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Relationship of Large Hepatitis B Surface Antigen Polypeptide to Human Serum Albumin

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A significant proportion (20 to 40%) of highly purified 22-nm hepatitis B surface antigen (HB_sAg) particles contain human serum albumin (HSA) as demonstrated by specific precipitation of radioiodinated particles by anti-HSA. Preparations of the isolated major HBsAg polypeptides (P-1, P-2, and P-6) were iodinated and analyzed by radioimmunoprecipitation for reactivity with rabbit antisera to human plasma proteins. Only the P-6 fraction (molecular weight, 68,000) was precipitated and only by anti-HSA; specific precipitation was observed with guinea pig antisera to P-6 and native HB_sAg and goat or rabbit antisera to HSA. Coprecipitation of P-6 with antiserum to HB_sAg and with anti-HSA, compared to precipitation with each antiserum alone, indicated that the HB_sAg and HSA determinants were on separate molecules. The P-6 polypeptide may represent a precursor protein of the hepatitis B virion.

The association of human serum proteins with hepatitis B surface antigen (HB_sAg) has been the subject of much controversy (reviewed by Schuurs and Wolters [13]). The major question has been whether serum proteins are intimately associated with the particulate HB_sAg structures or simply represent contaminants of individual preparations. In our laboratory, we used zonal centrifuge procedures (5, 6) to purify the 22-nm form of HB_sAg from serum. Although direct analysis of such preparations by conventional techniques failed to reveal human serum proteins, animals hyperimmunized with these preparations produced antibodies to human serum albumin (HSA) which were then removed by affinity chromatographic techniques (4). We report here the association of HSA with a subpopulation of HB_sAg particles. Furthermore, we analyzed the isolated major polypeptides of HB_sAg for the presence of individual normal serum proteins by double-antibody radioimmunoprecipitation (RIP) assays.

MATERIALS AND METHODS

HB_sAg and HB_sAg polypeptides. The purification of the 22-nm form of HB_sAg and isolation of its structural polypeptides were previously described (5, 14). Briefly, HB_sAg was purified from the plasma of chronic HB_sAg carriers by a four-step zonal centrifuge technique involving isopycnic bandings in CsCl and rate sedimentation in sucrose gradients (5). The individual polypeptide preparations were isolated from purified HB_sAg by preparative polyacrylamide gel electrophoresis after solubilization of the particles in 2-mercaptoethanol and sodium dodecyl sulfate (14). Analysis of the purity of the polypeptide preparations and immunoprecipitates of labeled antigen and antibody was performed by analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (14).

Antisera. Guinea pig antiserum to HB_sAg/*adw* (anti-HB_s) with anti-HB_s/*a* and anti-HB_s/*d* activities was the reagent prepared for the Research Resources Branch, National Institute of Allergy and Infectious Diseases (reagent V801-502-058). The antiserum was purified by affinity chromatography using Sepharose 2B-coupled normal human serum (4) and was free of activity to normal human serum components. Guinea pig antisera to the structural polypeptides of HB_sAg/*adw* were prepared and characterized previously (14, 17). Antiserum to guinea pig immunoglobulin was prepared by repeated immunization of a rabbit with purified guinea pig immunoglobulin G. Rabbit and goat antisera to HSA (anti-HSA) were purchased from Cappel Laboratories (Cochranville, Pa.) and Miles Laboratories (Elkhart, Ind.), respectively, and were free of anti-HB_s by Ausab assays (Abbott Laboratories, N. Chicago, Ill.). Rabbit antisera to individual human plasma proteins were purchased from Behring Diagnostics (Somerville, N.J.); the activity of each of these was confirmed by immunoelectrophoresis of normal human serum and the established mobility of the serum component as designated by the manufacturer. Goat antisera to rabbit immunoglobulin G heavy and light chains and rabbit antiserum to goat immunoglobulin G were purchased from Cappel Laboratories.

RIP and competitive inhibition assays. The preparations of HB_sAg and individual HB_sAg polypeptides were radiolabeled by chloramine-T or lactoperoxidase procedures (15, 17). The double-antibody RIP assays were performed in polystyrene tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) as previously described (14, 17). Phosphate-buffered saline (0.85% NaCl-0.01 M phosphate, pH 7.2) containing 1% bovine serum albumin, 0.25% Tween-80, and 10 mM ethylenedia-

minetetraacetic acid was used as the diluent. Briefly, 3,000 to 8,000 cpm of labeled antigen and 50 μl of the appropriately diluted antiserum were incubated in a final volume of 250 μl for 18 h at 4°C; antibody dilutions beyond 1:50 were made in a 1:50 dilution of normal animal serum in the diluent. Second antibody was added in 20-fold excess to undiluted first antibody, and the sample was incubated for a further 4 h at room temperature. The sample was then centrifuged at 1,600 × *g* for 30 min, and the supernatant was removed. The amount of radioactivity in the pellet was used to calculate the percent precipitation. Competitive inhibition assays were performed, either as described above, with the addition of unlabeled HSA before that of a constant amount of first antibody, or as previously described (17); HSA (Miles Laboratories) was negative for HB_sAg by Ausria II assay (Abbott Labs).

RESULTS

Association of HSA and HB_sAg. Four-step-purified HB_sAg particles were radioiodinated with $Na^{125}I$ by either the chloramine-T or lactoperoxidase method and further purified by two cycles of isopycnic banding in CsCl after removal of free iodate by gel filtration on Sephadex G-200. The iodinated HB_sAg preparations were analyzed by RIP assays using guinea pig anti-HB_s and rabbit anti-HSA (Table 1). Whereas 92% of the HB_sAg iodinated by the chloramine-T method was precipitated by guinea pig anti-HB_s, a significant amount (33%) of the ^{125}I-ligand was also precipitated by rabbit anti-HSA. The specificity of the reaction was confirmed by blocking the rabbit anti-HSA reaction with purified unlabeled HSA; HSA did not block the reaction between [^{125}I]HB_sAg and guinea pig anti-HB_s. The existence of two populations of HB_sAg particles, one precipitated by anti-HB_s alone and another by anti-HB_s or anti-HSA, was confirmed by the stepwise addition of rabbit anti-HSA followed by guinea pig anti-HB_s. The sum of the two-step precipitation (85.1%) was in good agreement with the one-step total precipitation obtained with guinea pig anti-HB_s alone (91.7%). Similar results were obtained with HB_sAg labeled by the lactoperoxidase method (Table 1). Other preparations of purified HB_sAg, both *adw* and *ayw* subtypes, were analyzed in a similar manner; 20 to 40% of the HB_sAg particles in these preparations were precipitable by anti-HSA. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown) of the immunocomplex between rabbit anti-HSA and [^{125}I]HB_sAg, the immunocomplex between the supernatant of that reaction and guinea pig anti-HB_s, and the [^{125}I]HB_sAg alone revealed identical patterns.

HB_sAg polypeptides and plasma proteins. The iodinated major polypeptides of HB_sAg (P-1, P-2, and P-6) were analyzed for reactions with rabbit antisera to individual human plasma components by double-antibody RIP (Table 2). Guinea pig anti-HB_s precipitated 64.7, 90.2, and 37.2% of the P-1, P-2, and P-6 preparations, respectively. No reactions were observed between the major P-1 and P-2 polypeptides and antiserum to any of the 19 plasma components. However, reactions were observed between P-6 and antisera to HSA (29.9%) and α_2-macroglobulin (13.6%); the reaction between [^{125}I]P-6 and anti-α_2-macroglobulin was not significant (5%) at higher dilutions (1:5) of antibody, and this reaction was not investigated further.

HSA as a component of P-6. The reactions of P-6 preparations with anti-HSA were further evaluated. Titrations of rabbit and goat anti-HSA, guinea pig anti-HB_s, and guinea pig anti-P-6 with iodinated P-6 as ligand are shown in Fig. 1. The lowest dilutions of the anti-HSA sera precipitated about 30% of the ligand; guinea pig anti-HB_s and anti-P-6 precipitated 28 and 43%, respectively. To determine whether the activi-

TABLE 1. *Radioimmunoprecipitation of HBsAg*

Antigen[a]	First precipitation		Second precipitation[b]		Sum (% ppt)
	Antiserum[c]	% ppt	Antiserum	% ppt	
HBsAg-CT	GP anti-HB_s	91.7	—	—	91.7
	GP anti-HB_s + 0.1 mg of HSA	90.1			
	Rb anti-HSA	32.7	GP anti-HB_s	52.4	85.1
	Rb anti-HSA + 0.1 mg of HSA	4.8			
HBsAg-LP	GP anti-HB_s	74.1	—	—	74.1
	GP anti-HBs + 0.1 mg of HSA	75.3			
	Rb anti-HSA	26.6	GP anti-HB_s	44.5	71.1
	Rb anti-HSA + 0.1 mg of HSA	2.9			

[a] Purified HBsAg iodinated by the chloramine-T (CT) or lactoperoxidase (LP) method.

[b] After the first precipitation, the supernatant was recovered for second precipitation. The percentage of precipitation (% ppt) of the second precipitation was calculated based on initial total input radioactivity.

[c] GP, Guinea pig; Rb, rabbit.

TABLE 2. *RIP of HB_sAg polypeptides with rabbit antiserum to human plasma proteins*

Antiserum	Polypeptide antigen[a]		
	P-6	P-2	P-1
Preserum	(2.0)	(1.5)	(5.1)
Albumin	29.9	0.9	−1.4
Prealbumin	1.8	1.4	0.4
Hemopexin	1.3	1.7	−0.2
C-reactive protein	0.6	1.0	−2.3
α_1-Antitrypsin	4.8	2.3	2.5
Fibrinogen	0.5	1.5	0.8
α_1-Lipoprotein	7.8	2.2	0.3
β-Lipoprotein	3.2	1.6	0
β_{1E}-Globulin (C4)	2.9	1.8	0.1
β_1C/β_{1A} Globulin (C3/$C3_c$)	0.7	1.6	−1.4
Transferrin	2.0	1.8	−0.6
Ceruloplasmin	0.7	1.6	−1.2
Haptoglobin	1.3	1.2	0
α_2-Macroglobulin	13.6	1.5	−0.9
α_{2HS}-Glycoprotein	0.7	1.3	−1.3
α_1-Acid glycoprotein	0.7	0.7	−1.6
β_2-Glycoprotein I	0.2	2.9	−0.8
β_2-Glycoprotein II	3.5	2.4	1.7
β_2-Glycoprotein III	1.0	1.4	−1.1

[a] Values represent the percent of precipitable counts different from that of the preserum control. Antiserum was tested at the final concentration of 1:30 dilution. At 1:60 dilution, guinea pig anti-HB_s precipitated 37.2, 90.2, and 64.7% of the counts for P-6, P-2, and P-1, respectively.

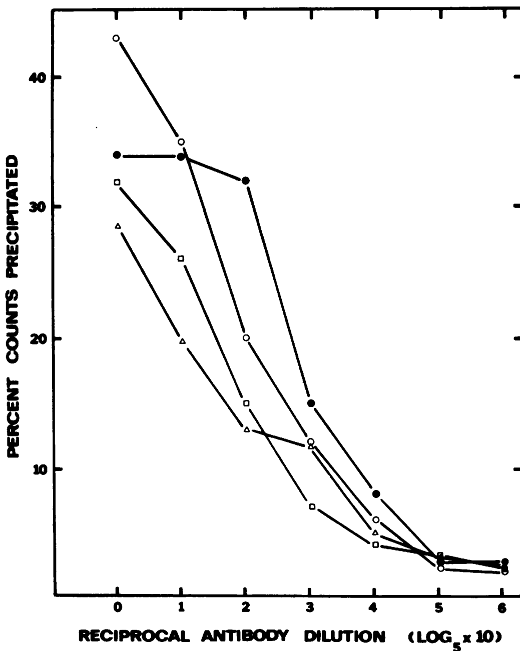

FIG. 1. *Precipitation of [^{125}I]P-6 by rabbit anti-HSA (●), goat anti-HSA (□), guinea pig anti-P-6 (○), and guinea pig anti-HB_s (△).*

ties were on separate molecules, coprecipitation experiments with two different [^{125}I]P-6 preparations were performed (Table 3). The amount of ligand precipitated by the combination of guinea pig anti-HB_s and rabbit anti-HSA was equal to the sum of each antiserum alone, indicating that the HSA and HB_sAg activities were expressed on separate molecules. The conclusion was further supported by competitive inhibition assays with unlabeled HSA (Fig. 2). The reaction between [^{125}I]P-6 and rabbit anti-HSA was completely inhibited by HSA; HSA did not inhibit the reaction of the ligand with guinea pig anti-HB_s. Guinea pig antiserum to P-6 was partially blocked by added HSA; in the presence of excess HSA, 32% of the precipitable P-6 reacted with guinea pig anti-P-6.

DISCUSSION

Analysis of purified HB_sAg confirmed the association of serum albumin with the 22-nm

TABLE 3. *Coprecipitation of [^{125}I]P-6*

Antiserum[a]	% Precipitation[b]	
	Expt 1	Expt 2
GP anti-HB_s + Rb preserum	35.3	24.9
GP preserum + Rb anti-HSA	28.2	14.1
GP anti-HB_s + Rb anti-HSA	56.6	38.1

[a] GP, Guinea pig; Rb, rabbit.

[b] Percent precipitation was determined as previously described (17). The reaction mixture (250 µl) contained [^{125}I]P-6 (5,000 cpm in 100 µl of buffer), 25 µl each of a 1:10 dilution of guinea pig and rabbit serum, and either 50 µl each of the appropriate second antibody (undiluted) or 100 µl of 25% trichloroacetic acid. Supernatants (150 µl) from tubes with presera in place of first antibody served as the total counts control, and those with trichloroacetic acid served as the trichloroacetic acid-soluble counts control.

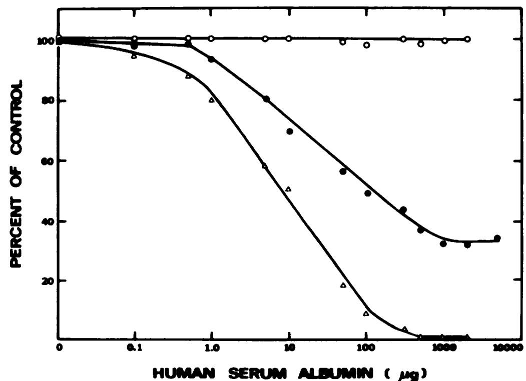

FIG. 2. *Precipitation of [^{125}I]P-6 in the presence of HSA by rabbit anti-HSA (△; 1:50), guinea pig anti-HB_s (○; 1:10), and guinea pig anti-P-6 (●; 1:50).*

HB_sAg particles as reported by others (1, 10). Radioiodination and extensive purification of HB_sAg/*adw* and HB_sAg/*ayw* preparations followed by RIP analysis with goat or rabbit anti-HSA revealed that 20 to 40% of the HB_sAg particles contained HSA as part of their structure; either 60 to 80% of the HB_sAg particles did not contain HSA or the HSA was not accessible to antibody. Serum albumin has anion-binding properties and probably binds to the lipoprotein HB_sAg particle in serum; the lack of HSA on most of the particles might simply represent the dissociation of HSA and its removal during HB_sAg purification. We have noted that prolonged storage or mild detergent treatment of purified HB_sAg results in the release of HSA from the particulate HB_sAg. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the particles revealed a comparable reduction of protein from the P-6 (68,000 molecular weight) region of the gel. However, we could not demonstrate the exchange of the radioiodinated soluble fraction with P-6 of freshly prepared HB_sAg or its reassociation with the HB_sAg particle from which it was released (unpublished data). Although several laboratories have now reported that some HB_sAg particles contain receptors for polymerized albumin (reviewed by Hollinger and Dressman [7]; 8, 11), the relationship between these receptors and the association of HSA with HB_sAg as reported here is not clear.

Structural analysis of HB_sAg reveals seven polypeptides ranging in molecular weight from 23,000 to 97,000 (15). The major polypeptides (P-1, P-2, and P-6) were previously shown to stimulate antibodies to HB_sAg in guinea pigs (14) and to contain both group- and subtype-specific determinants as part of their structures (17). Preparations of HB_sAg from different donors vary in the relative amount of P-6 (15). Analysis of amino acid composition by the method of Metzger et al. (9) revealed that isolated P-6 from HB_sAg with a high amount of P-6 differed markedly from the P-1 or P-2 fractions, whereas P-6 from HB_sAg with a low amount of P-6 was similar to P-1 and P-2 (16). In contrast, comparisons of the P-6 preparations with published values for HSA revealed that P-6 from HB_sAg with a high proportion of P-6 was similar to HSA in composition (difference index = 13.6), whereas that from HB_sAg with low P-6 was quite different (difference index = 23.0). Preparations of the isolated major polypeptides were analyzed by RIP for the presence of individual serum proteins; only the P-6 fraction was reactive, and significant amounts of ligand were precipitated only by anti-HSA. Therefore, the compositional analysis suggests, and the antigenic analysis confirms, the presence of both HSA and HB_sAg in isolated P-6 preparations. The low percent precipitability by antibody of the P-6 ligand apparently results from variable denaturation and renaturation of the determinants during the isolation procedure (17) and prevents an accurate estimation of the relative proportions of HSA and HB_sAg in the P-6 fraction. Since the amount of 68,000-molecular-weight material in HB_sAg preparations varies, and the relative proportion of HSA and P-6 is not known, direct comparison of the P-6 fraction and HSA by peptide mapping could overlook the presence of the HBV gene product. Importantly, the sum of the precipitable [^{125}I]P-6 by anti-HB_s and anti-HSA alone was equal to that of both antibodies combined, indicating that the HB_sAg and HSA determinants were not present on the same molecule, such as might occur if the hepatitis B virus genome were integrated into an appropriate region of the hepatocyte deoxyribonucleic acid.

The P-1, P-2, and P-6 polypeptides do not appear to represent unique gene products of hepatitis B virus; they share common determinants, and the P-2 probably represents a glycosylated form of P-1 (12, 18). Some preparations of the 22-nm HB_sAg form and all preparations of the hepatitis B virion (Dane particle) examined thus far appear to lack the P-6 component (J. W.-K. Shih, G. Hess, P. M. Kaplan, and J. L. Gerin, J. Virol. Methods, in press). P-6 may represent a precursor polypeptide that is subsequently cleaved and glycosylated to produce the P-1 and P-2 structural proteins. Several laboratories have recently cloned the hepatitis B virus deoxyribonucleic acid in *Escherichia coli* (2, 3, 18), and sequence analysis of the deoxyribonucleic acid and gene products should provide insight into the process of hepatitis B virus maturation.

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