

# Duality of hepatitis B e antigen in serum of persons infected with hepatitis B virus: Evidence for the nonidentity of e antigen with immunoglobulins

(type B hepatitis/infectivity/IgG/affinity chromatography)

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**ABSTRACT** Hepatitis B e antigen (HBeAg) is detected in the serum of some persons infected with hepatitis B virus. Owing to a close correlation of HBeAg and hepatitis B virus in the serum, it has been used as a practical indicator of infectivity. Two entities of HBeAg activity physicochemically different from each other were demonstrated in the serum of persons infected with hepatitis B virus. One was associated with a molecule that precipitated in 1.33 M ammonium sulfate solution, was larger than IgG, and had an electrophoretic mobility in the  $\beta$ - to  $\gamma$ -globulin regions and an isoelectric point of approximately pH 5.7. In contrast, the other HBeAg activity was associated with a molecule that was soluble in 1.33 M ammonium sulfate solution, was smaller than IgG, and had an electrophoretic mobility in the  $\alpha$ -globulin region and an isoelectric point at pH 4.8. In spite of their marked physicochemical differences, a line of antigenic identity was clearly observed for them when they were tested against antibody to HBeAg by a double immunodiffusion method. The HBeAg activity associated with the large molecule was completely removed by an affinity column of anti-IgG, whereas the activity of the small molecule was not. These results indicate that, in the serum, HBeAg exists as a molecule smaller than IgG and also in association with IgG.

Three antigens have been recognized in association with hepatitis B virus (HBV) infection. Hepatitis B surface antigen exists on the surface of 42-nm double-shelled Dane particles (1), presently accepted HBV, and also as 20-nm spherical and tubular particles. The core of the Dane particle, hepatitis B core antigen, has an antigenicity different from that of hepatitis B surface antigen (2, 3). In 1972, Magnus and Espmark (4) described another antigen which has been referred to as hepatitis B e antigen (HBeAg). HBeAg exists as a molecular entity separate from Dane particles (5) or 20-nm spherical and tubular particles (4), and it bears a distinct antigenicity. Among these three antigens related to HBV, HBeAg has attracted increasing attention of virologists and physicians because its presence in the serum closely correlates with the infectivity (6-8) and therefore can be used as a practical indicator of HBV. Studies of HBeAg, however, have been hampered by the low sensitivity of the methods for its detection. For the past 5 years since the original description of HBeAg by Magnus and Espmark (4), immunodiffusion has practically remained as the sole method available for its determination. We have recently developed a sensitive method to determine HBeAg by means of R-PHA which has proved to be at least 300 times more sensitive than the conventional immunodiffusion method (9).

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Recently, Neurath, and Strick (10) have reported that HBeAg had physicochemical and immunologic properties of an human immunoglobulin, predominantly of the IgG4 subclass. This paper describes the two different entities of HBeAg activity in the serum of persons infected with HBV and the nonidentity of HBeAg with immunoglobulins.

## MATERIALS AND METHODS

**Serum Containing HBeAg.** The serum samples of asymptomatic carriers of hepatitis B surface antigen were screened for HBeAg by immunodiffusion (4), and those with a high antigenic activity were selected.

**Determination of HBeAg.** HBeAg was determined by the reversed passive hemagglutination (R-PHA) method (9). Sheep erythrocytes were fixed by glutaraldehyde and coated with specifically purified antibody to HBeAg. Test samples were diluted in a microtiter plate, and 25  $\mu$ l of a suspension of detector cells was added. After incubation of the plate for 1 hr at 24°, the pattern of hemagglutination was read. The result was expressed by the highest 2-fold dilution ( $2^N$ ) of the sample that showed hemagglutination.

**Salt Precipitation.** Thirty milliliters of serum containing HBeAg (R-PHA titer,  $2^7$ ) was diluted with an equal volume of Tris-HCl, pH 7.5/0.1 M NaCl/0.01 M EDTA (Tris-buffered saline), and precipitated by adding 30 ml of 4 M  $(\text{NH}_4)_2\text{SO}_4$  at 24° (final concentration, 1.33 M). The precipitate was separated by centrifuging the solution at 10,000 rpm for 15 min and washed twice with 1.33 M  $(\text{NH}_4)_2\text{SO}_4$ . The supernatant was further precipitated by adding 30 ml of 4 M  $(\text{NH}_4)_2\text{SO}_4$ , and the formed precipitate was separated by centrifugation. Each of these two precipitates was dissolved in 5 ml of Tris-buffered saline and extensively dialyzed against the same buffer.

**Gel Filtration.** A column of Sephadex G-200 ( $2.6 \times 76$  cm; bed volume, 400 ml) was equilibrated with Tris-buffered saline. After application of the sample, the column was eluted with the same buffer and 9.5-ml fractions were collected in the cold.

**Isoelectrofocusing.** The sample was added to the Ampholine carrier ampholyte solution (1%, LKB-Produkter AB, Sweden), and a gradient of pH ranging from 3.5 to 10.0 was established in a column (LKB 7900 Uniphor,  $25 \times 400$  mm; bed volume, 200 ml) by applying electricity at 900 V for 40 hr. Three-milliliter fractions were collected and tested for pH and, after dialysis against Tris-buffered saline, for HBeAg activity.

**Disc Electrophoresis in Acrylamide.** Disc electrophoresis

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; R-PHA, reversed passive hemagglutination.

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was carried out in acrylamide gels according to the method described by Davis (11). Pyrex glass tubes measuring  $5 \times 100$  mm were used. After application of the sample, a current of 1.5 mA per tube was run for 4 hr. The gel was then cut into 3.2-mm slices, and each slice was eluted by mincing extensively in 200  $\mu$ l of Tris-buffered saline.

**Affinity Chromatography on Immobilized Anti-IgG.** Goat anti-human IgG( $\gamma$ -chain) antiserum was purchased from Hyland (Costa Mesa, CA) and conjugated with Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), according to the method of Porath *et al.* (12). The gel was packed in a  $9 \times 30$  mm column. After application of 5 ml of sample, the column was washed with 50 ml of Tris-buffered saline. The elution was performed with 3 M NaI. Fractions (0.5 ml) were collected at a rate of 4.5 ml/hr during application, washing, and elution. They were dialyzed against Tris-buffered saline and tested for HBeAg.

**Gel Diffusion and Immunoelectrophoresis.** Ouchterlony double immunodiffusion was performed in 0.9% agarose dissolved in Tris-buffered saline containing 2% dextran T-500 and 2% polyethylene glycol (6000). Immunoelectrophoresis was carried out as described by Grabar *et al.* (13). The gel consisted of 1% agarose and 2% dextran T-500 in barbital buffer (pH 8.6,  $\Gamma/2$  0.05). After application of the sample, a current of 2.5 mA/cm was run for 120 min, and then antisera were delivered into the troughs.

## RESULTS

**Salt Precipitation of HBeAg in the Serum.** Thirty milliliters of serum containing HBeAg was fractionated by precipitation in 1.33 M  $(\text{NH}_4)_2\text{SO}_4$ . HBeAg activity contained in the original serum, as well as the activity recovered in the precipitate and supernatant of 1.33 M  $(\text{NH}_4)_2\text{SO}_4$ , is given in Table 1. Approximately 67% of HBeAg activity in the original serum was precipitated and 17% remained in the supernatant.

**Gel Filtration of HBeAg in the Serum.** Ten milliliters of serum containing HBeAg was fractionated by gel filtration on Sephadex G-200 (Fig. 1A). HBeAg appeared in fractions 13–28, distributing widely through three protein peaks that were identified as macroglobulin, IgG, and albumin, in order of decreasing size. The peak activity was in fractions 15–17, between protein peaks of macroglobulin and IgG, with a wide shoulder between the peaks of IgG and albumin. Fractions corresponding to the peak of HBeAg and those corresponding to the shoulder (no. 22–25) were pooled separately, concentrated, and subjected to gel filtration on the same Sephadex G-200 column (Fig. 1B and C). HBeAg activity appeared as a single peak for both the large and small molecular HBeAg preparations, indicating that HBeAg in the original serum was associated with two separate molecules of different sizes. When several serum samples containing HBeAg were analyzed by gel filtration, they were found to contain small and large molecular HBeAg activities with a variable ratio. Some of them had predominantly large molecular HBeAg, and others contained mainly small molecular HBeAg.

In order to correlate the solubility in salt with the molecular size, HBeAg activities soluble and insoluble in 1.33 M  $(\text{NH}_4)_2\text{SO}_4$  (Table 1) were chromatographed on the same Sephadex column. HBeAg activity in the precipitate was found to be associated with the large molecule, the peak of which appeared at the identical position as the large HBeAg activity in the serum. In contrast, HBeAg activity in the supernatant appeared as a single peak at the identical fraction as the small HBeAg activity in the serum.

Table 1. Salt precipitation of HBeAg in serum

Sample	Volume, ml	HBeAg activity	
		R-PHA titer*	Total activity
Original serum	30	128	3840
Salt precipitation in 1.33 M $(\text{NH}_4)_2\text{SO}_4$			
Precipitate	5	512	2560
Supernatant	5	128	640

\* HBeAg was determined by the R-PHA method for the serial 2-fold dilution of the sample (9).

**Isoelectrofocusing of HBeAg.** Small and large molecular HBeAg preparations partially isolated by salt precipitation and then by gel filtration were tested for difference in isoelectric point (Fig. 2). Large molecular HBeAg showed a major peak at approximately pH 5.7 but, in addition, a minor peak was observed at pH 4.8. Small molecular HBeAg showed a single peak at pH 4.8, identical to the position of the minor peak in large molecular HBeAg preparation. Accordingly, the minor peak in Fig. 2A was considered to be contamination or dissociation of small molecular HBeAg in the large molecular HBeAg preparation. Therefore, fractions of large molecular HBeAg corresponding to the peak at pH 5.7 were pooled and concentrated. Similarly, fractions of small molecular HBeAg corresponding to the peak at pH 4.8 were pooled and concen-

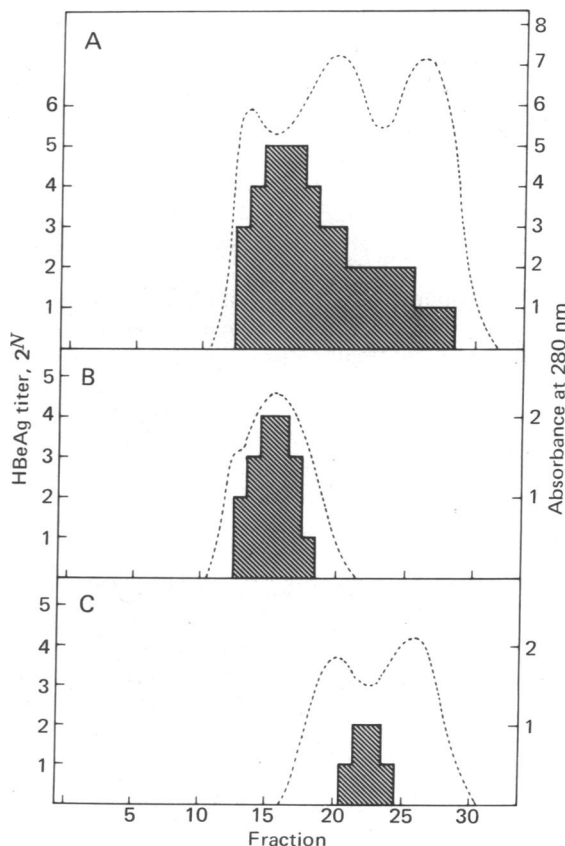


FIG. 1. Gel filtration of HBeAg. Shaded areas represent HBeAg activity tested by the R-PHA method. Dotted lines represent absorbance at 280 nm. (A) Pattern of whole serum. Fractions corresponding to the peak (no. 15–17) and to the shoulder (no. 22–25) of HBeAg activity were subjected to gel filtration on the same column (B and C).

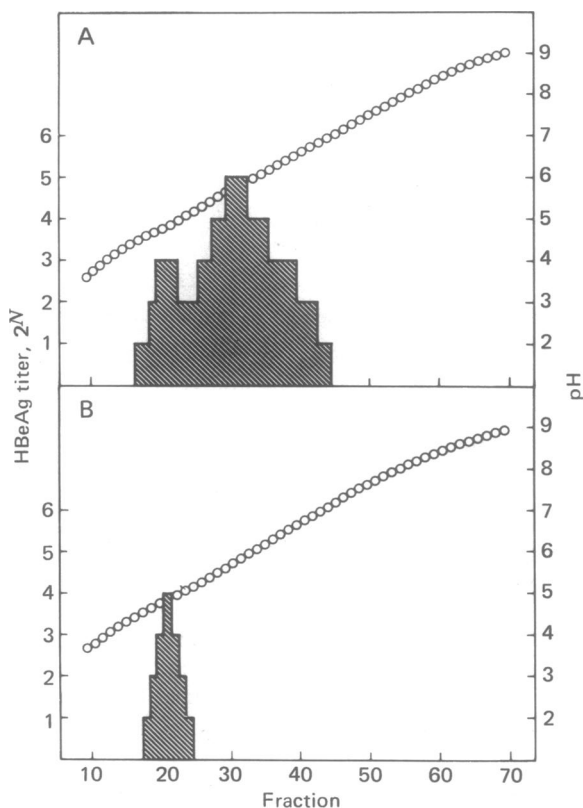


FIG. 2. Isoelectrofocusing of large (A) and small (B) molecular HBeAg preparations partially isolated by salt precipitation and gel filtration. Shaded areas represent HBeAg; open circles represent pH.

trated. Fig. 3 depicts the Ouchterlony immunodiffusion of these purified large and small molecular HBeAg preparations against antibody to HBeAg. A line of identity was observed for them. They were further subjected to disc electrophoresis, immunoelectrophoresis, and affinity chromatography on anti-IgG column.

**Disc Electrophoresis of Large and Small HBeAg.** Fig. 4 illustrates the disc electrophoretic patterns of preparations containing large and small molecular HBeAg, together with the pattern of original serum. HBeAg activity in the original whole serum appeared in two peaks completely separated from each other (Fig. 4A). Large HBeAg migrated to a position corresponding to one peak in the whole serum (Fig. 4B). Small HBeAg moved faster than large HBeAg, to the position corresponding to the other peak in serum (Fig. 4C).

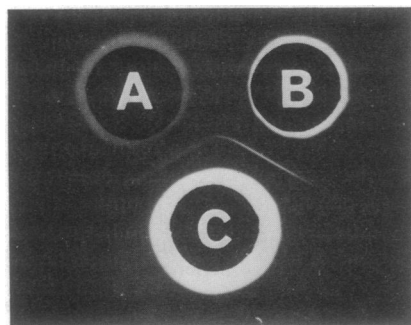


FIG. 3. Ouchterlony double immunodiffusion of preparations containing large and small molecular HBeAg. Wells: A, large molecular HBeAg; B, small molecular HBeAg; C, human serum containing antibody to HBeAg.

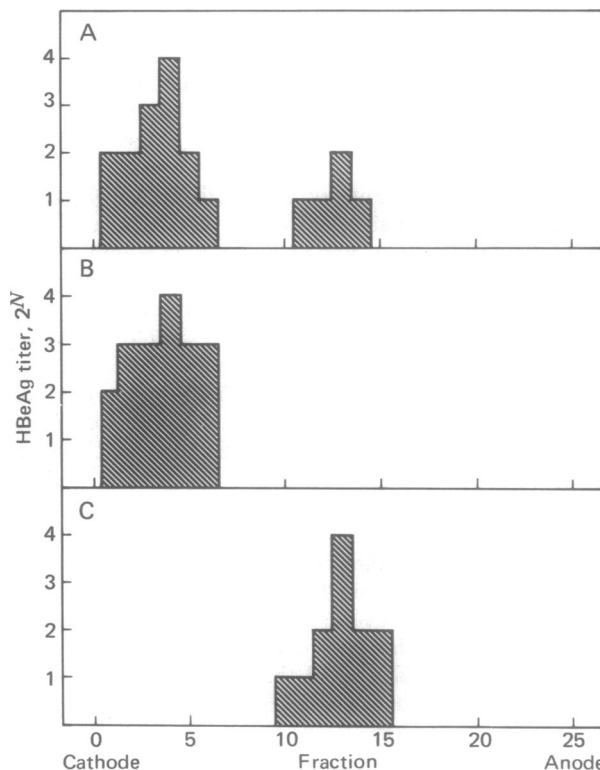


FIG. 4. Disc electrophoresis of whole serum (A) and large (B) and small (C) molecular HBeAg. Shaded areas represent HBeAg.

When large and small molecular HBeAg preparations were subjected to immunoelectrophoresis, a single arc was obtained for each of them. The arc of large HBeAg was between the  $\beta$ - and  $\gamma$ -globulin regions, whereas that of small HBeAg was in the  $\alpha$ -globulin region (Fig. 5).

**Affinity Chromatography of Large and Small HBeAg on Anti-IgG Column.** When large molecular HBeAg was applied to an affinity column of immobilized anti-IgG, all the activity was bound to the column; the bound HBeAg was recovered by elution with 3 M NaI (Fig. 6A). In contrast, little, if any, of small molecular HBeAg was bound to anti-IgG column (Fig. 6B).

### DISCUSSION

The clinical and epidemiological significance of HBeAg has become increasingly evident. Although it exists as a molecular

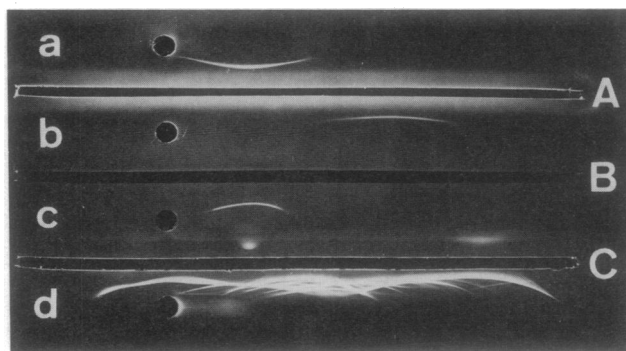


FIG. 5. Immunoelectrophoresis of large and small molecular HBeAg. Wells: a, large molecular HBeAg; b, small molecular HBeAg; c, purified hepatitis B surface antigen; d, normal human serum. Troughs: A, human serum containing antibody to HBeAg; B, rabbit anti-hepatitis B surface antigen antiserum; C, horse anti-whole human serum antiserum.

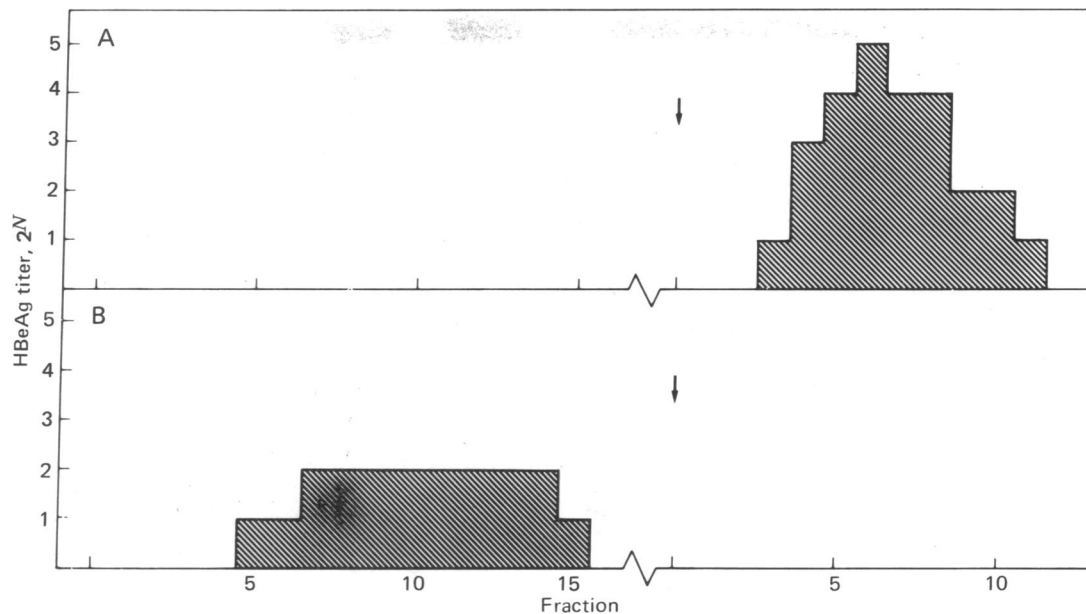


FIG. 6. Affinity chromatography of large (A) and small (B) molecular HBeAg on a column of anti-IgG. Shaded areas represent HBeAg activity. The arrow indicates the start of elution with 3 M NaI.

entity separate from Dane particles (presently accepted HBV) (5) or 20-nm hepatitis B antigen particles (4), a close correlation of HBeAg and infectivity has been reported both in vertical (6, 7) and horizontal (8) transmission of HBV. This view is supported by the facts that antigenic activity of the core of Dane particles (14), hepatitis B antigen-associated DNA polymerase (15, 16), and also DNA hybridizable to a  $^{32}\text{P}$ -labeled probe prepared from Dane particles (17) were found in the hepatitis B antigen-positive serum samples containing HBeAg but not, or rarely, in those containing antibody to HBeAg.

The present study has made it unambiguous that HBeAg does not have properties of IgG as previously reported by Neurath and Strick (10). Instead, HBeAg exists in the serum as two kinds of molecular entities physicochemically different from each other in terms of molecular size, solubility in a salt solution, electrophoretic mobility, and isoelectric point. Large molecular HBeAg appears to be in close association with IgG, because its activity was completely fixed by an affinity column of anti-IgG. In contrast, small molecular HBeAg was not bound by the anti-IgG column. It can be assumed that HBeAg originally exists as the small molecular form, and that large molecular HBeAg is the small molecular HBeAg bound to IgG, probably the antibody against HBeAg. Because large molecular HBeAg exists in the presence of free HBeAg, such antigen-antibody complexes would have been formed in the antigen excess region; large molecular HBeAg would still retain antigenic sites to react with antibody to HBeAg and allow its detection by various immunologic methods. To verify this hypothesis, however, antibody to HBeAg must be obtained after the dissociation of HBeAg from large molecular HBeAg.

Magnius (18, 19) found that HBeAg has a S value of 11.6 and a molecular weight of 300,000. Neurath and Strick (9) reported that HBeAg has the physicochemical and immunochemical properties of an immunoglobulin, predominantly of the IgG4 subclass, and suggested that the HBeAg determinant would represent idiotypic determinants on antibodies that are formed in response to HBV infection. In the light of the present study, the HBeAg activity observed by Magnius as well as by Neurath and Strick appears to be large molecular HBeAg. Indeed, Neurath and Strick reported the isoelectric point of HBeAg as

pH 5.0–5.7, very close to that of large molecular HBeAg in our study.

The failure of the former workers to identify small molecular HBeAg may partly be attributable to the low sensitivity of the immunodiffusion method they used. But, in addition, the HBeAg-positive serum samples they studied could have contained predominantly large molecular HBeAg with little small molecular HBeAg, which apparently escaped the detection. We found a variable ratio of small to large molecular HBeAg in the serum. Seroconversion from HBeAg to antibody to HBeAg has been recognized (20, 21). If the large molecular HBeAg were assumed to be an immune complex involving small molecular HBeAg and corresponding antibody, large and small molecular HBeAg would be formed and circulate in HBeAg-positive persons in variable ratios depending on the stage of HBV infection, humoral antibody responses of the host against HBeAg, and amount of HBeAg produced by HBV. The determination of HBeAg in the serum with a special attention to its duality, in terms of large and small molecular HBeAg, will further reinforce its clinical usefulness as an indicator of infectivity and prognosis in HBV infection.

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