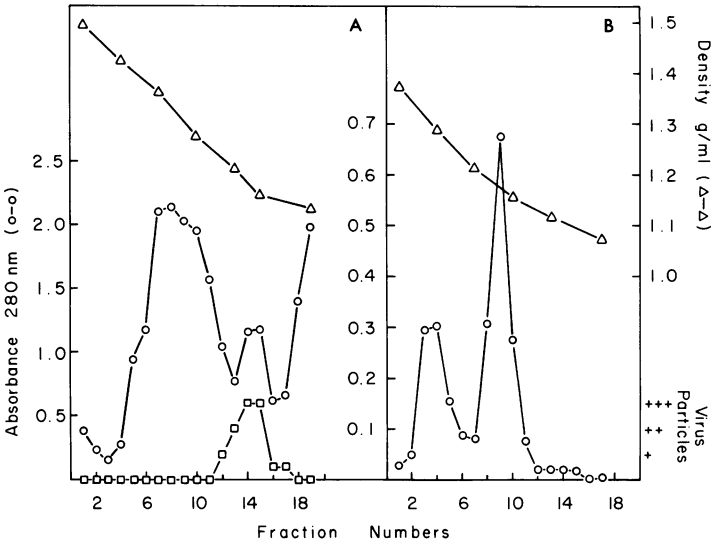

FIG. 1. Purification of DHBsAg particles from serum by CsCl density gradient centrifugation. After pelleting of particles from the serum of duck Y26 through 10% sucrose, two successive CsCl gradient purification steps were carried out as described in the text. The fractions were examined for absorbance at 280 nm after a 10-fold dilution with TNE (A) and after a twofold dilution with TNE (B), and densities were estimated by reading the refractive indices. The presence of virus particles (□) was monitored as described in the text and rated as follows (number of particles per milliliter): +, ca. 10^6; ++, 10^7; and +++, 10^8.

absorbance at 280 nm is also present at the same density range. The majority of protein representing normal duck serum proteins was found at a buoyant density of 1.3 g/ml and was present in gradients containing uninfected duck serum taken through similar steps of purification. In ducks with lower titers of DHBsAg particles, the absorbance peak at 1.17 g/ml in the first CsCl gradient was absent or visible as a shoulder only. Fractions 13 to 16 were pooled, brought to a buoyant density of 1.2 g/ml with CsCl, and centrifuged again to equilibrium. The optical densities of fractions collected from this gradient are shown in Fig. 1B, where the predominant peak of absorbance at 280 nm is at a buoyant density of 1.175 g/ml. Uninfected animals had no 280-nm absorbing material in their sera at this buoyant density.

Electron microscopy of CsCl-purified DHBsAg particles. Electron microscopic examination of fractions with a density of 1.175 g/ml from the second CsCl gradient diluted fivefold with PBS revealed numerous empty irregular-shaped particles with diameters ranging from 42 to 55 nm (Fig. 2a). Incubation of these particles with a 1:5 dilution of rabbit antiserum (rabbit no. 2) raised to DHBsAg particles by William Mason for 1 h at 37°C caused the formation of aggregates of heavily coated particles (Fig. 2b). Incubation of the particles with the antiserum of a rabbit that had been injected and boosted with material pelleted from normal duck serum, as in the initial purification step of the DHBsAg particles, failed to aggregate the particles but appeared to have coated them (Fig. 2c).

SDS-PAGE of DHBsAg. SDS-PAGE of radioiodinated DHBsAg preparations, including fraction 9 of the experiment shown in Fig. 1B, revealed a major band at 17,500 daltons, with additional material migrating faster than 14,400 daltons (Fig. 3, lane b). In contrast, the pattern of purified radioiodinated HBsAg polypeptides, shown in Fig. 3, lane a, is a pair of major bands at 25,000 and 29,000 daltons (the band at 66,200 daltons represents human serum albumin, a contaminant of this experiment). Serum not containing DHBV was subjected to the same purification procedure, and the 1.175-g/ml density fraction was radioiodinated. Although very little incorporation was observed, the resulting radiolabeled material was analyzed. No 17,500-dalton band was detectable; only a small amount of ^{125}I was observed at the top of the stacking gel (Fig. 3, lane c).

The pattern of DHBsAg polypeptides was also analyzed by silver staining after SDS-PAGE. The 17,500-dalton band appeared as the predominant polypeptide with this method of detection (Fig. 4, lane b), with very little material appearing below 14,400 daltons. The very-fast-migrating radioiodinated material was thus probably made during the iodination procedure and was not native to the DHBsAg particles. A much less intense band appeared at 35,800 daltons and may

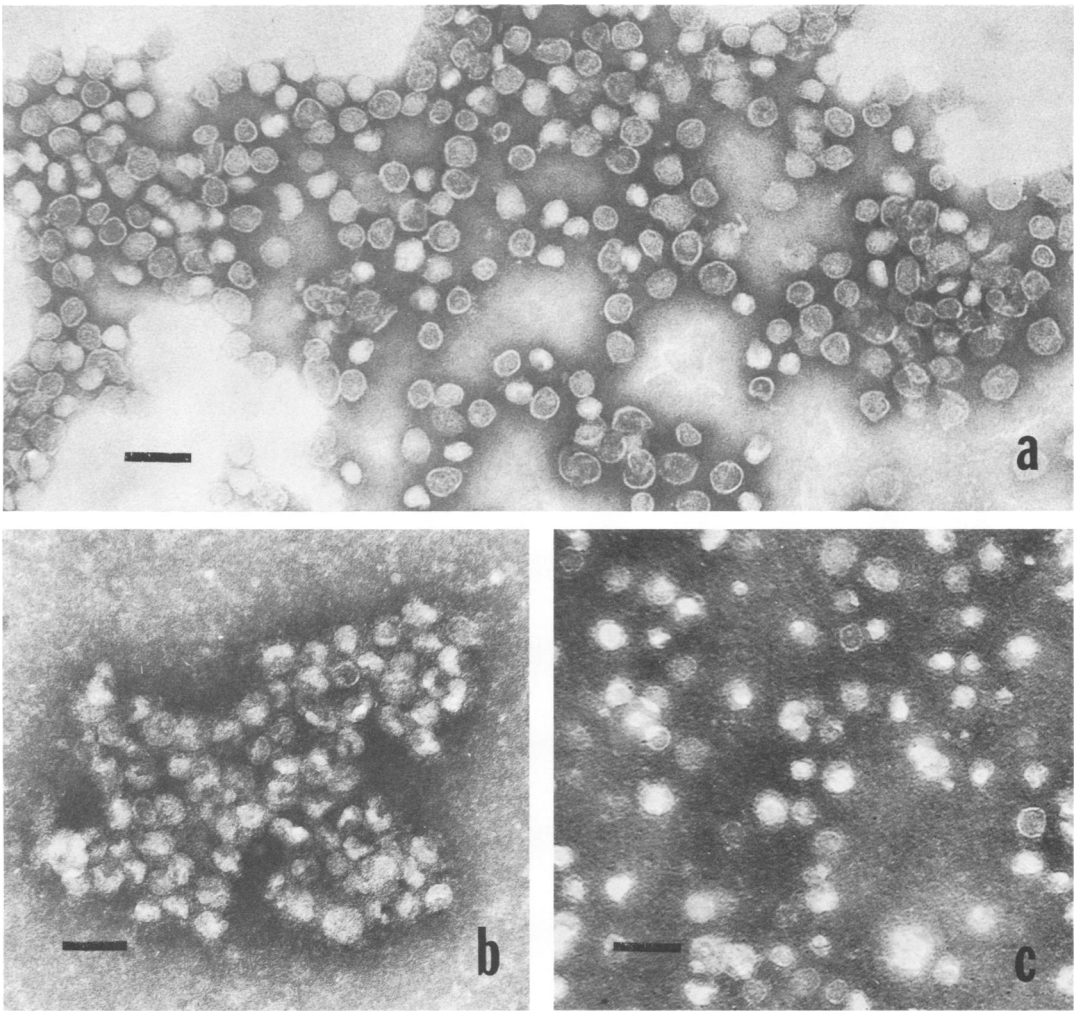

FIG. 2. Electron micrographs of purified particles from DHBV-containing duck serum. Purified DHBsAg particles were incubated for 1 h at 37°C with (a) PBS, (b) a fivefold dilution of rabbit anti-DHBsAg, or (c) a fivefold dilution of rabbit anti-normal duck serum, spotted onto grids, stained, and examined as described in the text. Bar = 100 nm.

represent a dimer of the 17,500-dalton polypeptide. Coomassie blue staining revealed only a 17,500-dalton band (data not shown). The polypeptides of purified GSHsAg particles are shown in Fig. 4, lane a, with the characteristic major double bands of polypeptides of the mammalian virus surface antigen at 24,000 and 27,000 daltons.

Tryptic peptide mapping of the 17,500-dalton DHBsAg polypeptide and comparison with the major polypeptide of HBsAg *adw* particles. To further characterize the 17,500-dalton DHBsAg polypeptide and to detect any similarity to mammalian surface antigen polypeptides, tryptic peptide maps of the major polypeptides of two preparations of DHBsAg were generated and compared with mappings of the mammalian viral antigens. Tryptic peptide maps derived from Bolton-Hunter-labeled polypeptides have a relatively large number of spots due to the chemical heterogeneity introduced by the acylation procedure, as described in previous work in which this technique was used to characterize the mammalian virus surface antigens (3, 5). The heterogeneity introduced by this labeling method is quite reproducible since different radiolabeled preparations of a protein give identical peptide maps. The pattern of spots in the peptide maps of the two duck surface antigen preparations (one from California ducks and one from the serum of an East Coast DHBV-infected duck given to us by William Mason) were similar (Fig.

5A and B). These also resembled the pattern repeatedly obtained with the 24,000-dalton protein of HBsAg preparations from the *adw* or *adr* subtype (Fig. 5C) more than did any other mammalian virus antigen previously studied (3). Each peptide map of the 17,500-dalton DHBsAg polypeptide shared approximately 50% of its spots with the other. In contrast, a similar comparison of the peptide maps of the major polypeptides from three HBsAg *adw* subtypes (data not shown) revealed 78% homology, suggesting that the DHBsAg preparations analyzed, coming from widely separated regions of the country, may represent genetic variants. Like a map of the major polypeptides of GSHsAg and WHsAg (3), the peptide map of the major DHBsAg component contained a significantly greater number of spots than did the major polypeptide of most HBsAg preparations of the *adw* or *adr* subtype.

Serological testing for anti-DHBsAg. Purified DHBsAg was used in assays for anti-HBsAg. For detection of antibody to DHBsAg raised in rabbits and mice, purified antigen was bound to microtiter wells; then antiserum was incubated in the wells. When ^{125}I-labeled DHBsAg was used to detect the bound antibody, *P/N* values (see Table 1) were low and the assay was quite variable. A more satisfactory assay was obtained for mouse anti-DHBs by replacing radiolabeled DHBsAg with ^{125}I-labeled monoclonal anti-mouse IgG2a heavy chain. *P/N* values in this assay reached 155, and background levels of nonimmune sera remained at or below 100 cpm (Table 1). This assay appeared to be equally specific, with blocking by DHBV-bearing sera reaching 87% (Table 2). No blocking was noted with mouse no. 1 in this experiment, because the antibody titer of undiluted serum exceeded the blocking capability of the DHBV-positive serum.

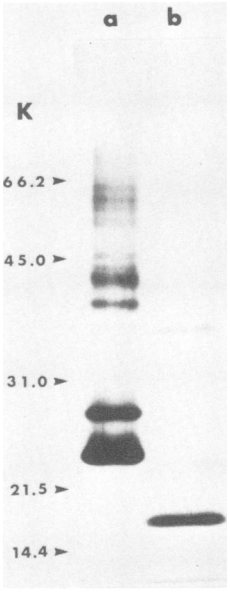

FIG. 4. SDS-PAGE analysis of DHBsAg and GSHsAg particles. Purified preparations of GSHsAg (a) and DHBsAg (b) were analyzed by SDS-PAGE and silver staining. Numbers at the left are the molecular weights of the protein standards expressed in thousands (K).

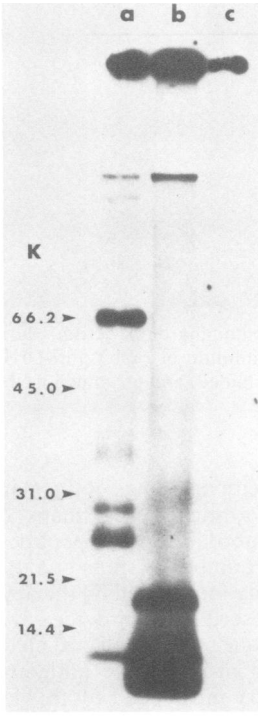

FIG. 3. SDS-PAGE analysis of DHBsAg and HBsAg particles. Purified preparations of HBsAg (a) and DHBsAg (b) were radioiodinated by the Iodogen method and analyzed by SDS-PAGE and autoradiography. Numbers at the left represent the molecular weights of protein standards (Bio-Rad) expressed in thousands (K).

DISCUSSION

We have isolated, from sera, the abundant incomplete 40- to 50-nm particles accompanying infection of Pekin ducks with DHBV (DHBsAg particles). In our preparations, from both East and West Coasts, these particles were found at a higher buoyant density in CsCl (1.175 g/ml) and had a less convoluted appearance than that

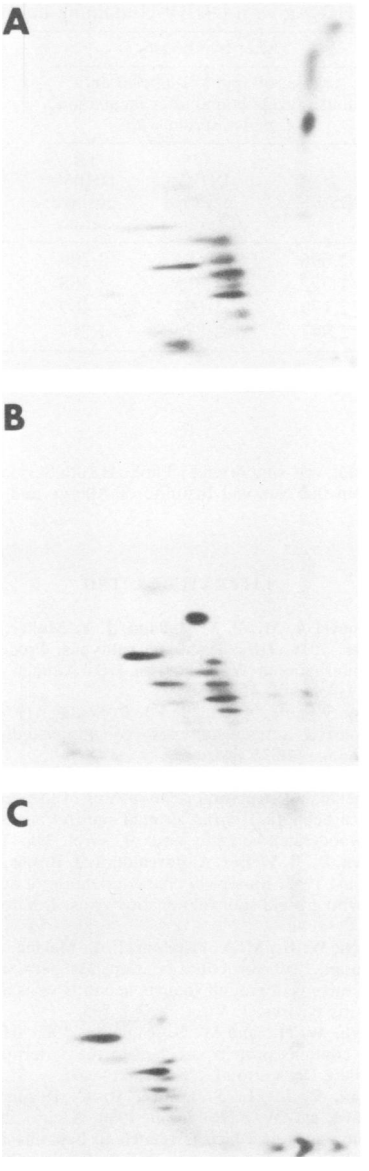

FIG. 5. Tryptic ^{125}I-labeled peptide maps of the major polypeptides of DHBsAg and HBsAg *adw* radioiodinated by the Bolton-Hunter method. (A) ^{125}I-labeled peptide map of the 17,500-dalton polypeptide of DHBsAg isolated from a California duck; (B) ^{125}I-labeled peptide map of the 17,500-dalton polypeptide of DHBsAg isolated from a duck from a flock in the eastern part of the United States; (C) ^{125}I-labeled peptide map of the 25,000-dalton polypeptide of HBsAg *adw*.

described by Mason and co-workers (9). These differences are possibly due to use of a chelating agent (EDTA) in our solutions. The DHBsAg particles were nevertheless of a buoyant density lower than that of either of HBsAg (1.190 g/ml) or GSHsAg (1.183 g/ml) particles prepared in a similar fashion with the same reagents (3).

Analysis of the polypeptides of DHBsAg by SDS-PAGE revealed only one major polypeptide of ca. 17,500 daltons instead of the major pair of polypeptides of larger size seen with the three mammalian viruses. In HBsAg particles, there is evidence that the 29,000-dalton polypeptide is a glycosylated version of the 25,000-dalton polypeptide (13), and it is thought that the 27,000-dalton polypeptides of GSHsAg and WHsAg are probably glycosylated versions of the 23,000-dalton polypeptide, as their tryptic maps are very similar (M. A. Feitelson, P. L. Marion, and W. S. Robinson, Virology, in press). It is not yet known whether the DHBsAg particle polypeptide is glycosylated. The smaller size of the duck virus polypeptide compared with the nonglycosylated polypeptide of the mammalian viruses parallels the smaller size of the duck virus genome compared with those of the mammalian viruses. However, as the gene structure of the DHBV genome is not yet known, the smaller size of the DHBsAg protein could be due to a smaller open reading frame or to the processing of a larger polypeptide.

The tryptic maps of the 17,500-dalton polypeptide of DHBsAg indicate a genetic variation between the viruses found in ducks from various geographic locations. This would not be surprising in view of the known variation of HBsAg (1, 2). Whether this variation in DHBsAg polypeptide sequence is associated with antigenic variation awaits more extensive serological analysis. We observed a resemblance of the DHBsAg 17,500-dalton polypeptide maps to those of the HBsAg *adw* major nonglycosylated polypeptide. This may be fortuitous or due to the similarity of

TABLE 1. Detection of mouse antibody to DHBsAg by solid-phase radioimmunoassay

Material assayed	Time (wk) after initial injection	Avg cpm of ^{125}I-labeled anti-mouse IgG2a bound	P/N^a
PBS/BSA[b]		19	
Mouse no. 1	0	62	
Mouse no. 2	0	31	
Mouse no. 3	0	109	
Mouse no. 4	0	46	
Mouse no. 1	11	2,696	43.5
Mouse no. 2	11	1,772	57.2
Mouse no. 3	11	2,179	20.0
Mouse no. 4	11	2,947	64.1

[a] P = counts per minute of ^{125}I-anti-mouse IgG2a bound after incubation with mouse antiserum. N = counts per minute of ^{125}I-anti-mouse IgG2a bound after incubation with preinoculation mouse serum.

[b] PBS/BSA, BSA (0.1%) in PBS.

TABLE 2. Blocking of a radioimmunoassay for mouse anti-DHBsAg with DHBV-containing duck serum

Animal no.	After one boost			% Blocking[a]	After two boosts			% Blocking
	Avg no. of cpm ^{125}I-labeled anti-mouse IgG2a bound after incubation of mouse serum with:				Avg no. of cpm ^{125}I-labeled anti-mouse IgG2a bound after incubation of mouse serum with:			
	(1) PBS/BSA[b]	(2) DHBV-negative serum	(3) DHBV-positive serum		(1) PBS/BSA	(2) DHBV-negative serum	(3) DHBV-positive serum	
1	ND[c]	2,715	2,371	13	2,696	2,594	3,209	0
2	ND	1,815	243	87	1,772	2,015	368	82
3	ND	1,901	369	81	2,179	1,694	469	72
4	ND	2,358	447	81	2,947	2,870	1,264	56

[a] Percent blocking = $\{[(2) - (3)]/(2)\} \times 100$.
[b] PBS/BSA, BSA (0.1%) in PBS.
[c] ND, Not determined.

structure engendering similar amounts of charge and hydrophobicity, or there may be some actual amino acid sequence homology. No serological cross-reaction with the mammalian virus antigens has been observed by us or others (17). We also have not detected any cross-hybridization of DHBV DNA with the mammalian hepadnavirus DNA (data not shown).

Work on the serology of this antigen has only begun. A simple radioimmunoassay for antibody to DHBsAg particles, using radioiodinated DHBsAg, was much less successful than similar assays used by us for antibody to HBsAg and GSHsAg (3). When the use of radiolabeled DHBsAg was avoided by using a radiolabeled anti-mouse IgG, however, a very satisfactory assay for mouse anti-DHBsAg was obtained. All assays were successfully blocked with sera containing DHBV but not with normal sera, indicating the specificity of this assay for DHBsAg. It should be possible, therefore, to develop sensitive assays, similar to that used for mouse anti-DHBsAg, for rabbit and duck anti-DHBsAg by using a high-titer anti-rabbit IgG and anti-duck IgG, preferably monoclonal antibodies.

Finding this distinct variation in the polypeptide pattern of DHBsAg compared with those of the mammalian surface antigens adds to the list of differences between the avian virus and the mammalian viruses and suggests that DHBV could be dissimilar to HBV in other aspects of its growth and biology as well. Study of the dissimilarities of DHBV from its mammalian relatives, e.g., replication in the pancreas and presence of more completely double-stranded DNA in its virions than in those of mammalian viruses, could lead to the detection of new variants of this virus family in either humans or animals important to us.

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