Confirmatory Evidence for the Association of Hepatitis B Surface Antigen with Antigenic Determinants Reactive with Antibodies Present in Some Anti-HBe-positive Sera

By A. R. NEURATH, N. STRICK and C.-Y. HUANG
The Lindsley F. Kimball Research Institute of The New York Blood Center, New York, New York 10021, U.S.A.

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SUMMARY

Spherical 20 nm diam. particles of hepatitis B surface antigen (HBsAg), purified from some sera positive for hepatitis B e-antigen (HBeAg), are associated with antigenic determinants reacting with antibodies frequently present in sera containing anti-HBe. Treatment of HBsAg with the detergent Sarcosyl increased the exposure of these determinants which could be differentiated from HBeAg on the basis of immunological specificity and sensitivity to reduction and alkylation. The presence of these determinants appeared to be restricted to a subpopulation of HBsAg associated with IgG and albumin.

INTRODUCTION

The following antigens related to hepatitis B virus (HBV) have been identified using human convalescent sera and sera from HBV-infected individuals: hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg; Almeida et al. 1971) and a group of heterogeneous proteins collectively designated as e-antigen (HBeAg; Magnius & Espmark, 1972; Williams & LeBouvier, 1976; Murphy et al. 1978; Trepo et al. 1978). A predominant component of the HBeAg system is a protein with a mol. wt. of approx. 35000, detected by radioimmunoassay (RIA) in sera, either in free form or in association with itself or with other proteins (Neurath et al. 1979; Tedder & Bull, 1979). This protein is also a constituent of the nucleoprotein core of HBV (Neurath & Strick, 1979a; Takahashi et al. 1979; Ohori et al. 1979). On the other hand, earlier studies (Neurath et al. 1976) suggested the presence, on the envelope of Dane particles and tubular forms of HBsAg, of antigenic sites reacting with antibodies present in a serum containing anti-HBe. These findings were not confirmed by the experiments of Gerin et al. (1978) and Takahashi et al. (1978b) but Vnek et al. (1979) detected HBeAg in all morphological forms of HBsAg after treatment with detergents. However, since HBeAg determinants are associated with HBcAg, it was necessary to reassess the possible association of HBsAg with HBeAg-like determinants using preparations which did not contain HBcAg in either free or cryptic form. The results presented here show that HBsAg purified from HBeAg-positive sera may indeed be associated with antigenic determinants reactive with antibodies present in some anti-HBe-positive sera. The nature of these determinants has been explored in experiments described in this report.
METHODS

Purification of HBsAg. Spherical HBsAg particles approx. 20 nm in diam. were purified either from pellets enriched with respect to Dane particles and tubular forms of HBsAg, prepared as described (Neurath et al. 1976), or from pellets obtained after re-centrifuging the Dane particle-free supernatant fluid at 30000 rev/min for 24 h in the Spinco rotor 35 (Beckman Instruments, Palo Alto, Calif., U.S.A.). In the first case, the pellet resuspended in 0.14 M-NaCl-0.01 M-tris, pH 7.2 (TS), was layered on top of a 10 to 25% (w/w) sucrose gradient and centrifuged for 16 h at 16000 rev/min in the SW25.1 rotor. Fractions collected from the gradient were screened by electron microscopy and those containing only 20 nm particles were pooled, dialysed against TS and used for subsequent tests. In the second case, the pellets were resuspended in 34% (w/w) KBr, placed in centrifuge tubes for the SW25.1 or SW27 rotor, overlaid with a linear gradient of 31 to 19% KBr and centrifuged at 25000 rev/min for 16 h. Fractions collected from the gradient were tested for HBsAg by radioimmunoassay (RIA) at a dilution of 1:100 in normal human serum. Twenty µl aliquots of the fractions were each treated with 2 µl of a 1% solution of N-lauroyl sarcosine (sodium salt, Sigma Chemical Company, St Louis, Mo., U.S.A.; Sarcosyl) for 1 h at 37 °C, diluted with 300 µl of TS and 100 µl of normal human serum and tested by the RIA test for HBeAg. Fractions corresponding to the peak of both HBsAg and ‘HBeAg-like’ activity were pooled and dialysed against TS.

To demonstrate further the association of HBsAg with ‘HBeAg-like’ determinants, samples were submitted to rate zonal centrifugation in 10 to 30% (w/w) sucrose gradients in TS adjusted to 0.5M-NaCl for 2 h at 58000 rev/min in the SW65 rotor, or to isopycnic centrifugation at 39000 rev/min for 48 h in CsCl gradients using the same rotor.

Affinity chromatography. Chromatography on insolubilized Concanavalin A, anti-IgG, anti-albumin (using 1 ml columns for each adsorbent) was performed as described (Neurath & Strick, 1977; Neurath et al. 1978b). Approx. 100 µg of HBsAg were applied to each column.

HBeAg was partially purified by affinity chromatography on columns of insolubilized anti-HBe (Neurath et al. 1976).

RIA tests. HBsAg was determined by the AUSRIA test (Abbott Laboratories, North Chicago, Ill., U.S.A.). HBeAg (as well as the ‘HBeAg-like’ determinants), anti-HBe and HBcAg were assayed by recently developed techniques (Neurath et al. 1979; Neurath & Strick, 1979a). Albumin-binding sites on HBsAg were assayed as reported (Neurath & Strick, 1979b).

For double-immunoprecipitin tests, 125I-labelled proteins were extracted from slices of polyacrylamide gels after electrophoresis using the Model 1750 electrophoretic sample concentrator (ISCO, Lincoln, Neb., U.S.A.). Phosphate buffers, 0.03M and 0.01M, pH 8.0, were used in the electrode compartment and sample cup, respectively. Bovine albumin (5 mg in 100 µl of TS) was added to the gel slices to improve the recovery of eluted radioactive material. Electrophoretic elution was for 5 h at 3 W. Samples of the eluted proteins (800 to 12000 ct/min) were mixed with 20 µl of rabbit antisera to the appropriate human serum protein, incubated for 1 h at 37 °C and at 4 °C overnight. Optimal volumes, established in preliminary experiments, of goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind., U.S.A.) were added to the mixtures, followed by incubations as described above. Finally, the suspensions were centrifuged in 03105 tubes in the SS-34 rotor (Sorvall, Newtown, Conn., U.S.A.) for 30 min at 15000 rev/min, and the pellets and supernatant solutions were counted in a γ-counter. In preliminary experiments, 89, 47 and 87 % of radioactivity extracted from gels and corresponding to albumin and IgG heavy and light chains, respectively, was precipitated by the appropriate antisera.
'HBeAg-like' antigens on HBsAg

Fig. 1. Rate zonal centrifugation of 20 nm spherical HBsAg particles in a 10 to 30% (w/w) sucrose gradient at 58000 rev/min for 2 h. Fraction 1 = bottom of gradient. •–•, HBsAg determined by the AUSRIA test; ⊘, RIA for HBeAg with Sarcosyl-treated fractions.

Other methods. Molecular exclusion chromatography on Sephadex G-100 in 3M-NaSCN and purification of the 35000 mol. wt. HBeAg was carried out as described previously (Neurath et al. 1979). Chromatography on Sepharose 6B CL (Pharmacia, Piscataway, N.J., U.S.A.) was performed under the same conditions. Electron microscopy; reduction and alkylation in the absence of urea; polyacrylamide gel electrophoresis; immunization of rabbits with different forms of HBeAg and with HBsAg preparations enriched in Dane particles and tubules, and the removal of anti-HBs from the resulting antiserum were all performed as before (Neurath et al. 1976, 1978a, 1979; Neurath & Strick, 1979a).

'HBeAg-anti-HBe' precipitin lines labelled in the antigen moiety were prepared as follows: HBeAg (300 µl) partially purified by affinity chromatography and dialysed against 0.05M-borate, pH 8.5 or 0.025M-sodium carbonate, pH 9.5, were labelled with 1 mCi of 125I-labelled Bolton-Hunter reagent (Neurath et al. 1979) or with 250 µCi of 3H-dansyl chloride (each from New England Nuclear, Boston, Mass., U.S.A.), respectively. Labelling with the latter reagent was carried out overnight at 4°C and the excess of free dansyl chloride was removed by prolonged dialysis against TS. The labelled antigens were used in rheophoresis with IgG from an anti-HBe-positive serum. After the appearance of the precipitin lines, the agarose discs were extensively washed (mixed for 2 to 3 days with 2 l of TS) to remove labelled proteins which were not part of the precipitin line. Finally, the lines were excised, counted for radioactivity, extracted with 8M-urea-1% sodium dodecyl sulphate in 0.0625M-tris, pH 7.2, and used for polyacrylamide gel electrophoresis. The recovery of radioactivity in the precipitin lines corresponded to 0.3 and 2% of counts present originally in the 125I- and 3H-labelled antigens, respectively.

RESULTS

Association of HBsAg with antigenic determinants reacting with antibodies from an anti-HBe-positive serum

Spherical HBsAg particles about 20 nm in diam., purified from an HBeAg-positive serum were tested by RIA for HBeAg. The test results were positive. Pre-treatment of the particles with Sarcosyl resulted in a five- to sixfold increase of counts in the RIA test for HBeAg. RIA tests for HBcAg were negative. Addition of anti-HBc to the detergent-treated HBsAg
failed to cause any inhibition in the RIA test for HBeAg. This indicates the absence of HBcAg from the preparation of HBsAg, confirming the results of electron microscopy which identified only spherical particles about 20 nm in diam.

The major portion of antigen giving a positive test for HBeAg co-sedimented with HBsAg during rate zonal centrifugation (Fig. 1) and isopycnic banding in CsCl gradients (Fig. 2b and c). HBsAg and the 'HBeAg-like' activity had a buoyant density of 1.225 g/ml, which increased to about 1.25 g/ml after treatment with Sarcosyl. In comparison, HBeAg with a mol. wt. of 35000, purified as described (Neurath et al. 1979), had a density of about 1.30 g/ml (Fig. 2a). Treatment with Sarcosyl decreased the counts obtained with HBeAg (density of 1.30 g/ml) which was present as a minor contaminant in the preparation of HBsAg (Fig. 2c). Treatment with the detergent did not result in disintegration of HBsAg particles (data not shown).
Distinctiveness of HBeAg and of HBsAg-associated determinants reacting with antibodies present in some anti-HBe-positive sera

HBsAg was purified from seven distinct HBeAg-positive sera. The level of 'HBeAg-like' determinants associated with HBsAg was unrelated to the concentration of HBsAg or HBeAg in serum. In fact, no such determinants were detected in two of the sera with the highest levels of HBeAg (positive in RIA up to a dilution of 1:10000). This suggested that HBsAg does not acquire the determinants by mere adsorption of HBeAg. HBsAg purified from two anti-HBe-positive sera was not associated with the 'HBeAg-like' determinants.

Thirty-five sera positive for anti-HBe by rheophoresis were tested for inhibitory activity in HBeAg RIA tests with Sarcosyl-treated HBsAg. Five of the sera did not show any inhibitory activity. Two of the latter sera and six sera with inhibitory activity were tested for anti-HBe by RIA using the 35,000 mol. wt. HBeAg in the tests. Six sera had anti-HBe titres >1:512; two sera, each reacting with Sarcosyl-treated HBsAg, had titres of 1:128. This indicated that the reactivity of sera with HBsAg was not related to the level of anti-HBe. Rabbit anti-HBe did not react with Sarcosyl-treated HBsAg.

Furthermore, the 35,000 mol. wt. HBeAg can be distinguished from the 'HBeAg-like' determinants associated with HBsAg on the basis of: (a) affinity chromatography on Con-
canavalin A linked to Sepharose. HBeAg did not adsorb to the lectin while HBsAg and the 'HBeAg-like' activity did; (b) inactivation of the determinants by reduction with mercaptoethanol followed by alkylation, in contrast with the resistance of HBeAg to such treatment (Neurath et al. 1979).

The 'HBeAg-like' determinants were inactivated by the following treatments (all carried out for 1 h at 37 °C) of HBsAg: exposure to 0.2 M-acetate, pH 4.0; or to 0.2 M-glycine-HCl, pH 2.2; to trypsin (100 μg/ml, pH 7); and pepsin (100 μg/ml, pH 4). None of these treatments affected HBsAg as measured by AUSRIA tests.

Evidence that determinants reacting with antibodies present in some anti-HBe-positive sera are associated with a subpopulation of HBsAg containing host proteins

When Sarcosyl-treated HBsAg was chromatographed on columns of insolubilized antibodies to human albumin and IgG, respectively, a portion of HBsAg, but most of the antigen reacting with anti-HBe, was adsorbed to the columns and subsequently eluted at pH 10.9 (Fig. 3). When intact HBsAg was chromatographed, most of the 'HBeAg-like activity' failed to adsorb to the column, indicating that treatment of HBsAg with Sarcosyl reveals both 'HBeAg-like' (Fig. 2 c) and host-related antigenic determinants. This suggests that 'HBeAg-like' determinants were detected in a subpopulation of HBsAg associated with both albumin and IgG. The presence of these host proteins in HBsAg was also demonstrated by polyacrylamide gel electrophoresis of 125I-labelled HBsAg, followed by double immunoprecipitin tests with radioactive extracts from the appropriate regions of the gel (data not shown).

HBsAg particles have receptors for polymerized human albumin, the expression of these being related to the presence of HBeAg in serum serving as the source for HBsAg (Imai et al. 1979; Neurath & Strick, 1979b). Therefore, it was of interest to estimate the albumin-binding capacity of HBsAg subpopulations which either failed to adsorb to the immunosorbent columns or became attached to the columns and were subsequently eluted under conditions dissociating antigen–antibody bonds. The appropriate column fractions were pooled, adjusted to the same levels of HBsAg as determined by AUSRIA assays and tested for albumin binding sites. Most (80 to 90%) of the albumin-binding capacity was associated with HBsAg which failed to adsorb to either anti-albumin of anti-IgG columns. Thus, HBsAg with 'HBeAg-like' determinants, which is already associated with host protein (albumin and/or IgG), has a diminished reactivity with polymerized albumin.

Search for 'HBeAg-like' antigens not associated with HBsAg

The void volume fractions obtained after gel filtration on Sephadex G-100 in 3 M-NaSCN of HBeAg, partially purified by affinity chromatography on anti-HBe were shown to contain a minor portion of antigens giving a positive RIA test for HBeAg, while the major portion corresponded to the 35000 mol. wt. HBeAg (Neurath et al. 1979). A sample of pooled void volume fractions was re-chromatographed on a column of Sepharose 6B CL using 3 M-NaSCN as eluant. Antigen(s) giving a positive RIA test for HBeAg were recovered in the same fractions as IgG detected by immunodiffusion with an anti-IgG serum. Reduction and alkylation of the void volume fractions after chromatography on Sephadex G-100 resulted in a 97% loss of activity in the RIA test for HBeAg similar to the inactivation observed with the antigen associated with HBsAg. Repeated gel filtration on Sephadex G-100 in 3 M-NaSCN revealed that the residual antigenic activity was associated with molecules having a mol. wt. of 35000 and less, possibly representing contaminants resulting from incomplete separation during the initial gel filtration, or antigens released from larger complexes as a result of cleaving disulphide bonds.
The association of 'HBeAg-like' activity with IgG and albumin is supported also by results of polyacrylamide gel electrophoresis of proteins extracted from precipitin lines obtained after immunodiffusion of labelled antigens (partially purified by affinity chromatography of HBeAg-positive serum on a column of insolubilized anti-HBe) against unlabelled anti-HBe. The major labelled proteins detected after electrophoresis had the mobility (Fig. 4) and immunological specificity of albumin and the heavy and light chains of IgG, since 75, 46 and 50% of labelled components extracted from the appropriate regions of the gel were precipitated by antibodies to albumin and the heavy and light chains of IgG, respectively.

**DISCUSSION**

Our earlier studies demonstrated that Dane particles and tubular forms of HBsAg, isolated from sera positive for HBeAg, reacted with antibodies obtained from an anti-HBe-positive serum and from serum of rabbits immunized with Dane particles and tubules, and adsorbed on 20 nm spherical HBsAg particles to remove anti-HBs (Neurath et al. 1976). The small spherical HBsAg particles used in the study were prepared from sera negative for HBeAg as determined by immunodiffusion. The possibility of antigenic differences between these particles depending on the serum sources, as later indicated by Vnek et al. (1979) and confirmed in this study, was not considered in our original work. Subsequent studies (Gerin...
et al. 1978; Takahashi et al. 1978b) failed to detect HBeAg on the surfaces of the morphological forms of HBsAg, while Vnek et al. (1979) reported that the association with HBeAg could be demonstrated after treatment of HBsAg particles with detergents.

The present study contributes to reconciliation of these apparently divergent results by demonstrating that the additional antigenic sites associated with HBsAg are: (1) distinct from HBeAg, as characterized in preceding studies (Neurath et al. 1979; Tedder & Bull, 1979), and defined as a component of the nucleoprotein core of Dane particles (Neurath & Strick, 1979a; Ohori et al. 1979; Takahashi et al. 1979); (2) restricted to a subpopulation of HBsAg associated with albumin and IgG; (3) fully exposed for interaction with antibodies after treatment with a detergent by analogy with HBsAg-associated IgG (Stannard & Moodie, 1976).

Our results indicate the presence in HBeAg-positive sera of an antigen; (a) existing in both free form or in association with HBsAg; (b) reacting with antibodies present in anti-HBe-containing sera; (c) from which HBeAg proper cannot be cleaved off by 3 M-NaSCN; and (d) having the size of IgG (in 3 M-NaSCN), i.e. having properties described earlier (Neurath & Strick, 1977). Thus the duality of HBeAg discovered by Takahashi et al. (1978a) may not be only a reflection of association of HBeAg proper with host serum proteins but also a result of immunological heterogeneity of the 'e-system' recognized originally by Magnus & Espmark (1972).

The occurrence of both albumin and IgG in HBsAg associated with 'HBeAg-like' determinants, as well as in 'HBeAg-anti-HBe' precipitin lines may offer a clue to the nature of these determinants.

We considered the possibility that HBsAg might become associated with the albumin precursor proalbumin during biosynthesis in hepatocytes and that the 'pro-piece' of proalbumin (Brennan & Carrell, 1978) may correspond to the 'HBeAg-like' determinants. This possibility was excluded, since purified proalbumin did not have any inhibitory effect in the RIA test for these determinants (own unpublished data).

The presence of albumin in HBsAg is probably a reflection of the in vivo reaction of HBsAg-associated receptors (Neurath & Strick, 1979b; Imai et al. 1979) with albumin polymers. The HBsAg-albumin complexes may react with autoantibodies to albumin present in normal human sera (Wadsworth et al. 1973; Wager & Teppo, 1978) and specially in sera of individuals with liver disease (Hollinger & Dreesman, 1979). The resulting HBsAg-albumin-IgG complexes might elicit an antibody response to IgG idiotypes present in the complex (Klaus, 1978). Experimental verification of this hypothesis will depend on the availability of sufficient volumes of consecutive serum specimens from HBsAg-carriers who converted from HBeAg- to anti-HBe-positivity and developed antibodies to 'HBeAg-like' determinants associated with HBsAg.

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