

## Sites That Bind Polymerized Albumin on Hepatitis B Surface Antigen Particles: Detection by Radioimmunoassay

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Received for publication 3 July 1979

Antibodies to polymerized human albumin (poly-HSA) could not be detected by using sensitive methods (enzyme-linked immunosorbent assay and radioimmunoprecipitation) in sera from chronic carriers of hepatitis B surface antigen (HBsAg) or in serial bleedings from one chimpanzee infected with type A hepatitis virus and one infected with non-A, non-B hepatitis virus. By a solid-phase radioimmunoassay, receptor sites for poly-HSA could be detected on HBsAg particles from sera containing either hepatitis B "e" antigen (HBeAg) or anti-HBe. Blocking experiments showed that monomeric HSA did not bind to this receptor. In general, the HBsAg particles from sera with HBeAg had more poly-HSA receptor sites or relatively more particles carrying this receptor compared with HBsAg from sera with anti-HBe. Microtiter plates coated with poly-HSA bound HBsAg from sera containing HBeAg with greater efficiency than did anti-HBs coupled to a solid phase (Ausria II beads), whereas with sera positive for anti-HBe, the two assays were equally sensitive. Decreased ability of HBsAg to bind to poly-HSA was seen in some sera which had been stored for a few years at 4°C, whereas the binding to anti-HBs was unaffected. It is possible that polymers of albumin on the surface of hepatocytes could function as receptors for hepatitis B virus.

It has been reported that polymerized human albumin (poly-HSA) reacts with certain sera from patients with hepatitis (5, 8, 9). The results were obtained by gel diffusion and passive hemagglutination tests. The reacting agent(s) was interpreted to be antibodies, although they were thought to be of different immunoglobulin classes. Recently, Imai et al. (6) and Neurath (11) found that hepatitis B surface antigen (HBsAg), predominantly from sera positive for hepatitis B "e" antigen (HBeAg), reacted with poly-HSA.

It was considered to be of interest to try to confirm, with sensitive methods, the findings of anti-poly-HSA in patients with hepatitis, especially in light of the reports of reaction between poly-HSA and HBsAg. To further study the interaction between HBsAg and poly-HSA, a solid-phase radioimmunoassay was developed, using microtiter plates coated with poly-HSA. The binding of HBsAg to poly-HSA in this radioimmunoassay was compared with results obtained by a commercial radioimmunoassay for HBsAg (Ausria II).

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### MATERIALS AND METHODS

**HBsAg.** Purified HBsAg of subtype *ayw* was purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, Md. Sera from 20 unselected chronic HBsAg carriers positive for HBeAg by gel diffusion tests and 20 positive for anti-HBe by the same method were obtained from materials previously used for other studies.

Preparations of 22-nm spherical and filamentous HBsAg particles and of Dane particles purified by a combination of isopycnic and rate zonal centrifugations were kindly provided by John Gerin.

Weekly serum samples were obtained from two chimpanzees, one (no. 714) inoculated with hepatitis B virus type *adw* and the other (no. 755) inoculated with type *ayw* in other studies (R. H. Purcell, unpublished data).

**Hepatitis A and non-A, non-B sera.** Serial bleedings were obtained from a chimpanzee (no. 753) experimentally infected with hepatitis A virus (2) and one (no. 884) with a strain of non-A, non-B hepatitis virus (S. M. Feinstone, unpublished data).

**Poly-HSA.** HSA (electrophoretic purity, 100% [Behringwerke, A. G., Marburg/Lahn, West Germany]), was cross-linked by glutaraldehyde by the modification of Lenkei et al. (8) of the method of Avrameas and Ternynck (1). Twenty milligrams of HSA was dissolved in 0.9 ml of 0.1 M phosphate buffer, pH 6.8, and 0.1 ml of 2.5% glutaraldehyde was added. The mixture was incubated at room temperature for

2 h and then dialyzed against phosphate-buffered saline for 3 h. This glutaraldehyde-treated solution was mixed with 40 mg of untreated HSA dissolved in 1 ml of 0.2 M carbonate buffer, pH 9.0, and further incubated for 1 h at 37°C and overnight at 4°C. The poly-HSA was chromatographed on a Sephadex G-200 column (2.6 by 34 cm). Fractions of 4.6 ml were saved.

**Assays for anti-poly-HSA.** Immuno-electro-osmophoresis (4), enzyme-linked immunosorbent assay (3), and radioimmunoprecipitation assay (7) were used as tests for anti-poly-HSA.

**PAA.** For the polymerized albumin assay (PAA), the wells of polyvinyl microtiter plates (Cooke Engineering Co., Alexandria, Va.) were coated overnight at room temperature with 60  $\mu$ l of poly-HSA in 0.08 M carbonate-bicarbonate buffer, pH 9.6 (coating buffer). After the coating solution had been removed, the plates were postcoated for another day at room temperature with 1% normal guinea pig serum in the same buffer. The plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20, and 50- $\mu$ l amounts of the samples to be tested for HBsAg were added. After incubation overnight at 37°C, the plates were again washed four times with phosphate-buffered saline-0.05% Tween 20. The amount of HBsAg bound was determined by adding 50  $\mu$ l of  $^{125}$ I-labeled anti-HBs (routinely, human  $^{125}$ I-anti-HBs [Ausria II reagent antibody; Abbott Laboratories, North Chicago, Ill.] was used, but also guinea pig  $^{125}$ I-anti-HBs was tried) per well and incubating the plate for 4 h at 37°C. Unbound tracer was removed by washing four times with phosphate-buffered saline-0.05% Tween 20, and the wells were cut apart with scissors and transferred to gamma counting tubes. The amount of HBsAg bound to the individual wells was expressed as counts per minute or as a quotient between the counts per minute of the test sample (P) and the mean counts per minute of wells that had received negative control samples (N): P/N. Values of 2.1 or greater were considered positive.

**Ausria II test.** The Ausria II (Abbott Laboratories) test was used as recommended by the manufacturer.

**HBsAg and anti-HBe assays.** HBsAg and anti-HBe were assayed by a solid-phase radioimmunoassay (10).

**Passive hemagglutination tests.** Unfixed human group O erythrocytes coated with poly-HSA, monomeric HSA, or bovine serum albumin plus uncoated cells were obtained from D. De Souza, Electro-Nucleonics Laboratories. Serial dilutions of sera with or without HBsAg were tested for their abilities to agglutinate the albumin-coated erythrocytes.

## RESULTS

**Anti-poly-HSA.** Anti-poly-HSA could not be detected in any of the sera tested by any method: 12 sera from chronic carriers of HBsAg tested by immuno-electro-osmophoresis, 25 sera from chronic HBsAg carriers and serial bleedings from one chimpanzee infected with hepatitis A and one infected with non-A, non-B hepatitis virus tested by enzyme-linked immunosorbent assay, and 27 sera from chronic HBsAg carriers tested by radioimmunoprecipitation assay.

**PAA. (i) Coating of microtiter plates.** Fractionation on a Sephadex G-200 column of the glutaraldehyde-treated HSA solution showed one peak of poly-HSA in the void volume and one peak of monomeric HSA (Fig. 1).

A portion from each fraction was diluted in coating buffer to contain 50  $\mu$ g of protein per ml and was used for coating the wells of a microtiter plate. A 1/50 dilution of a serum containing HBsAg was used to investigate the ability of the HSA from the different fractions to bind HBsAg. The peak fraction of the poly-HSA had the greatest specific activity (binding capacity per microgram of protein) for binding HBsAg.

Poly-HSA and monomeric HSA were applied in different concentrations on microtiter plates, which were subsequently used for testing different dilutions of a HBsAg-positive serum. It was found that the binding of HBsAg to the plate with poly-HSA increased with increasing amount of protein which had been used for coating the plate, up to a concentration of 50  $\mu$ g of protein per ml of coating buffer (Fig. 2). HBsAg was also bound to the plate coated with monomeric HSA, but to a lesser extent than to the one with poly-HSA. Furthermore, only when the highest concentration of HBsAg was tested for binding to monomeric HSA did the amount of antigen which bound to the solid phase increase with increasing concentration of coating HSA (Fig. 2). Microtiter plates coated with dilution series of monomeric and polymerized bovine serum albumin were also tested, but no

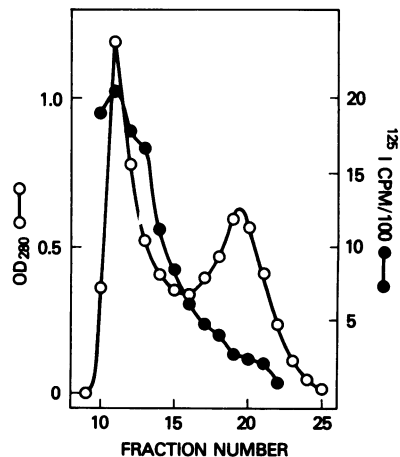


FIG. 1. Poly-HSA solution separated on a Sephadex G-200 column (2.6 by 34 cm). A microtiter plate was coated with 50  $\mu$ g of protein per ml from the individual fractions and used to test a HBsAg-positive serum diluted 1/50. The amount of HBsAg bound is shown as counts per minute. OD<sub>280</sub>, Optical density at 280 nm.

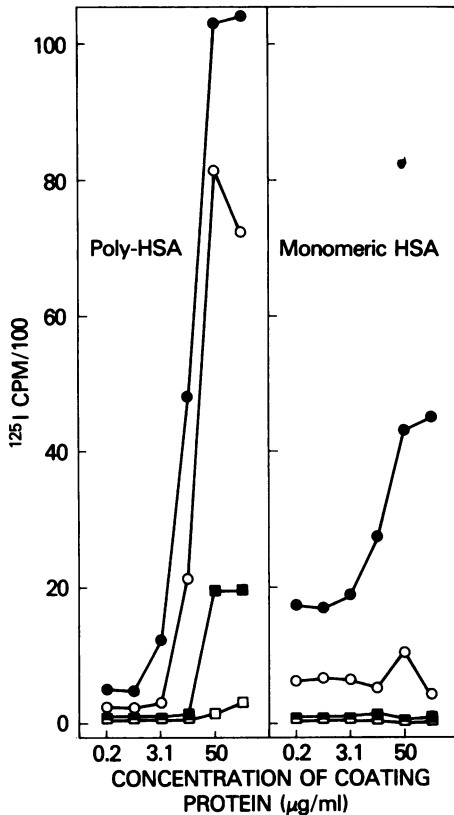


FIG. 2. Binding of HBsAg from four 10-fold serum dilutions to a microtiter plate coated with a dilution series of poly-HSA or monomeric HSA. The amount of HBsAg bound is shown as counts per minute. Serum dilutions: ●,  $10^{-2}$ ; ○,  $10^{-3}$ ; ■,  $10^{-4}$ ; □,  $10^{-5}$ .

specific binding of HBsAg to these plates could be demonstrated.

(ii) **Blocking tests.** To investigate the specificity of the binding of HBsAg to poly-HSA, different blocking tests were performed. A final dilution of  $10^{-3}$  of purified HBsAg was incubated overnight at room temperature in dilutions of monomeric or polymerized HSA or bovine serum albumin. When subsequently tested by PAA, only poly-HSA blocked the binding of HBsAg to the solid-phase poly-HSA (Fig. 3a). The binding of HBsAg to the anti-HBs solid phase of the Ausria test was not inhibited by any of the albumin solutions (Fig. 3b).

(iii) **Titration experiments.** When three 10-fold dilutions from each of 20 sera containing HBsAg and anti-HBe were screened by PAA and Ausria II, 3 sera reacted poorly with poly-HSA compared with anti-HBs. The same result was obtained with 1 of 20 sera with HBeAg. All of these sera had been kept unfrozen for a few years. Serum samples from the same bleedings,

which had been stored frozen, were tested by PAA and Ausria II. The HBsAg in one of the three sera with anti-HBe bound as well to poly-HSA as to anti-HBs, and the HBsAg from the HBeAg-positive serum bound even better. The remaining two sera with anti-HBe showed the same low degree of HBsAg-binding to the poly-HSA solid phase as did the samples which had been kept unfrozen.

Fourfold dilutions in phosphate-buffered saline of 10 of the sera containing HBsAg and anti-HBe and 10 with HBsAg and HBeAg were tested in parallel by PAA and Ausria II. The same preparation of  $^{125}\text{I}$ -labeled anti-HBs was used in both tests. Titration curves representative of an HBeAg-positive serum are shown in Fig. 4. Pronounced prozoning ("high-dose hook effect") was seen with the Ausria tests. The P/N values in the lower dilutions were approximately 10 times higher for PAA than for Ausria. The linear portions of the titration curves were essentially parallel in all of the comparative tests made, and the curves were linear until below a P/N of 2.1. The endpoint titers measured as P/N = 2.1 on the titration curves of HBeAg-positive and anti-HBe-positive sera are plotted in Fig. 5. By Ausria II, the geometric mean titer of HBsAg was  $4^{-6.06}$  for the anti-HBe sera and  $4^{-7.14}$  for the HBeAg samples ( $P < 0.01$ ). When tested by PAA, the mean endpoint titers for the two groups of sera were  $4^{-6.04}$  and  $4^{-8.75}$ , respectively ( $P < 0.01$ ). The PAA titers covered a wider range than those for Ausria II. Due to the parallelism of the titration curves, the titer differences between PAA and Ausria II achieved when making the comparisons at P/N levels of, for example, 10 or 20 were the same as those at P/N levels of 2.1 (except for two sera which did not reach P/N = 10 by PAA).

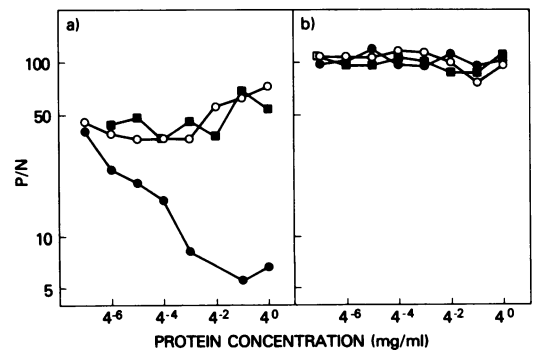


FIG. 3. Binding of purified HBsAg ( $10^{-3}$  dilution) to (a) solid-phase poly-HSA and (b) solid-phase anti-HBs (Ausria II beads). The HBsAg was diluted in different concentrations of poly-HSA (●) or monomeric HSA (○) or in bovine serum albumin (□). The amount of HBsAg bound is presented as P/N.

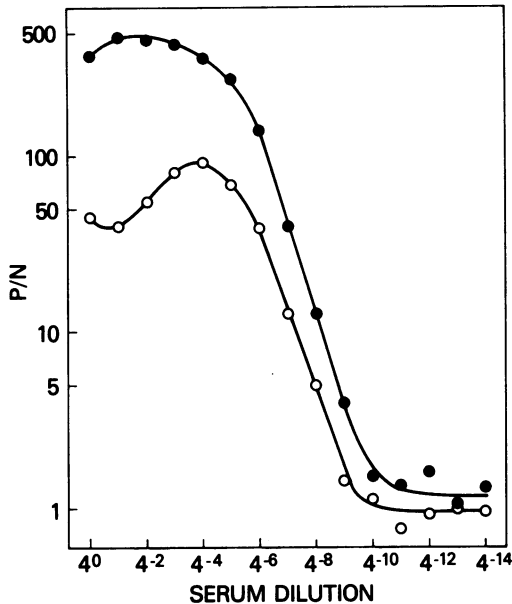


FIG. 4. Fourfold dilutions of a serum containing HBsAg tested by PAA (●) and Ausria II (○). The amount of HBsAg bound is expressed as P/N.

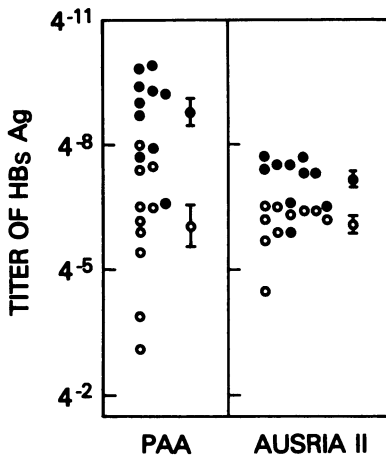


FIG. 5. Titers of HBsAg in 10 sera containing HBeAg (●) and 10 sera containing anti-HBe (○) as determined by PAA and Ausria II. Circles with bars on the right side of individual plotted results correspond to the geometric mean values. The lengths of the bars indicate the magnitudes of the standard errors of the means.

Examination of the different purified morphological forms of HBsAg did not reveal any difference in their ability to bind to poly-HSA. All three showed approximately 100 times higher endpoint titers by PAA than by Ausria II.

(iv) **Chimpanzee sera.** Serial bleedings from the two chimpanzees infected with hepatitis B

virus were tested for HBsAg by PAA and Ausria II. In the PAA, 50  $\mu$ l of a 1/10 dilution of each serum was used, and in the Ausria assay 200  $\mu$ l of a 1/40 dilution of each serum was used; i.e., the same amount of serum (5  $\mu$ l) was tested in the two assays. The same preparation of radio-labeled anti-HBs was used in both PAA and Ausria.

In chimp 714, HBsAg (*adu*) became detectable by PAA on week 16 after inoculation and remained positive until week 38 (Fig. 6). By Ausria, HBsAg could be detected from week 19 until week 41. HBeAg was found from week 21 to week 36 in a biphasic pattern. Anti-HBe appeared during week 22 in coexistence with HBeAg and rose in titer in subsequent sera.

Chimp 755 became positive for HBsAg (*ayw*) by both PAA and Ausria 7 weeks after inoculation (Fig. 6). PAA was positive until week 16, and Ausria was positive until week 14. HBeAg was found between weeks 8 and 13, and anti-HBe appeared first on week 13.

**Passive hemagglutination tests.** The erythrocytes coated with poly-HSA were agglutinated by most sera containing HBsAg, but the titers were considerably lower than those obtained by PAA (data not shown). The cells coated with monomeric HSA also were agglutinated by HBsAg, but less frequently and to lower titers. The agglutinations of both kinds of erythrocytes could be blocked by preincubating the sera with poly-HSA but not with monomeric HSA. Bovine serum albumin-coated and uncoated control cells were not agglutinated by any of the sera tested.

## DISCUSSION

The enzyme-linked immunosorbent and radioimmunoprecipitation assays, which presumably are more sensitive than the gel diffusion and passive hemagglutination tests used in previous studies (5, 8, 9), did not detect anti-poly-HSA in any serum. It therefore is probable that the reaction between poly-HSA and HBsAg has been misinterpreted as a reaction between poly-HSA and antibodies.

Although HBsAg was shown to bind to a certain extent to microtiter plates coated with monomeric HSA (Fig. 1 and 2), only poly-HSA could block the binding of HBsAg to solid-phase poly-HSA (Fig. 3). Conceivably, monomeric HSA, when bound to a plastic surface, can to some extent be recognized as a polymer. The results of the blocking tests also rule out that the receptor sites on the HBsAg particles are recessed and not easily reached by single HSA molecules bound to the solid phase.

The addition of poly-HSA to HBsAg particles

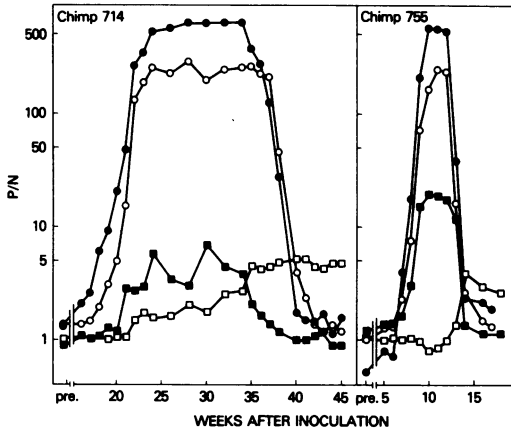


FIG. 6. Serial bleedings from chimpanzees 714 and 755 infected with hepatitis B virus *adw* and *ayw*, respectively, tested by PAA (●) and Ausria II (○); the same amount of serum (5  $\mu$ l) was used in both tests. HBeAg (■) and anti-HBe (□) were determined by a solid-phase radioimmunoassay. The results are shown as P/N.

did not change their ability to bind to anti-HBs, indicating that different structures on the HBsAg particles react with poly-HSA and anti-HBs. The specific reaction between HBsAg and poly-HSA could also be demonstrated by the ability of HBsAg to agglutinate erythrocytes coated with poly-HSA. However, we have not been able to reverse the assay, i.e., to detect any binding of poly-HSA to HBsAg bound to a solid phase (directly or with anti-HBs as intermediate) either by using radiolabeled poly-HSA or by using unlabeled poly-HSA and radiolabeled anti-HSA as the tracer. The reason for this inability of solid-phase HBsAg to react is not yet understood.

The sensitivity of PAA in detecting HBsAg was demonstrated to be approximately the same as that of Ausria II for sera containing anti-HBe, but PAA yielded significantly higher HBsAg titers than did Ausria in sera positive for HBeAg ( $P < 0.01$ ) (Fig. 5). In the chimpanzees infected with hepatitis B virus, HBsAg was detected for about the same length of time by the two tests (Fig. 6). The higher P/N values of the PAA indicated that the poly-HSA solid phase used had capacity to bind relatively more HBsAg than the anti-HBs solid phase.

The high-dose hook effect often seen in solid-phase radioimmunoassays and thought to be an effect of antibody heterogeneity of the solid phase or incomplete washing or both (12) was observed in the Ausria tests but scarcely in the PAA tests. It seems reasonable to presume that the difference between the two tests is caused

by a variety of different equilibrium constants governing the anti-HBs-HBsAg reaction, in contrast to only one constant or a small number with similar values in the reaction between poly-HSA and HBsAg.

The lower reactivity with poly-HSA of HBsAg from sera with anti-HBe compared to sera containing HBeAg could be explained by relatively fewer receptor sites for poly-HSA on the HBsAg particles or by blocking of receptor sites by aggregated albumin present in the serum. That the latter mechanism in at least some cases might be of importance was supported by the fact that the HBsAg in some sera was demonstrated to lose the ability to bind to poly-HSA by storage. Thus, it seems probable that aging of sera can produce substances, such as aggregates of albumin, which can block the poly-HSA receptors of the HBsAg particles.

Whether there is any biological implication of the ability of the HBsAg to bind to poly-HSA is not known, but if structures which can be recognized as polymeric albumin are exposed on the surface of hepatocytes, they might function as receptors for the hepatitis B virus.

#### ACKNOWLEDGMENTS

We thank Lacy Overby for performing the radioimmunoassay tests for HBeAg and anti-HBe and Harvey Alter, Vincent McAuliffe, and the American Red Cross for supplying sera from chronic carriers of HBsAg.

This work was supported by grants from Anna-Lisa och Sven-Erik Lundgrens stiftelse for medicinsk forskning and Alfred Osterlunds stiftelse.

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