Maternofetal Transmission of IgG-Bound Hepatitis B e Antigen

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Summary

Utilizing a highly sensitive radioimmunoassay, hepatitis B e antigen (HBeAg) was detected in the cord serum of eight out of nine babies born to mothers who carried hepatitis B surface antigen together with HBeAg. The titer of HBeAg in the cord serum was much lower than that in the corresponding maternal serum, and escaped the detection by the conventional micro-Ouchterlony immunodiffusion. HBeAg in maternal and cord sera was fractionated into free and IgG-bound moieties by salt precipitation, and their proportion was compared in four pairs. In every pair tested, both free and IgG-bound HBeAg were found in the maternal serum, but only IgG-bound HBeAg was detected in the corresponding cord serum. Based on these results, IgG-bound HBeAg can be transmitted through the placenta, but free HBeAg may barely pass the placenta despite its much smaller size.

Speculation

Among macromolecules present in the maternal circulation, only IgG is known to be transmitted into fetal circulation. The presence of IgG-bound HBeAg, but not of free HBeAg, in the cord serum of babies born to HBeAg-positive mothers suggests that IgG would be responsible for the maternofetal transmission of HBeAg.

Hepatitis B virus (HBV) transmits vertically, from infected mothers to their children. We have previously reported that children born to mothers who carried hepatitis B surface antigen (HBsAg) and were seropositive for hepatitis B e antigen (HBeAg) developed persistent HBV infection within 6 months after delivery, whereas children born to carrier mothers seropositive for antibody to HBeAg (anti-HBe) escaped persistent infection (13). Despite the apparent mother to child transmission of HBV, the exact route by which children acquire HBV infection remains uncertain. It has been repeatedly reported that HBsAg was not or rarely detectable in the cord serum of babies who were born to carrier mothers and later developed persistent HBs antigenemia (12, 14). We, as well as others, (3, 24) have not been able to find HBeAg even in the cord serum of babies who were born to HBeAg-positive carrier mothers and acquired persistent HBV infection.

Previous failure to identify HBeAg in the cord serum may relate to the insensitivity of the methods applied. By means of a sensitive radioimmunoassay, we have detected HBeAg in the cord serum of babies born to carrier mothers seropositive for HBeAg. Furthermore, HBeAg bound to IgG, but not free HBeAg, was found to be capable of passing the placenta.

MATERIALS AND METHODS

SUBJECTS

Eight pregnant women were identified who were asymptomatic carriers of HBsAg and seropositive for HBeAg. Their prepartum

serum and cord serum of their babies were tested in pairs for HBV-associated antigens and antibodies. One of the mothers (Case 6) labored twice, while she kept HBsAg and HBeAg at the age of 25 and 27, and was tested on 2 occasions. In total therefore, nine pairs of maternal and cord sera were available for the test. Criteria for asymptomatic carriers of HBV consisted of: (1) the presence of HBsAg in the serum by tests performed at least 6 months apart; and (2) no signs and symptoms of hepatitis with serum transaminase levels within normal limits. All of the babies were born after full-term of pregnancy, and free of any anomalies on visual inspection at birth. Out of nine babies in the series, four (Cases 2, 6-1, 7 and 8) developed the persistent carrier state of HBV within the first 6 months of their lives.

SEROLOGICAL TESTS

HBsAg was determined by reversed passive hemagglutination method (23). Antibody to hepatitis B core antigen (anti-HBc) was determined by immune adherence hemagglutination method (22). HBeAg was determined by immunodiffusion method. The gel for micro-Ouchterlony was made of 0.9% agarose dissolved in Tris-HCl buffer (0.01 M, pH 7.6) containing 0.1 M NaCl, 0.1% NaN₃, 0.02 M ethylene diaminetetraacetic acid, 2% dextran T500 (Pharmacia Fine Chemicals, Uppsala) and 2% polyethylene glycol-6000 (Iwai Kagaku, Tokyo). Wells of 3 mm in diameter were cut 2 mm apart in a hexagonal arrangement. The undiluted sample was tested against standard anti-HBe and HBeAg reagents and the fusion of precipitin lines was observed under an inverted microscope.

HBeAg was determined also by a modification of the microtiter solid phase radioimmunoassay described previously (9). Human anti-HBe to coat a polyvinyl microtiter plate (Cooke Engineering Co., Alexandria, VA.) and for radiolabeling was purified by affinity column chromatography and gel filtration in Sephadex G-200 (17). Into wells of the microplate coated with anti-HBe, 50 μ l of the test serum, undiluted or diluted 2- to 100-fold, were transferred. After an incubation at 24°C for 2 h and then at 4°C for overnight, the plate was washed 5 times with isotonic saline. One hundred μ l of a solution containing anti-HBe labeled with ¹²⁵I (100 ng, 7 μ Ci/ μ g) were delivered into each well and the plate was further incubated at 24°C for 2 h and at 4°C overnight. The plate was washed 5 times with isotonic saline, and the bound radioactivity was counted. Twenty-five serum samples from normal individuals without HBsAg or anti-HBs were tested for HBeAg by the radioimmunoassay, and their average binding was determined. The result was expressed by CPM of the test sample divided by the average CPM of normal sera. The sample was considered to be positive for HBeAg when the ratio exceeded a cut-off value of 2.1. Figure 1 depicts the binding curve of a HBeAg-positive serum serially diluted up to 10,000-fold. The serum showed a steep slope of binding and the ratio exceeded the cut-off value of 2.1 up to a 3,000-fold dilution. The other HBeAg-positive sera showed similarly steep binding curves.

Fig. 1. Microtiter solid phase radioimmunoassay of hepatitis B e antigen. The binding curve of a serum sample containing HBeAg is shown. The binding was expressed by the ratio of CPM of the sample to the average CPM of normal sera. HBeAg was regarded to be positive when the ratio exceeded the cut-off value of 2.1.

SEPARATION OF IgG-BOUND HBeAg FROM FREE HBeAg

HBeAg in the serum has been shown to occur in two physico-chemically and antigenically different forms, *i.e.*, free "small" HBeAg and IgG-bound "large" HBeAg (18). Free and IgG-bound moieties of HBeAg in the test serum were separated from each other by a salt precipitation in $(\text{NH}_4)_2\text{SO}_4$. To determine the concentration of $(\text{NH}_4)_2\text{SO}_4$ for an efficient separation, an aliquot of the serum containing both free and IgG-bound HBeAg was mixed with a varying volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution at 24°C. The formed precipitate was separated by centrifugation at $6,600 \times g$ for 15 min. Precipitate was then dissolved in a portion of water. Both precipitate and supernatant were dialysed extensively against Tris-HCl saline, concentrated, and subjected to disc electrophoresis in polyacrylamide gel. After electrophoresis, the gel (5×100 mm) was cut into 3.2 mm slices. Each slice was eluted with an aliquot of buffer, and the HBeAg activity was detected by the inhibition on hemagglutination of sheep erythrocytes coated with purified HBeAg by a limited amount of anti-HBe (17). Patterns of precipitate and supernatant of the serum fractionated at 33.3% saturation with $(\text{NH}_4)_2\text{SO}_4$ (A), 34.9% (B), 36.5% (C) and 38.4% (D), are illustrated in Figure 2. At low saturation with $(\text{NH}_4)_2\text{SO}_4$, some IgG-bound HBeAg activity remained in the supernatant (A and B), whereas at higher saturation, free HBeAg contaminated in the precipitate (D). Based on these observations, 36.5% saturation (C) was adopted for an efficient separation of free HBeAg from the IgG-bound form.

RESULTS

Table 1 summarizes the results of tests for HBV-associated antigens and antibodies in nine pairs of maternal and cord sera.

All the maternal sera were positive for HBsAg with titers ranging from 2^9 to 2^{12} . None of cord sera of their babies, however, revealed any HBsAg detectable by the hemagglutination method. The titers of anti-HBc in maternal sera were high (2^{10} - 2^{16}), in accordance with their persistent carriage of HBV (6). Cord sera were also positive for anti-HBc with titers essentially unchanged from those of corresponding mothers.

All the maternal sera were positive for HBeAg, but none of cord sera were positive as tested by micro-Ouchterlony immunodiffusion test. HBeAg in serum samples from mother and cord was tested at appropriate dilution by the radioimmunoassay. Except for Case 8, the same dilution was maintained within each pair to facilitate comparison; HBeAg in the cord serum of Case 8 was too low to be tested at the same dilution as the corresponding maternal serum. Except for Case 3, all of cord sera were positive for HBeAg by the radioimmunoassay, albeit the activity was much lower than that in the corresponding maternal serum. The maternal serum of Case 3 showed a low activity of HBeAg, therefore it had to be tested undiluted. The cord serum of Case 3, even when tested undiluted, was negative for HBeAg. A wide variation in the difference between maternal and cord sera indicated that not a constant fraction of HBeAg, but a particular population of HBeAg in the maternal serum would be able to pass the placenta.

Because there are two forms of HBeAg in the serum, free and IgG-bound HBeAg (18), we studied which form of HBeAg could pass the placenta more easily than the other by comparing the proportion of free and IgG-bound HBeAg activities between paired maternal and cord sera. Table 2 gives the results of such determination in four pairs for which enough amount of serum was available. HBeAg in the maternal serum was tested at 10-fold dilution, but owing to the low concentration of HBeAg, the cord serum was tested undiluted, with the provision that the dilution would not affect the ratio of free HBeAg to IgG-bound form. Both

Fig. 2. Separation of free and IgG-bound HBeAg in the serum by salt precipitation. The serum containing HBeAg was precipitated at 33.3% saturation with $(\text{NH}_4)_2\text{SO}_4$ (A), 34.9% (B), 36.5% (C), and 38.4% (D). Thereafter, both precipitate and supernatant were tested for free and IgG-bound HBeAg by disc electrophoresis in polyacrylamide gel. Shaded areas represent HBeAg determined by the inhibition of passive hemagglutination, and arrows indicate the positions of free and IgG-bound HBeAg.

Table 1. *Hepatitis B virus-associated antigens and antibodies in maternal and cord sera*

Case No.	Age	Maternal serum					Cord serum				
		HBsAg ¹	Anti-HBc ²	HBeAg			HBsAg ¹	Anti-HBc ²	HBeAg		
				ID ³	RIA ⁴	Tested at			ID ³	RIA ⁴	Tested at
1	25	10	13	+	10.7	1:100		14	5.9		1:100
2	27	11	10	+	12.4	1:100		10	2.8		1:100
3	32	9	10	+	23.6	undiluted		9	1.7		undiluted
4	30	10	16	+	25.4	1:2		16	7.8		1:2
5	28	10	12	+	26.8	1:2		13	3.9		1:2
6 a	25	12	15	+	15.4	1:100		16	6.9		1:100
6 b	27	9	16	+	13.6	1:100		16	2.5		1:100
7	24	11	10	+	10.0	1:100		10	2.4		1:100
8	30	11	15	+	12.6	1:100		15	11.2		1:10

¹ Hepatitis B surface antigen was determined by reversed passive hemagglutination method (23).

² Antibody to hepatitis B core antigen was determined by immune adherence hemagglutination method (22).

³ Hepatitis B e antigen was determined by micro-Ouchterlony immunodiffusion method.

⁴ Hepatitis B e antigen was also determined in the serum diluted as indicated by microtiter solid phase radioimmunoassay (see "Materials and Methods").

Table 2. *IgG-bound and free hepatitis B e antigen in maternal and cord sera*¹

Case No.	HBeAg in maternal serum			HBeAg in cord serum		
	IgG-bound	Free	Tested at	IgG-bound	Free	Tested at
1	13.0	2.7	1:10	21.0	<1	1:1
2	18.0	7.4	1:10	14.0	<1	1:1
7	12.2	7.4	1:10	14.6	<1	1:1
8	16.0	4.1	1:10	12.1	<1	1:1

¹ Serum samples were precipitated at 36.5% saturation with (NH₄)₂SO₄. Both precipitate and supernatant were reconstituted to the original volume and tested for HBeAg by microtiter solid phase radioimmunoassay. Maternal serum was tested at 1:10 dilution, but cord serum was tested undiluted due to low concentration of HBeAg contained.

IgG-bound and free forms of HBeAg were found in the maternal serum. In remarkable contrast, free HBeAg was not detectable in the cord serum, despite the detection of IgG-bound HBeAg with a titer comparable to that in the corresponding maternal serum. The possibility still remains that free HBeAg might have been present in the cord serum at a concentration too low to be detected by the method employed. It can be reasonably assumed, however, that the ratio of free HBeAg to IgG-bound form was much lower in the cord serum than that in the corresponding maternal serum. On the basis of these observations, IgG-bound HBeAg in the maternal circulation can be transmitted through the placenta into fetal circulation, but free HBeAg may barely pass the placenta in spite of its much smaller size.

DISCUSSION

The presence of HBeAg in the serum of mothers who carry HBV was closely associated with the vertical transmission of HBV (13). The presence of HBeAg in HBsAg-positive sera also signaled the infectivity in horizontal transmission of HBV (1). The experimental transmission of HBV infection to chimpanzees, sera from asymptomatic carriers containing HBeAg were at least 10⁶ times more infectious than those containing anti-HBe (15). These findings are reinforced by the correlation of HBeAg with immunologic and biochemical tags of Dane particles [the presently accepted HBV (2)], such as HBsAg and HBsAg-associated DNA polymerase (7, 11, 16). Recently, direct evidence for the association of Dane particles and HBeAg was provided by demonstrating polypeptides with HBeAg activity, P19 and P45, in their cores (19).

HBeAg has been shown to occur in the serum both free and in association with IgG molecules (18). There have been accumulating lines of evidence to suggest that IgG molecules bound to HBeAg would be antibodies directed to HBeAg. In a chimpanzee experimentally infected with HBV, free HBeAg appeared in the circulation first, gradually shifted to the IgG-bound form. Toward the end of infection, free HBeAg disappeared and the IgG-bound form represented all of the HBeAg activity. Finally, even IgG-bound HBeAg disappeared to be taken over by anti-HBe (20). Another line of evidence in support of the immune complex nature of IgG-bound HBeAg would be its phlogistic activity to induce membranous glomerulonephritis in children who carry HBV (21).

Previous attempts to detect HBeAg in cord sera of babies born to carrier mothers who were seropositive for HBeAg have been unsuccessful (3, 24). By means of a sensitive radioimmunoassay, we could detect HBeAg activity in the cord serum of babies born to carrier mothers with circulating HBeAg. Furthermore, IgG-bound HBeAg was found to be capable of passing the placenta, while free HBeAg was not in spite of its much smaller size than the IgG-bound form.

The transmission of IgG-bound HBeAg, but not of free HBeAg, from mother to fetus may have to do with the selective transport of IgG molecules through the placenta. Gitlin *et al.* (4) reported that among macromolecules in the serum of mothers they studied, only IgG could pass the placenta into the cord serum with a concentration increasing to reach that in the maternal serum toward birth. The other molecules such as albumin, transferrin and IgM did not pass the placenta appreciably. They further extended their finding and demonstrated the binding of IgG, but not of the other plasma proteins, with membrane fractions obtained from the placenta *in vitro*, and proposed the presence of a receptor for IgG on the placenta (5). There is a possibility that IgG-bound HBeAg would adhere to the surface of placenta by means of the receptor for IgG to be transmitted into the fetal circulation. McNabb *et al.* (8) showed that subclasses of IgG were different in their affinity to the placenta. In their study, IgG₁ and IgG₃ were equally affinitive to the placenta, but IgG₄ was much less reactive. Neurath *et al.* (10) reported that the subclass of IgG in molecules with HBeAg activity was IgG₄. Weak affinity of IgG₄ for the placenta, a large size of IgG-bound HBeAg, and inability for free HBeAg to pass the placenta would be among the factors responsible for the observed difference of HBeAg activity between maternal and cord sera.

The majority of the cases of vertical HBV transmission is thought to occur at the perinatal period; such transmission may be prevented by hepatitis B immune globulin given to babies after birth. There are occasional cases, however, in whom the transplacental passage of HBV has to be postulated. In two out of seven

children born to carrier mothers who eventually acquired persistent HBV infection, HBsAg appeared in their circulation as early as on the 5th and 13th day, respectively, after birth (12). The possibility of Dane particles being capable of passing the placenta when bound to IgG, just as we have observed for HBeAg, would deserve further considerations as a mechanism of the intrauterine infection of the fetus. Such IgG molecules could either be anti-HBs or heretofore unidentified antibodies directed to Dane particles. Dane particles bearing IgG, if present, might be trapped onto the placenta by means of the receptor for IgG, taken up by pinocytosis, and transmitted into fetal circulation to initiate HBV infection *in utero*.

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25. Abbreviations used in this paper: hepatitis B virus, HBV; hepatitis B surface antigen, HBsAg; antibody to HBsAg, anti-HBs; hepatitis B core antigen, HBcAg; antibody to HBcAg, anti-HBc; hepatitis B e antigen, HBeAg; antibody to HBeAg, anti-HBe.
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