Identification of Additional Antigenic Sites on Dane Particles and the Tubular Forms of Hepatitis B Surface Antigen

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SUMMARY

Additional antigenic sites, distinct from those present on spherical 20 nm diam. particles of hepatitis B surface antigen (HBsAg), are exposed on the surface of Dane particles and tubular forms of HBsAg. The immunological relationship of these sites to e-antigen, an antigen detected earlier in HBsAg-positive sera from patients with chronic hepatitis, cirrhosis or acute hepatitis but not in healthy HBsAg-carriers, was established by immune electron microscopy and affinity chromatography. These findings suggest that e-antigen may be potentially useful in active immunization against hepatitis B.

INTRODUCTION

A new antigen(s) associated with infection caused by hepatitis B virus (HBV), but distinct from the spherical 20 nm diam. particles of hepatitis B surface antigen (HBsAg) in both immunological and physicochemical properties has been identified recently (Magnius & Espmark, 1972 a, b; Magnius, 1975). One of the immunological determinants of this antigen was designated ‘e’, and the name e-antigen (eAg) was chosen for this newly discovered marker of HBV infection. The presence of eAg seemed to be correlated with the infectivity of HBsAg-positive sera (Magnius & Espmark, 1972 b) in agreement with observations that: (1) only eAg-positive sera contained Dane particles (the candidate hepatitis B virus) in relatively high concentrations (Nielsen, Dietrichson & Juhl, 1974; our own unpublished data); (2) Dane particles were not detected in sera containing antibodies to eAg (eAb; Nielsen et al. 1974) and (3) blood from HBsAg- and eAb-positive donors could not be implicated so far in any reported case of post transfusion hepatitis (Magnius et al. 1975).

To explain these three findings, we considered the possibility that: (1) additional antigenic sites, distinct from HBsAg determinants, were exposed on the surface of Dane particles and (2) eAb or other antibodies possibly present in sera containing eAb reacted with the postulated antigenic sites. Recent data indicate that the different morphological forms associated with HBsAg (Dane, Cameron & Briggs, 1970; Almeida, 1972) vary in the density of exposed HBsAg determinants (Chairez et al. 1974) suggesting possible immunological differences between their surface determinants. This is supported by the occurrence in HBsAg-positive serum of complexes consisting of either Dane particles only (Moodie, Stannard & Kipps, 1974; Couleru et al. 1973) or of Dane particles and tubular (tadpole) forms (Field & Cossart, 1971; Yamada, 1974). The results presented here confirm the

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presence of additional antigenic determinants distinct from HBsAg on the surface of Dane particles and tubules, and indicate that these determinants are related to eAg.

METHODS

Purification and determination of HBsAg. Spherical HBsAg particles approx. 20 nm in diam. were purified from sera of HBsAg-positive blood donors by precipitation with polyethylene glycol 6000, followed by chromatography on insolubilized concanavalin A, molecular exclusion chromatography, rate zonal and isopycnic sedimentation as described before (Neurath, Prince & Lippin, 1974). HBsAg was determined by radioimmunoassay (RIA; Ausria II-125, Abbott Laboratories, North Chicago, Illinois). The relative concentration of HBsAg was determined from calibration curves relating radioactivity (ct/min) to the reciprocal of the dilution of a standard serum containing HBsAg in human serum negative for both HBsAg and antibodies to HBsAg (HBsAb).

HBsAg preparations enriched with respect to Dane particles and tubular forms were obtained by centrifuging 160 ml of human or chimpanzee sera positive for HBsAg and eAg at 20000 rev/min for 16 h in the Spinco rotor SW 25·2 (Beckman Instruments, Palo Alto, California). The pellets were resuspended in 28 ml of 0·14 M-NaCl-0·01 M-tris, pH 7·2 (TS), layered on top of 28 ml of 3·6 M-glycerol in TS and recentrifuged under the same conditions. The pellet was resuspended in 1 ml of TS, layered on top of 3·8 ml of 3·6 M-glycerol in TS and recentrifuged for 3 h at 35000 rev/min in the rotor SW 65. The final pellet, corresponding to approx. 106 RIA units of HBsAg, was resuspended in 1 ml of TS. The preparations consisted of 4 to 8% Dane particles, 19 to 48% tubular forms and 44 to 77% 20 nm spherical particles.

Detection and purification of eAg. Reference standards for identification of eAg and eAb were obtained from Dr L. Magnius, Karolinska Institutet, Stockholm, Sweden. eAg was detected by agar gel diffusion using 'Aus-tec' reophoresis plates from Abbott Laboratories. The peripheral wells were filled with 50 μl samples and the centre well with 20 μl of human eAb. The plates were kept at 24 °C for 24 h followed by additional incubation in a humid chamber at 24 °C for 24 to 72 h. Human eAb was prepared from serum positive for HBsAg and eAb from which most of lipoproteins had been removed by adding MgSO4 and sodium dextran 500 (Pharmacia, Uppsala, Sweden) to final concentrations of 0·1 M and 4 mg/ml, respectively. The serum was clarified by centrifuging and mixed with a saturated solution of (NH4)2SO4 in a volume ratio of 3 to 2. The precipitate was pelleted, resuspended in TS in a vol. corresponding to 1/5 of the vol. of the original serum and dialysed against TS. To separate eAb from HBsAg, 16 ml samples of the dialysed preparation were layered on top of 40 ml of 3·6 M-glycerol in TS and centrifuged at 22000 rev/min for 20 h in the rotor SW 25·2. The top 16 ml portions from each centrifuge tube were pooled and dialysed against 0·14 M-NaCl.

Purification of eAg was accomplished by affinity chromatography on columns of eAb linked to Sepharose 4B. The immunoabsorbent was prepared as described (Neurath et al. 1974). A column containing 250 mg of protein from the eAb preparation bound to 7·5 g of CNBr-activated Sepharose 4B (Pharmacia) was used for chromatography. Eleven ml of serum containing eAg, from which Dane particles and tubules had been removed by ultrasedimentation, was applied to the column. The column was incubated for 30 min at 37 °C and 1 h at 4 °C and then washed with 80 ml of TS. The adsorbed eAg was eluted with 0·2 M-NaHCO3-0·5 M-NaCl, pH 10·9. Fractions corresponding to the peak of optical density at 280 nm (OD280), which contained 0·43% of protein applied to the column, were
pooled, concentrated to 1 ml by ultrafiltration, layered on top of 1 ml of 3.6 M-glycerol and centrifuged at 21000 rev/min for 16 h in the rotor SW 65 to pellet trace amounts of HBsAg unspecifically adsorbed to and eluted from the column. The final preparation contained 0.01% of HBsAg applied to the column and gave a precipitin line with eAb in immunodiffusion tests.

**Antisera.** One rabbit was immunized three times with 1 ml samples of HBsAg preparations enriched in Dane particles and tubules, prepared as described above, and mixed with an equal volume of complete Freund's adjuvant. The 2nd and 3rd injections of HBsAg followed 8 and 35 d, respectively, after the initial immunization. Blood samples were taken for antibody testing 18 and 46 d after the first injection. In order to separate HBsAb and antibodies to normal human serum proteins, possibly formed in the course of immunization, from eAb, 4 ml samples of the sera were applied to a column, pre-washed with TS, which had been prepared by linking 2 mg of 20 nm spherical HBsAg particles and 50 mg of human serum proteins to 3 g of CNBr-activated Sepharose 4B. The column was kept for 30 min at 37 °C and 1 h at 4 °C and washed with 40 ml of TS followed with 30 ml of 0.2 M-NaHCO3-0.5 M-NaCl, pH 10.9, to elute HBsAb. Fractions corresponding to the peaks of OD280 after elution with the respective buffers were adjusted to pH 7.2 when necessary, and pooled separately. Ammonium sulphate was added to each of the pools to a final concentration of 1.5 M. The precipitated globulins were pelleted by centrifuging, dissolved in 1 ml of TS and dialysed against TS. HBsAb was determined in the final preparations by RIA (Ausab test, Abbott Laboratories).

Another rabbit was injected twice with eAg mixed 1:1 with complete Freund's adjuvant. Each dose of eAg corresponded to the yield from a single affinity chromatography column. The second injection of eAg was given 2 weeks following the first one. Blood samples were withdrawn 10 d after each injection. Sera were fractionated by affinity chromatography as described before.

The IgG fraction from pooled sera of chimpanzees immunized with purified 20 nm spherical HBsAg particles was used to agglutinate all morphological forms of HBsAg. The HBsAb level of this preparation, tested by RIA at a 1:4000 dilution, corresponded to 72000 ct/min.

**Immune electron microscopy.** Samples (0.4 ml) of preparations containing IgG prepared by precipitation with (NH4)2SO4 from various antisera or from control sera were clarified by centrifuging for 10 min at 20000 rev/min in the rotor SW 65 and mixed with 0.1 ml of HBsAg suspensions enriched in Dane particles and tubules, which had been similarly centrifuged. The mixtures were incubated for 30 min at 37 °C and for 16 to 48 h at 4 °C, layered on top of 1 ml of 3.6 M-glycerol in TS and centrifuged for 20 min at 20000 rev/min in the rotor SW 65. The pellets were resuspended in 0.1 ml TS and observed under the electron microscope as already described (Neurath et al. 1975a). Sodium phosphotungstate (2% w/v, pH 6.8 to 7.0) was used as negative stain.

**Affinity chromatography of HBsAg on columns of insolubilized eAb.** Sepharose 2B was activated at pH 11.0 with CNBr dissolved in acetonitrile (Neurath et al. 1975b). Antibodies to eAg were linked to the activated gel as described for Sepharose 4B. Three ml columns were used for affinity chromatography. Purified 20 nm spherical HBsAg particles (106 RIA units) or an equivalent amount of a mixture of all morphological forms of HBsAg, suspended in 1.5 ml of lamb serum to suppress non-specific HBsAg binding, were applied to the immunosorbent. The columns were kept at 37 °C for 30 min, at 4 °C for 1 h and then washed with 45 ml of 0.5 M-NaCl-0.01 M-tris, pH 7.2, followed with the same vol. of 0.2 N-glycine-HCl, pH 2.2. Two ml fractions were collected and assayed for HBsAg after
fourfold dilution in a 5 % (w/v) solution of bovine serum albumin. HB,Ag was also chromato-
graphed on control columns prepared by linking bovine IgG to Sepharose 2B.

RESULTS

Evidence that human sera containing eAb react with an envelope component of Dane particles and of tubular forms

Immune electron microscopy appeared to represent a convenient approach to determine whether Dane particles react with sera containing eAb. Only eAg-positive sera contain Dane particles in concentrations high enough for electron microscopical and biochemical studies. Therefore, it was necessary to separate these particles from soluble eAg, which was expected to compete with Dane particles for eAb. This was accomplished by repeated ultrasedimentation. The resulting preparations of HB,Ag, obtained from 4 different sera, contained, in addition to Dane particles, tubular forms and the 20 nm diam. spherical particles. All three morphological forms of HB,Ag were agglutinated together by addition of HB,Ab (Fig. 1 a). Occasionally, aggregates of a single Dane particle with several 20 nm particles were seen (Fig. 1 b). Similar results were obtained with all four HB,Ag preparations. On the other hand, addition of eAb free of any HB,Ab detectable by RIA to the same HB,Ag preparations resulted in the formation of small aggregates consisting of Dane particles and tubules (Fig. 1 c–f). Such aggregates were not seen when eAb was replaced with normal human or rabbit IgG, with 5 % (w/v) bovine serum albumin or with eAb pre-incubated for 30 min at 37 °C and for 16 h at 4 °C with an equal volume of eAg partially purified by affinity chromatography.

In order to obtain independent evidence for the interaction between eAb and Dane particles and tubules, purified 20 nm particles and a preparation containing all morphological forms of HB,Ag were chromatographed in separate experiments on columns of insolubilized eAb. Results presented in Fig. 2 show that only a minor proportion of the 20 nm spherical particles was adsorbed. On the other hand about 50 % of HB,Ag, as determined by RIA, was adsorbed to the column when a preparation consisting of all three morphological forms was chromatographed. This result indicated that, unlike the 20 nm spherical particles, Dane particles and tubules reacted with the insolubilized antibodies. To confirm that this reaction was immunologically specific, the same multicomponent HB,Ag preparation was chromatographed also on a column of insolubilized bovine IgG. Only 10 % of HB,Ag adsorbed non-specifically to this column. The fractions containing HB,Ag which did not absorb to either the eAb or the bovine IgG column were pooled separately and centrifuged three times for 3 h at 35,000 rev/min in the rotor SW 65. The final pellets were resuspended in 0.05 ml of TS and observed under the electron microscope. Dane particles and tubular forms were present abundantly in the concentrated effluent from the column of insolubilized bovine IgG while only very few tubular forms were seen in the effluent from the eAb column. The fractions eluted from each of the columns at pH 2.2 were not suitable for electron microscopical investigations since Dane particles and tubules are disrupted at this pH (Dreesman et al. 1972).

Additional evidence for antigenic sites distinct from HB,Ag on Dane particles and tubules, obtained with animal antisera

The reaction of antibodies from human sera containing eAb free of HB,Ab detectable by RIA with Dane particles and tubules indicated that each of these morphological forms had exposed antigenic determinants distinct from HB,Ag. To confirm this finding, a rabbit
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Fig. 1. Antigen-antibody complexes formed after addition of various antibodies to preparations containing all three morphological forms of HBAg. (a and b) HBAb; (c to f) human eAb; (g) rabbit antibody to all morphological forms of HBAg from which HBAb was removed by immunoadsorption (see Fig. 3); (h), rabbit eAb.
was immunized with preparations containing all particle types of HB_Ag. The resulting antisera were passed through a column consisting of insolubilized normal human serum and 20 nm spherical HB_Ag particles. Results in Fig. 3 show that HB_Ab adsorbed to the column and was subsequently eluted at pH 10.9. The eluted HB_Ab, as expected, agglutinated all morphological forms of HB_Ag. On the other hand, antibodies which did not adsorb to the column and did not contain any HB_Ab detectable by RIA (Fig. 3) caused a selective aggregation of Dane particles and tubules (Fig. 1g). The possibility that this aggregation was caused by residual HB_Ab undetectable by RIA was excluded by the finding that HB_Ab diluted to a concentration equivalent to 2000 ct/min (Fig. 3) failed to cause any aggregation of HB_Ag particles observable by electron microscopy. Similar results were obtained with sera from a rabbit immunized with a single dose or with 2 doses of soluble eAg partially purified by affinity chromatography (Fig. 1h). These sera reacted in immunodiffusion tests with eAg and with an HB_Ag preparation containing all particle forms, which had been pre-treated for 20 min at 24 °C with a 2 % (v/v) solution of the detergent Nonidet P-40 (Shell Chemical Co., Chicago, Illinois). Additional immunodiffusion tests revealed that eAb mixed 1:1 with the multicomponent HB_Ag preparation failed to react with soluble eAg. IgG from serum samples obtained before immunization of the rabbits did not cause agglutination of Dane particles and tubules.
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Fig. 3. Radioimmunoassay of HBsAb. •—•, ■—■, pH 10.9 eluates after affinity chromatography of sera from rabbits immunized with two (•—•) or three (■—■) doses of an HBsAg preparation containing Dane particles, tubules and 20 nm spherical forms; ○—○, □—□, portions of the same antisera which did not bind to the immunosorbent consisting of insolubilized human serum protein and 20 nm spherical HBsAg particles.

DISCUSSION

The concentration of Dane particles appears to be much higher in sera of immunosuppressed HBsAg carriers, patients with persistent antigenaemia and chronic hepatitis, polyarteritis nodosa and anicteric (preicteric) hepatitis than in sera of apparently healthy HBsAg carriers (Couleru et al. 1973; Nielsen, Nielsen & Elling, 1973; Yamada, 1974; Woolf et al. 1975). Independent studies revealed the presence of eAg in sera of patients belonging to the group mentioned before but not in sera of healthy HBsAg carriers (Magnius & Espmark, 1972a, b; Nielsen et al. 1974; Magnius et al. 1975; Trepo, 1975). Fluorescent antibody tests indicate that eAg is specified by HBV and is not a host component released from liver cells (Trepo, 1975). The apparent correlation between the presence in sera of Dane particles and of eAg was confirmed by direct analysis of serum specimens (Nielsen et al. 1974; own unpublished data) and raised the question of a possible immunological relationship between Dane particles and eAg. Our results also suggested a positive correlation between the concentrations of tubules and of Dane particles in various specimens. The simultaneous presence of Dane particles and of antibodies to their core component (HBeAb) in some sera and the presence of HBeAb in HBsAg carriers with liver disease (Purcell et al. 1974; Moritsugu et al. 1975) is in variance with the suggestion that eAg and the core component are related (Nielsen, et al. 1974) since Dane particles were not detected in sera containing eAb. Instead, it seemed more attractive to postulate an asso-
association between additional antigenic determinants distinct from HBsAg on the surface of Dane particles (Moodie et al. 1974) and eAg. Such association, suggested by our results, appears to be of potential importance for designs to prevent post transfusion hepatitis B. Passive administration of HBsAb or immunization with HBsAg in the form of 20 nm spherical particles was successfully used for prevention or attenuation of hepatitis B infections transmitted by means other than transfusion (Ginsburg et al. 1972; Krugman & Giles, 1973; Hilleman et al. 1975; Purcell & Gerin, 1975). However, the prevention of post transfusion hepatitis B by HBsAb still remains uncertain (Holland et al. 1969; Prince & Trepo, 1975). Immunity to eAg in recipients of blood containing HBV may perhaps be essential in preventing infection when HBsAg determinants (present predominantly on the most frequent 20 nm spherical particles) in the donor’s blood would overwhelm the recipient’s immunity to HBsAg.

Earlier (Magnius & Espmark, 1972 b) and more recent data (G. Le Bouvier, personal communication) indicate that eAg is not a single entity. Further studies will be required to clarify in greater detail the relationship between the eAg system and the envelope components of Dane particles and tubules.

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