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CHARACTERIZATION OF A NEW ANTIGEN–ANTIBODY SYSTEM ASSOCIATED WITH HEPATITIS B

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SUMMARY

The occurrence of a new antigen–antibody system was recently described in hepatitis B surface antigen (HB_sAg) positive sera with different specificities, one of which was designated *e*. These specificities are related as demonstrated by the presently utilized *e* reagents not discriminating between them in immunodiffusion tests. Hence, they are collectively referred to as *e* in this report. The occurrence of *e* antibodies in sera containing antibodies against hepatitis B surface antigen (anti-HB_s) is reported. The *e* antibodies were found to move as 7S globulin on rate zonal centrifugation. The *e* precipitate was demonstrated on the cathode side of the HB_sAg precipitate on immunoelectrophoresis consistent with *e* antigen migrating in the gamma-globulin region. The *e* antigen is reported to have an S value of $11{\cdot}6 \pm 0{\cdot}6$ (s.d.) and a buoyant density of $1{\cdot}291 \pm 0{\cdot}003$ (s.d.), hence differing from HB_sAg in all respects studied thus far.

INTRODUCTION

During the past few years considerable attention has been focused on the antigenic specificities of hepatitis B surface antigen (HB_sAg) (Levene & Blumberg, 1969; Raunio *et al.*, 1970; Kim & Tilles, 1971; Le Bouvier, 1971; Bancroft, Mundon & Russell, 1972). These investigations have enabled the identification of the *a*, *d*, *y*, *w* and *r* determinants of HB_sAg (Le Bouvier, 1971; Bancroft *et al.*, 1972). However, in some reports data have occasionally been presented which might be taken as evidence for the existence of antigenic specificities in HB_sAg positive sera carried on particles other than HB_sAg, i.e. representing other hepatitis-associated antigens (Raunio *et al.*, 1970; Le Bouvier, 1971; Grob & Jemelka, 1972). Such specificities might be designated extraparticulate, as, in contrast to the determinants *a*, *d*, *y*, *w* and *r*, they did not appear to be carried on the 22 nm HB_sAg particles. Thus Raunio *et al.* (1970) reported multiple precipitates between HB_sAg-positive sera and certain human

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sera containing antibodies against hepatitis B surface antigen (anti-HB_s). They reported the occurrence of antibodies in HB_sAg-positive sera. Particles with *d* specificity devoid of the *a* determinant were demonstrated by Le Bouvier (1971). Multiple precipitin arcs were documented by Grob & Jemelka (1972) in testing HB_sAg-positive sera in crossed immunoelectrophoresis. However, no physicochemical or epidemiological data on the antigens implicated by these findings have been presented. Not until the recent description of the *e* system (Magnius & Espmark, 1972) has the existence of extra particulate specificities been made more definite.

In previous reports a more detailed description has been given of the epidemiological and possible pathological significance of these new specificities and the corresponding antibodies (Magnius & Espmark, 1972; Magnius *et al.*, 1975). In this study the occurrence of antibodies against these specificities is reported in human sera containing anti-HB_s and the characteristics for the *e* specificity obtained by immunoelectrophoresis, density gradient centrifugation and equilibrium density gradient centrifugation.

MATERIALS AND METHODS

HB_sAg reagents

Reference HB_sAg reagents possessing antibodies against the *a*, *d* and *y* determinants have been described previously (Magnius & Epsmark, 1972; Iwarson *et al.*, 1973). An anti-*w* serum was obtained by immunization of a rabbit with a partially purified HB_sAg of the *adw*-specificity according to procedures previously described (Iwarson *et al.*, 1973). This serum was kindly tested by Dr Gilbert R. Irwin, Walter Reed Army Institute of Research, Washington, District of Columbia, and found to contain antibodies against the *a* and *w* specificities. The antiserum was rendered monospecific for *w* by absorption with an *adr* antigen (Begley) kindly provided by Dr Paul Holland, National Institutes of Health, Bethesda, Maryland.

E reagents

Determination of *e* antigen and anti-*e* was performed by immunodiffusion (Magnius & Espmark, 1972). The anti-*e* used was a serum (At 102) derived from a long-term carrier of HB_sAg of the subtype *adw* with no previous history of hepatitis. This serum was 5-fold concentrated by the following procedure. Two parts of serum were precipitated with one part of 39% (w/v) polyethylene glycol, PEG (Carbowax 6000, Union Carbide Chemicals Company, New York) in 0·02 M disodium hydrogen phosphate buffer adjusted to pH 7·0 with citric acid. The precipitate was dissolved in one fifth of the original serum volume of 0·025 M sodium acetate buffer, pH 5·8. The source of *e* antigen was the serum of a patient (M.M.) with chronic hepatitis, who was a long-term carrier of HB_sAg of the *adw* subtype (Magnius & Espmark, 1972). The serum was 5-fold concentrated by means of PEG precipitation. These *e* reagents gave a single precipitin line in immunodiffusion which demonstrated a reaction of identity with the *e* precipitate between the reference *e* reagents. Antibodies previously reported to give a reaction of identity with the middle precipitate between the reference *e* reagents (Magnius & Espmark, 1972), gave a reaction of identity with the *e* reagents utilized in this study. Since the reagents did not discriminate between *e* antibodies and antibodies directed against the related specificities, all antibodies are for convenience referred to as *e* antibodies or anti-*e* in this report.

Immunodiffusion

The gel consisted of 0·9% (w/v) agarose (L'Industrie Biologiques Française S.A., Genevilliers, Seine, France) dissolved in 0·01 M Tris–HCl, pH 7·6, supplemented with 0·1 M NaCl, 0·02 M EDTA and 0·1% (w/v) protamine sulphate. The gel also contained 2% (w/v) Dextran 250 (Pharmacia, Uppsala, Sweden).

Specific antisera against human IgG and IgM were purchased from Behringwerke AG, Marburg, Western Germany.

Immunoelectrophoresis

Immunoelectrophoresis was performed as described by Grabar, Uriel & Courcon (1960). The gel consisted of 1% agarose with the addition of 2% dextran 250 dissolved in 0·1 M glycine–sodium hydroxide buffer, pH 9·5. The buffer compositions were as follows: 0·05 M Tris acetate, pH 4·5, 0·05 M Tris acetate, pH 6·5 and 0·05 veronal buffer, pH 8·6, and finally 0·1 M glycine–sodium hydroxide buffer, pH 9·5. The serum specimen was obtained from a patient suffering from hepatitis B with HB_sAg of the specificity *adw*. To increase the strength of the precipitates, wells 3 mm in diameter were used for the antigen and the wells were filled twice. Troughs 2 mm in width were utilized for the antisera. Electrophoresis was performed at 100 V for 60 min in a Microzone Electrophoresis Cell (Beckman Instrumentals Company, Fullerton, California). The slides were immersed in 0·2 M phosphate buffer, pH 7·4, for 5 min prior to rinsing and filling the antiserum troughs. The slides were incubated in a humid chamber at 37°C for 2 days. After soaking in PBS and distilled water slides were dried and sequentially stained with 0·1% (w/v) Sudan Black B (E. Merck AG, Darmstadt, Germany) and 0·25% (w/v) Coomassie Brilliant Blue R Michrome number 1137 (Gurr, London SW14).

Rate zonal centrifugation of material containing anti-e

0·2 ml of serum was layered on 13·6 ml gradient ranging from 10% to 37% (w/v) sucrose in PBS and subjected to centrifugation at 4°C and 39,000 rev/min in a SW:40 rotor in a Beckman L2 Ultracentrifuge (Spinco division, California) for 18 hr. The fractions were assayed for anti-*e*, IgG and IgM in immunodiffusion.

Rate zonal centrifugation of material containing e antigen

Sixty millitres of serum containing *e* antigen was precipitated with 13% PEG dissolved in 0·02 M sodium hydrogen phosphate buffer adjusted to pH 7·0 with citric acid. The precipitate was dissolved in 6 ml of 0·025 M sodium acetate buffer, pH 5·8. The material was layered on three 60-ml preformed gradients ranging from 10–37% (w/v) sucrose in PBS and subjected to a preparative rate zonal centrifugation with an SW 25:2 at 20,000 rev/min and 4°C for 18 hr (compare with Fig. 1). Three-millilitre fractions collected from the bottom of the tube were precipitated with 13% PEG. The precipitate from each fraction was dissolved in 0·2 ml of sodium acetate buffer, pH 5·8 and assayed for HB_sAg and *e* antigen in immunodiffusion. The *e* antigen-containing fractions were pooled and dialysed against PBS at 4°C overnight. 0·4 ml of the dialysed material were subjected to rate zonal centrifugation on six 13·5-ml preformed gradients ranging from 15–40% sucrose in PBS at 39,000 rev/min and 4°C for 24 hr. 0·4-ml fractions harvested from the bottom of the tube were concentrated by PEG precipitation and tested for *e* antigen in immunodiffusion. Prior to PEG precipitation fractions were assayed for IgG and IgM by radial immunodiffusion according to Mancini,

Carbonara & Heremans (1965) with partigen (Behringwerke AG, Marburg, Germany), for use as sedimentation markers.

FIG. 1. Immunodiffusion slide with consecutive fractions from a preparative rate zonal centrifugation of an *e*-positive material tested for HB$_s$Ag and *e* antigen. Note the occurrence of *e* antigen in fractions numbers 14–17 without the simultaneous presence of HB$_s$Ag. Observe that the human antiserum against HB$_s$Ag utilized also detected the *e* specificity accounting for the two precipitates with fraction number 13.

Equilibrium density gradient centrifugation

0·4 ml of serum materials containing *e* antigen were subjected individually to equilibrium density gradient centrifugation in CsCl dissolved in PBS giving a specific gravity of 1·3 in an SW:40 rotor at 30,000 rev/min and 4°C for 120 hr. The specific gravity of the fractions were determined by weighing 100 microlitre samples in constriction pipettes.

Serum specimens

Eight human sera containing high titre anti-HB$_s$ were tested for antibodies against the *e* specificity in immunodiffusion. These sera were diagnosed as anti-HB$_s$-positive by routine screening of patients and staff at clinics for drug addicts and hospitals for infectious diseases.

For rate zonal centrifugation of anti-*e* seven sera were used. Six of these were derived from healthy persistent carriers of HB$_s$Ag of subtype *adw* and another serum devoid of HB$_s$Ag and anti-HB$_s$ was derived from an individual (RA), who contracted hepatitis B during the trackfinders outbreak 10 years previously. HB$_s$Ag-negative blood donors were consecutive specimens sampled from material sent for routine screening of blood units for HB$_s$Ag.

The serum used in immunoelectrophoresis was obtained from a hepatitis B patient (WR) positive for HB_sAg with specificity *adw* and with a high titre *e* antigen. The *e*-positive serum material utilized for the determination of the S value for *e* antigen was derived from a patient (I.G.) with chronic hepatitis, who was a persistent carrier of HB_sAg of the *adw* specificity. For the determination of the buoyant density of *e* antigen three different materials were used: (1) the standard *e* antigen; (2) a partially purified material also derived from the standard *e* antigen source; (3) the serum from a patient (K.B.) with HB_sAg-positive hepatitis of subtype *ayw*.

RESULTS

Antibodies against e

When eight human sera containing anti-HB_s were tested in immunodiffusion, it was noticed that one of the eight antisera gave rise to two precipitin lines with HB_sAg positive sera containing *e* antigen. The precipitate, which was not due to HB_sAg (i.e. as hitherto observed the precipitate closest to the antiserum well) gave a reaction of identity with the *e* precipitate (Fig. 2a). With four of the other sera containing anti-HB_s there was a deviation of the *e* precipitate indicating presence of antibodies against the *e* specificity in the serum containing anti-HB_s (Fig. 2b). Hence, antibodies against the *e* antigen were found in five out

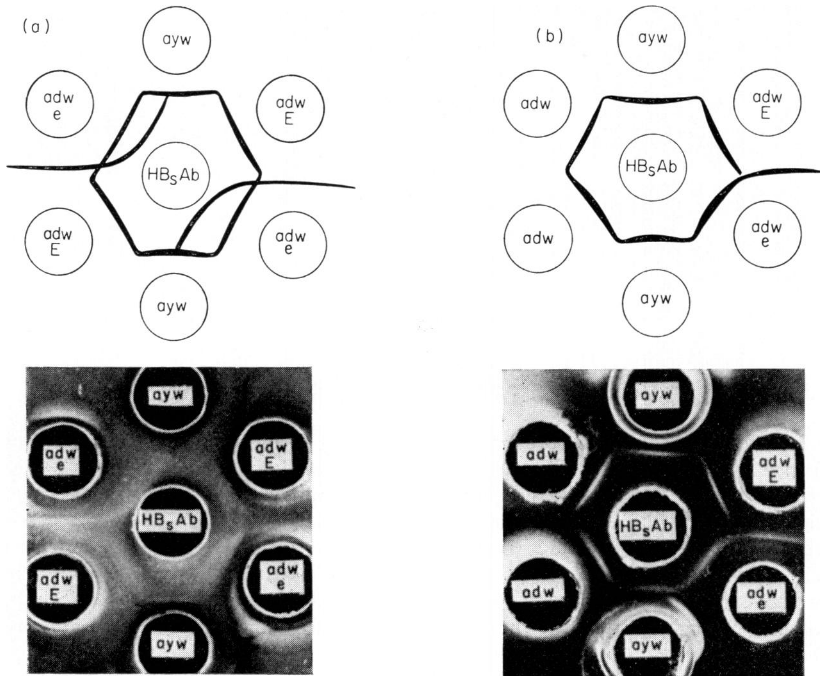

FIG. 2. Immunodiffusion slides demonstrating antibodies against the *e* specificity in human sera containing anti-HB_s. (a) Two precipitates are formed between the human serum containing anti-HB_s and the *e*-positive serum. Note the reaction of identity of the precipitate closest to the antiserum well with the *e* precipitate. (b) A human serum containing anti-HB_s gives a deviation of the *e* precipitate indicating presence of antibody against *e* in the antiserum against HB_sAg. The small roman letters signify antigenic specificities and E signifies anti-*e*.

of eight tested human sera containing anti-HB_s. However, in testing a thousand HB_sAg negative blood donors in the *e* system no *e* antibodies (nor *e* antigen) were encountered. Thus antibodies reactive against the *e* antigen in immunodiffusion do not seem to occur more frequently than anti-HB_s as detected by immunodiffusion in the particular donor population.

Since rheumatoid factor has been shown to occur frequently in hepatitis sera (Hoofnagle *et al.*, 1973), it seemed relevant to determine whether anti-*e* moved as 7S or 19S globulin on rate zonal centrifugation. Seven sera possessing antibodies reacting in the *e* system were centrifuged individually. In all seven sera the anti-*e* was found to move as 7S globulin.

The e antigen

To further substantiate that the *e* specificity was not carried on the HB_sAg particle, its behaviour was compared with that one of HB_sAg in immunoelectrophoresis, density gradient centrifugation and equilibrium density gradient centrifugation.

When comparing the *e* antigen with HB_sAg in immunoelectrophoresis the *e* precipitate was found on the cathode side of the HB_sAg precipitate at pH 4·5, 6·5, 8·6 and 9·5, i.e. at all pH values studied (Fig. 3).

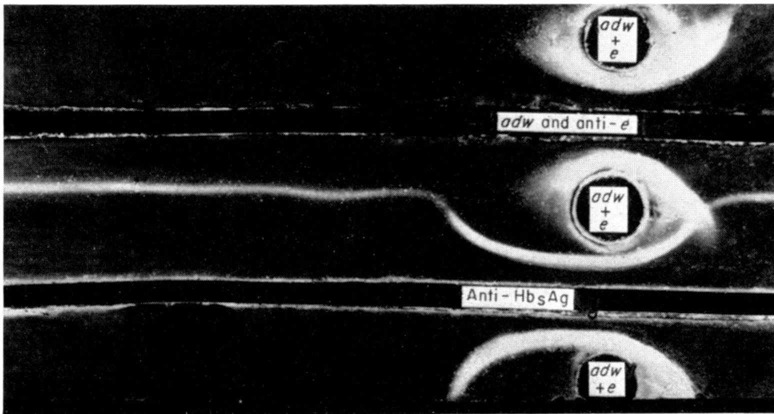

FIG. 3. Comparison of the *e* specificity with HB_sAg, subtype *adw*, in immunoelectrophoresis performed at pH 9.5. Cathode is to the right. The precipitate between the lateral troughs is due to the presence of HB_sAg in the serum containing antibodies against the *e* specificity. This precipitate demonstrates a reaction of identity with the HB_sAg precipitate formed with the tested sample. The *e* precipitate is found on the cathode side of the HB_sAg precipitate with which it demonstrates a reaction of non-identity. This stained slide was kindly photographed in dark field illumination by Dr Stig Jeansson, Virological Department, Karolinska Sjukhuset, Stockholm, Sweden, utilizing the fluorescent property of Coomassie brilliant blue (Jeansson, 1972).

In the rate zonal centrifugation studies on *e* antigen the S values were calculated from the positions of the *e* antigen-positive fractions in relation to the peaks of IgG and IgM according to the formula:

$$S_s = S_2 + [(h_s - h_2)(S_1 - S_2)]/(h_1 - h_2).$$

The S values for the internal references IgG and IgM were set to 6·8S and 19·6S respectively (Charlwood, 1963).

Due to the weak character of the *e* precipitate, it was not possible to obtain any titre of the *e* antigen. Providing that the peaks were symmetrical the S value obtained for the *e* antigen in six determinations would be $11{\cdot}6 \pm 0{\cdot}6$S (s.d.).

When three *e*-positive specimens were subjected to equilibrium density gradient centrifugation in CsCl the *e* specificity was found to band distinctly at a specific gravity of $1{\cdot}291 \pm 0{\cdot}003$ (s.d.) (Fig. 4).

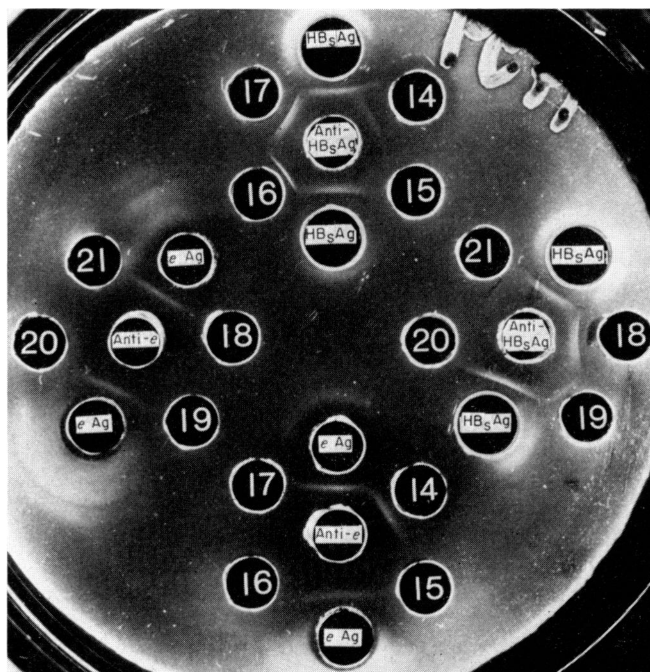

FIG. 4. Immunodiffusion slide with consecutive fractions from equilibrium density gradient centrifugation of *e*-positive material tested for HB_sAg and *e* specificity.

The *e* precipitate did not take stain with Sudan Black B, but we readily stained with Coomassie Brilliant Blue.

DISCUSSION

The physicochemical characteristics of the *e* antigen as reported herein all differed from the corresponding data of HB_sAg.

HB_sAg of the *ad* specificity was by Kim & Tilles (1971) reported to migrate as a β-globulin. This would agree with the *e* antigen migrating in the gamma-globulin region. Since double filling and large wells were used to allow the *e* precipitate to form in the immunoelectrophoresis, the separation was decreased. Therefore, it was not possible to rely on the direct demonstration that the *e* antigen migrated in the gamma-globulin region by filling anti-human IgG in the second lateral trough.

The considerably lower S value of *e* antigen indicated a smaller size than HB_sAg. The objection might be raised that the high density of the sucrose gradient utilized for the determination of the S value for *e* antigen might cause a retardation of the molecules sedimenting in the lower portion of the tube. However, such an effect did not seriously interfere with the S value determination, since by including the origin as a third point in the calculation an S value of $11{\cdot}9 \pm 0{\cdot}4$ (s.d.) was calculated, which is in good agreement with the determination utilizing only IgG and IgM as references.

The *e* antigen was not a lipoprotein as judged from its buoyant density of 1·29 in CsCl and non-staining with lipid stains, thus also in that respect differing from HB_sAg.

In a previous study (Magnius & Espmark, 1972) epidemiological evidence has been presented for the association of *e* antigen to hepatitis B, which has been considerably strengthened by the demonstration of *e* antigen in sera from patients incubating hepatitis B (Magnius *et al.*, 1975). This association does, however, not imply that the *e* antigen should necessarily be specified by the hepatitis B virus (HBV). Such an association might also reflect a specific host response to the replication of the HBV. Thus the causal relationship of *e* antigen to HBV remains unsettled.

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