Host specificity of a serum marker for hepatitis B: Evidence that "e antigen" has the properties of an immunoglobulin

(affinity chromatography/isoelectric focusing/IgG subclasses/idiotypes)

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ABSTRACT   A family of antigens, referred to collectively as e antigen (eAg), has been detected in sera of some individuals with liver disease who test positive for hepatitis B surface antigen. Studies on eAg partially purified by affinity chromatography on insolubilized antibodies to eAg revealed the following: (i) eAg has the physicochemical and immunologic properties of an immunoglobulin, predominantly of the IgG4 subclass, and (ii) specific antigenic sites, designated as e determinants, differentiate eAg from other immunoglobulins. We suggest that these determinants represent idiotypic determinants on antibodies that are formed in response to hepatitis B virus infection and that block the host's immune surveillance mechanisms by which virus proliferation is stopped.

Comprehensive evidence links infections caused by hepatitis B virus (HBV) with the occurrence in blood and liver of particles with common antigenic specificities—the hepatitis B surface antigen (HBsAg) (1–4). Some of these particles—first described by Dane et al. (5)—have a diameter of about 42 nm, contain an inner core (hepatitis B core antigen) enclosing DNA (6), and are generally believed to represent HBV. Magnius and Espmark (7) described a new complex of antigens, referred to collectively as "e antigen" (eAg). Though distinct from HBsAg in both immunologic and physicochemical properties (8), eAg is also specifically associated with HBV infections and appears to be a marker for the infectivity of HBsAg-positive sera (7, 9, 10); its persistence seems to be associated with chronic liver disease (11). Our attempts to characterize eAg, described here, led to the surprising finding that eAg has the properties of an immunoglobulin.

MATERIALS AND METHODS

eAg was partially purified by precipitation with polyethylene glycol 6000, followed either by affinity chromatography on columns of insolubilized antibodies to eAg (eAb) (12) or by chromatography on DEAE-Bio-Gel (13). eAb was prepared from human sera by sequential precipitation with Rivanol and Na_2SO_4 (14). Antibodies to individual human plasma proteins and to IgG subclasses were each linked to Sepharose 4B as described (15). Normal human serum or IgG was insolubilized by the same procedure.

Partially purified eAg (total amount of protein: 90–250 mg in 30 mM sodium citrate, pH 7.4) was submitted to isoelectric focusing (16). Fractions after isoelectric focusing were adjusted to pH 7, dialyzed against 0.14 M NaCl/0.01 M Tris·HCl at pH 7.2 (Tris/saline), concentrated about 10-fold, and tested for eAg. Identical results were obtained with either the cathode or the anode positioned in the bottom of the electrofocusing column.

To compare the sedimentation rates of eAg and an IgG

marker, 0.3-ml samples were layered on top of a 1.4–3.5 M glycerol gradient (4 ml) in an appropriate buffer. Centrifugation was performed at 65,000 rpm for 4 hr in the Spinco rotor SW 65. Fractions collected from the bottom of the centrifuge tubes were tested for eAg and protein.

F(ab')$_2$ and Fab fragments from samples containing eAg or eAb were prepared by digestion with pepsin (17) or papain (18), respectively. The F(ab')$_2$ fragments were isolated by chromatography on Sephadex G-150 (17). The Fab fragments were separated from Fc fragments by affinity chromatography on anti-Fab columns. F(ab'')$_2$ fragments from samples containing eAg were prepared by cleavage with CNBr (19), followed by affinity chromatography on anti-Fab columns.

eAg purified by affinity chromatography or proteins eluted with 50 mM $Na_2B_4O_7$ (pH 10.9) from extensively washed eAg-eAb precipitin lines excised from the agarose gel of rheophoresis plates were each labeled with ^{125}I (20) or with [^{125}I]-iodinated p-hydroxyphenylpropionic acid N-hydroxysuccinimide ester (Bolton-Hunter reagent; New England Nuclear, Boston, MA). Similar results were obtained by either of the two methods. Labeled eAg was further purified by isoelectric focusing. Fractions collected in the pH range of 4.9–5.7 were pooled, dialyzed against Tris/saline, mixed with 2 ml of lamb serum and with normal human serum proteins, precipitated from 10 ml of serum with polyethylene glycol 6000 (600 mg), and resuspended in 2 ml of Tris/saline. Labeled eAg from this mixture was repurified by affinity chromatography on a 15-ml column of insolubilized eAb.

The method for polyacrylamide gel electrophoresis described earlier (16) was used. Samples were dissociated in 6 M urea/87 mM sodium dodecyl sulfate/13 mM 2-mercaptoethanol and subsequently alkylated by dialysis against 25 mM Na_2HPO_4/6 M urea/87 mM sodium dodecyl sulfate/50 mM ICH_2CO_2H at pH 8.0 overnight.

Serial 2-fold dilutions of eAg-positive sera or of partially purified eAg were typed for Gm factors by the method of Borel et al. (21).

Methods for the determination of HBsAg by radioimmunoassay and of eAg and eAb by rheophoresis have been described in earlier reports (12, 13, 16).

RESULTS

Physicochemical Characterization of eAg. Results of molecular exclusion chromatography (Fig. 1) suggested heterogeneity of eAg. When 30 mM sodium citrate (pH 7.4) was used as eluant, eAg was detected in fractions in which globular proteins with molecular weights (M_r) between 300,000 and 650,000 would have been recovered. The apparent heterogeneity increased to a range of 160,000–650,000 M_r when 250 mM citrate (pH 7.4) was used as eluant. Increase of salt concentration thus resulted in an apparent partial conversion of eAg

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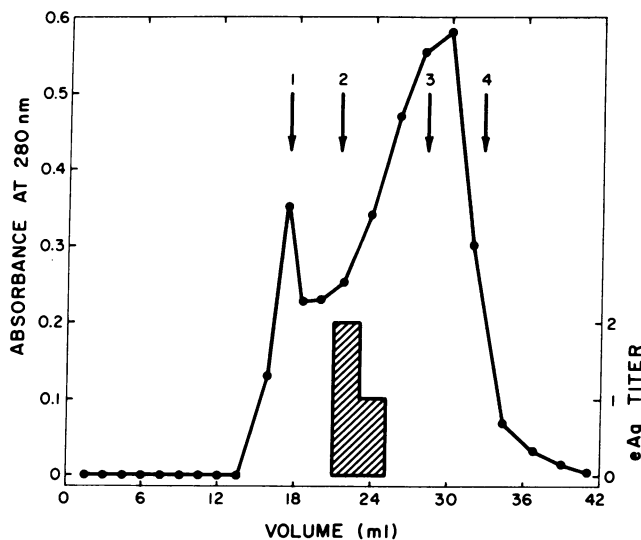
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FIG. 1. Molecular exclusion chromatography of eAg (1.5 ml) partially purified by precipitation with polyethylene glycol 6000 and ion exchange chromatography (13) on a 1.5 × 30 cm column of Ultrogel AcA 34 (LKB Instruments, Rockville, MD). 30 mM citrate (pH 7.4) was used for elution. One-milliliter samples were collected. Shaded area corresponds to fractions in which eAg was detected. Arrows indicate peaks of UV absorbance corresponding to the following M_r standards chromatographed in separate experiments: blue dextran (1), α_2-macroglobulin (2), IgG (3), and Hb (4).

to forms having lower M_r. This suggested that eAg was either associated with other serum proteins or was self-aggregated. It was expected that protein-protein associations would become suppressed either in alkaline solutions or at neutral pH and low protein concentration. To obtain more reliable estimates for the M_r of eAg, samples of eAg were adjusted to pH 10.9 and then chromatographed as before but with 50 mM $Na_2B_4O_7$ (pH 10.9) as eluant. Under these alkaline conditions eAg was recovered in the same fractions (adjusted to pH 7 for rheophoresis) as was an IgG marker chromatographed in a separate experiment. ^{125}I-Labeled eAg containing approximately $\frac{1}{10}^4$ the protein of

the unlabeled samples used before was chromatographed at neutral pH (pH 7.2) using Tris/saline for elution. The peak of radioactivity coincided with the UV absorbance peak corresponding to the unlabeled IgG marker.

Rate zonal sedimentations of unlabeled eAg at pH 10.9 and of labeled eAg at pH 7.2 revealed that the s values of eAg and of the IgG marker were similar. Two precipitin lines were observed when neutralized fractions were tested for eAg. This suggested that the two antigens e_1 and e_2 (22) have similar s values at pH 10.9.

Isoelectric focusing revealed that eAg was electrophoretically heterogeneous (Fig. 2). The distribution of eAg in the pH gradient varied for different serum samples and was within the pH range of 4.9–7.5. The largest portion of eAg was recovered between pH 5.0 and 5.7. Two distinct precipitin lines were observed when fractions within the latter pH range were tested for eAg.

Polyacrylamide gel electrophoresis of polypeptides extracted from eAg-eAb precipitin lines (labeled after extraction) revealed radioactivity to be predominantly associated with two distinct polypeptides having mobilities identical to those of heavy (H) and light (L) chains of an IgG standard (Fig. 3). Since eAb is an IgG (7), this suggested that eAg does not contain polypeptides distinct in M_r from those present in eAb. Polyacrylamide gel electrophoresis of labeled eAg purified by two affinity chromatography steps and by isoelectric focusing also revealed the presence of two predominant polypeptide species having the electrophoretic mobilities of IgG H and L chains.

Immunochemical Characterization of eAg. eAg did not adsorb to normal human IgG linked to Sepharose, but attached to columns of insolubilized eAb and eluted from these columns at pH 10.9. eAg was purified 17- to 60-fold in this way (Fig. 4). The major protein detected by immunoelectrophoresis in the pH 10.9 eluate was IgG. IgA was also detected by agar gel diffusion with anti-IgA. Immunoglobulins attached to the column even when HB_sAg- and eAg-negative sera were chromatographed. Since soluble eAb reacts neither with insolubilized normal human IgG nor with soluble IgG (in rheophoresis tests), antibodies other than eAb were probably involved in the
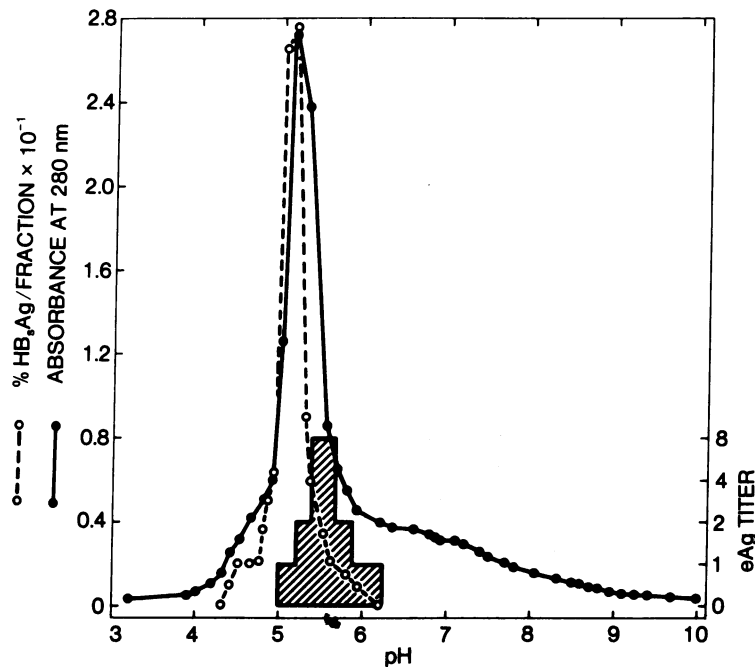

FIG. 2. Isoelectric focusing of eAg partially purified by precipitation with polyethylene glycol 6000 and ion exchange chromatography. Shaded area corresponds to fractions in which eAg was detected.
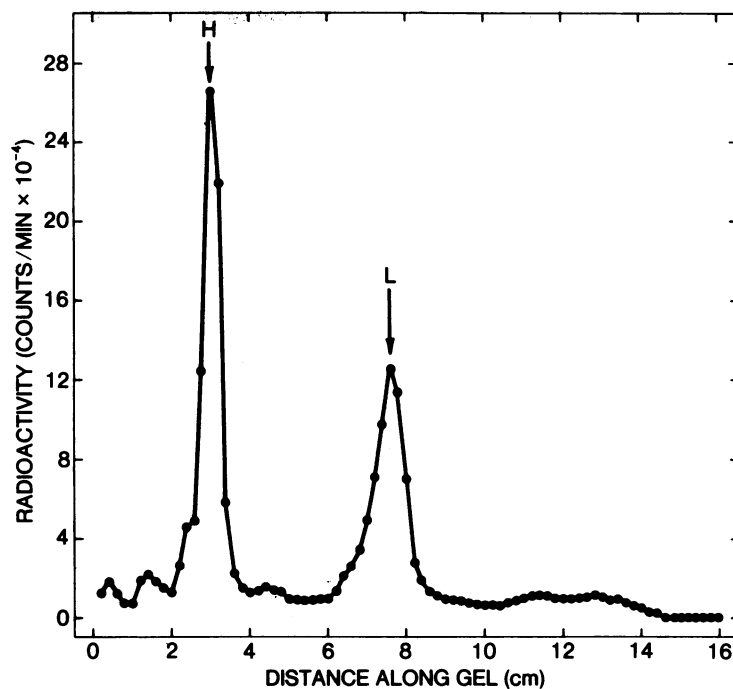
FIG. 3. Polyacrylamide gel electrophoresis of ^{125}I-labeled polypeptides from an eAg-eAb precipitate. Arrows indicate the positions of peaks of radioactivity corresponding to the heavy (H) and light (L) chains of IgG electrophoresed simultaneously in another portion of the gel slab.

adsorbtion of normal immunoglobulins. These antibodies may correspond to 7S rheumatoid factors present in normal and HB_sAg-positive sera (23, 24).

In apparent disagreement with the observation that antigenic determinants specific for eAg (referred to later as e determinants) are not present on normal serum proteins (7, 11), our experiments revealed that eAg has antigenic sites in common with a serum protein. This was discovered when attempts to separate serum proteins from eAg by affinity chromatography on insolubilized antibodies to normal human serum proteins were unsuccessful. eAg became adsorbed to such immunosorbent and was eluted at pH 10.9 together with other serum proteins. To identify the crossreacting plasma protein(s), we chromatographed eAg partially purified as described (Fig. 4) on columns of insolubilized antibodies to individual human plasma proteins (15). eAg was adsorbed to anti-IgG columns and, to a much smaller extent, to an anti-IgA and anti-IgE column. Anti-IgM columns did not adsorb eAg. eAg was also retained on an anti-Fab column. eAg was displaced from anti-IgG columns at 37° with a solution of pooled human IgG (15 mg/ml) in Tris/saline. These results suggest that e determinants are located on IgG molecules and probably also, to a much smaller extent, on IgA and IgE molecules.

eAg was retained on ion exchange resins with DEAE groups under conditions leading to elution of the bulk of IgG (13, 25), in accordance with the much lower average isoelectric point of eAg (Fig. 2) as compared with IgG (26). This suggested that the e determinants were predominantly located on a subpopulation of IgG with a low isoelectric point. The existence of such IgG subpopulation is evident from previous reports (25, 26). Comparative titration for Gm factors of an eAg-positive serum Gm(1,-2,4,12) and of a preparation of eAg partially purified from the same serum by affinity chromatography, and diluted to adjust the total IgG concentration to the level present in the serum, also suggested that e determinants are located on a subpopulation of IgG. The titers of Gm(1) and Gm(4) markers [located on IgG1 (27)] were both 20 times lower and the titer of Gm(12) [located on IgG3 (27)] was 2.5 times lower in partially

purified eAg than in the original serum. This indicated the relative enrichment of the eAg preparation with IgG having no Gm1, 4, and 12 factors, i.e., with IgG4 and/or IgG2. Since IgG4 has been reported to have the lowest average isoelectric point among the four subclasses of IgG (26), it seemed likely that e determinants are located on IgG4. Therefore, partially purified eAg was chromatographed on a column of insolubilized
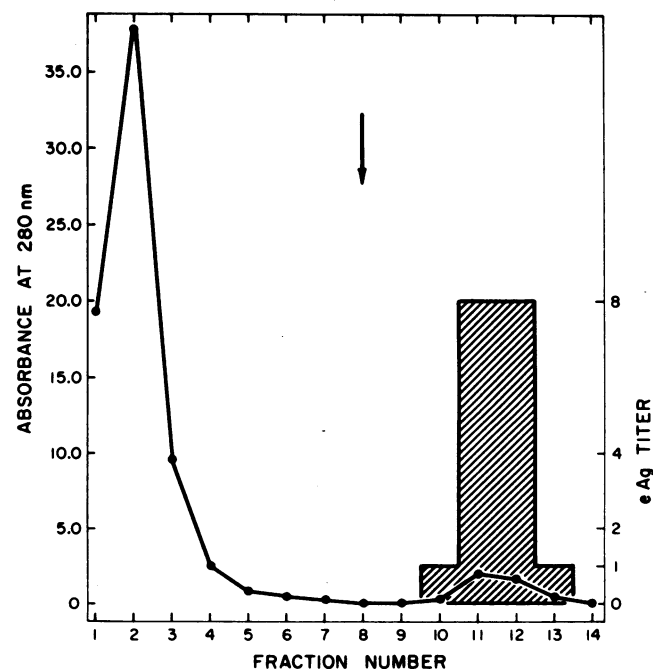

FIG. 4. Affinity chromatography of eAg on a column (5 × 6 cm) of IgG isolated from an eAb containing human serum and linked to Sepharose 4B. Shaded area corresponds to fractions in which eAg was detected. Arrow indicates the start of elution with 50 mM $Na_2B_4O_7$ (pH 10.9). eAg, precipitated with polyethylene glycol 6000 from 250 ml of serum and resuspended in 50 ml of Tris/saline, was applied to the column. Ten-milliliter fractions were collected.
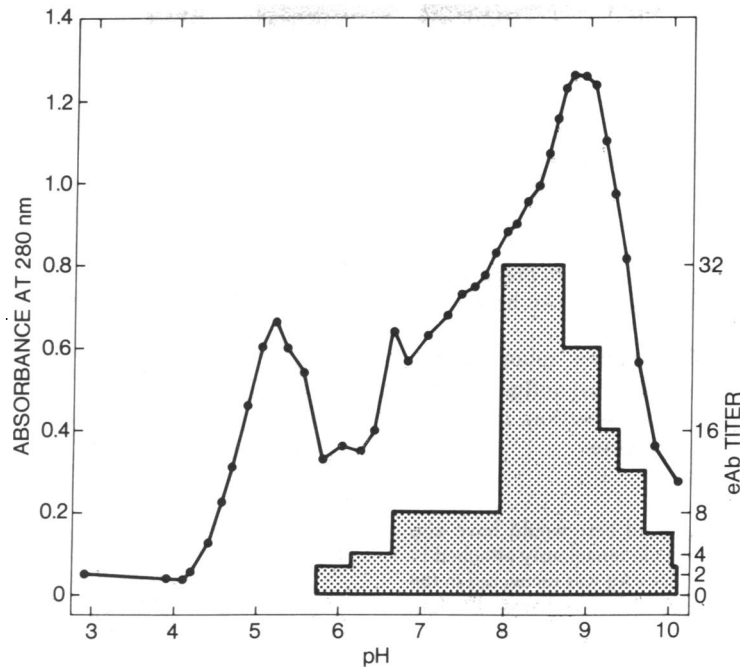
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FIG. 5. Isoelectric focusing of eAb from an eAb- and HB$_s$Ag-positive serum. Dotted area corresponds to fractions in which eAb was detected.

antibodies to IgG4. About 85% of eAg was adsorbed to the column and subsequently eluted at pH 10.9.

eAb was more heterogeneous than eAg and consisted of IgG molecules with much higher isoelectric points in comparison with eAg (Fig. 5). Very little, if any, of the eAb belongs to the IgG4 subclass, since no detectable attachment of eAb to an anti-IgG4 immunosorbent was observed.

The suggested presence on IgG of apparently HBV-specific e determinant suggests that the latter represent idiotypic determinants on antibodies formed in response to HBV infection. The anti-e sites on eAb could thus represent anti-idiotypic determinants. Since idiotypic and anti-idiotypic determinants are located in the variable region of immunoglobulins (27), the activity of fragments derived from preparations of eAg and eAb by cleavage with proteases or with CNBr was investigated. F(ab′)$_2$ fragments from eAb reacted with eAg in rheophoresis. The corresponding monovalent Fab fragments did not precipitate eAg, but inhibited the formation of precipitin lines between eAb and eAg. Attempts to demonstrate precipitating or inhibitory activity in F(ab′)$_2$ and Fab fragments derived from eAg using rheophoresis were unsuccessful, possibly due to the sensitivity of e determinants to proteases. However, F(ab″)$_2$ fragments derived from eAg by cleavage with CNBr retained their capacity to form precipitin lines with eAb.

DISCUSSION

The pathological manifestations of HBV infections are determined by differences in the host's immunologic response (28, 29). The association of eAg with active replication of HBV in HB$_s$Ag-positive liver disease and of eAb with the HB$_s$Ag carrier state without apparent liver disease (7, 9–11), except primary hepatic carcinoma (30), suggests that eAg plays a crucial role in determining the outcome of HBV infections. Our results suggest that eAg is an immunoglobulin with restricted heterogeneity. Such immunoglobulins may be formed as a consequence of repeated stimulation with the same antigen (31), as in the case of chronic infections (32, 33). Immunodeficiency can be a predisposing factor for the synthesis of such immu-

noglobulins (32). In this respect, it is of interest that hepatitis B may be associated with the synthesis of transient monoclonal immunoglobulins (32, 34).

The predominant association of e determinants with IgG4 may have special significance. Unlike other subclasses of IgG, IgG4 appears to attach neither to the C1q component of complement nor to macrophages (27). Therefore, IgG4 probably does not play a positive role in some of the immune mechanisms by which spread of viral infections is stopped (35), namely, antibody-mediated, complement-dependent virus neutralization and/or cytotoxicity against infected target cells and antibody-dependent destruction of virus or of infected cells by macrophages. On the other hand, antibodies of the IgG4 subclass, by competing with other antibodies for the same antigenic sites, could enhance the survival of infected cells or of virus released from the cells. For these reasons, eAg might play the role of antibodies protecting liver cells in which HBV replicates in analogy with antibodies enhancing the survival of allografts (36).

Both eAg and eAb appear to be antibodies formed in response to HBV infection. Seroconversion from eAg to eAb positivity was observed (37, 38). The fact that eAg and eAb react with each other and neither reacts with normal IgG suggests that their respective antibody-binding sites correspond to idiotypic and anti-idiotypic determinants. The presence of eAg in unrelated individuals would imply a broad crossidiotypic specificity for e determinants (27, 39).

Antibodies carrying idiotypes can induce the synthesis of autoanti-idiotypic antibodies within the same individual (40, 41). These antibodies may play a key role in the homeostasis of the immune response (42). The probable role of idiotype-anti-idiotype interactions in the development of human malignancies has been recently recognized (43). The importance of similar interactions for the outcome of HBV infections seems possible.

Antibodies reacting with the same antigen do not necessarily carry identical idiotypes (27, 40), and a shift of idiotypic characteristics may occur in the course of prolonged immunization (40). On the other hand, antibodies of identical idiotype

may belong to distinct immunoglobulin classes (44). These observations may be relevant to the reported heterogeneity of eAg (22).

Although eAg and eAb are each formed in response to HBV, the antigen actually inducing the synthesis of either eAg or eAb remains to be identified. Our recent findings that preparations of eAb free of antibodies to HB$_s$Ag agglutinated Dane particles and tubular forms of HB$_s$Ag (12) suggests the possibility that the antigen in question is located on these particles. However, it is also possible that rheumatoid factors present in preparations of eAb caused the agglutination since the particles have some IgG exposed to their surface (45).

The results presented here suggest another, more general, conclusion: immunoglobulins with individual antigenic specificity, synthesized in response to virus infection, may masquerade as genuine virus-specified antigens.

Note Added in Proof. The results described here would have also been obtained if eAg were a small molecule tenaciously attached to IgG. This possibility appears unlikely since eAg was recovered in the same fractions as IgG when chromatographed on Sepharose 6BCL in 4 M NaSCN or in 8 M urea.

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