Does Hepatitis B Virus Prenatal Transmission Result in Postnatal Immunoprophylaxis Failure?  
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The objective of this work was to evaluate whether postnatal hepatitis B immunization failure in children is caused by prenatal infections. A prospective study was conducted from October 2006 to September 2008. Fetal samples from HBsAg-positive mothers were retrieved by either amniocentesis or cordocentesis (percutaneous umbilical blood sampling [PUBS]). Hepatitis B virus (HBV) serologic markers (HBVM) and quantitative HBV DNA assays were performed to assess prenatal infection. All neonates were given combined HBV immunoprophylaxis after delivery. The newborns were followed up with HBV serologic testing at 1 year old. For the 252 pregnant women recruited, 16 fetuses were found to be HBV DNA positive, with all HBV DNA levels under $10^4$ copies/ml. HBsAg and HBV DNA detected in the uterus were uncommon and were expressed at low levels. In contrast to the case with prenatal statuses, neonatal serologies were more similar to their mothers'. The response rate of vaccination was 95%. Six children for whom immunoprophylaxis failed were born to HBeAg-positive mothers with high HBV DNA levels ($>10^8$ copies/ml), but only one of them was found to be positive for intrauterine HBV DNA ($8.5 \times 10^2$ copies/ml). The presence of intrauterine hepatitis B antigen and DNA does not indicate postnatal HBV infection and vaccination failure.

Perinatal hepatitis B virus (HBV) infection is the predominant mode of infection in most high-prevalence areas, such as Southeast Asia (3). Transmission from an HBV surface antigen (HBsAg)-positive mother to her infant may occur during labor by maternal blood contamination or in the prepartum period through transplacental leakage of the virus (10, 22, 24, 25, 27). The latter is considered a real intrauterine HBV infection, which might be a reason for postnatal vaccination failure (23).

Different kinds of fetal samples were used to assess intrauterine HBV infection in previous studies. Collection of amniotic fluids, cord blood, or placental tissues after labor to detect fetal infection is noninvasive and economical and is done in most studies (6, 24, 25, 27). Since postnatal samples were inevitably contaminated by maternal blood during labor, some authors have preferred using neonatal peripheral blood, which was collected prior to postnatal immunization, to detect intrauterine infection. This method was usually achieved by neonatal femoral venous puncture and might better reflect the intrauterine status (10, 19, 20, 21). However, this method cannot identify the time of infection (before or during labor) because maternal blood leakage into fetal circulation is unavoidable during labor (22). In fact, real intrauterine HBV transmission is hard to verify after labor.

Use of pure fetal samples obtained from invasive prenatal diagnosis to evaluate the fetal intrauterine status has been widely adopted. For infants of HBsAg-positive mothers, the risk of HBV transmission caused by invasive procedures is very low (1, 4, 5, 8). Furthermore, the invasive prenatal procedure seems to be an improved approach for extraction of purified fetal samples. Different procedures have been developed to avoid maternal blood contamination. For example, the first syringe is discarded due to the "blood-tip" possibility, and trans-placenta puncture is avoided if possible. Fetal blood samples (percutaneous umbilical blood sampling [PUBS]), collected from the floating cord, are verified by hemoglobin electrophoresis, which can identify the tiny maternal hemoglobin (HbA2) ingredient mixed into the fetal blood.

No study has been conducted to investigate the entire HBV status, including serologic markers and HBV DNA on the intrauterine samples. Because only a licensed prenatal diagnosis center is allowed to carry out this invasive technique, the relationship between prenatal HBV transmission and postnatal vaccination failure has never been reported (18). Although modern HBV immunoprophylaxis is very effective in preventing mother-to-infant transmission, the mechanism of vaccine failure is still uncertain. Intrauterine HBV infection is assumed to be a possible cause for postnatal immunoprophylaxis failure (23). The tolerogen theories supposed that the soluble antigens, such as HBsAg or HBeAg, might cross the placenta and induce immunological tolerance in utero, which is a possible reason for postnatal vaccination failure (11, 12, 19). However, all those hypotheses are based only on postnatal serologic investigations, which lack prenatal evidence.

The aim of our study was to address the relationship between intrauterine serological statuses and vaccine failure. For this purpose, fetal intrauterine samples obtained during prenatal diagnosis were tested for HBV markers (HBVM) and HBV DNA.

MATERIALS AND METHODS

Virological and clinical profile. This prospective longitudinal study was conducted at Taizhou Hospital of Zhejiang Province, a large prenatal diagnostic
TABLE 1. General characteristics of HBsAg-positive pregnant women

| Characteristic       | Mean value ± SD | No. (%) of positive carriers |
|----------------------|-----------------|-----------------------------|
| Maternal age (yr)    | 34.02 ± 5.63    |                             |
| Gestational wk at time of procedurea |                  |                             |
| Amniocentesis         | 20.81 ± 1.056   |                             |
| Cordocentesis         | 28.1 ± 3.68     |                             |
| Maternal HBV serologic status |        |                             |
| HBsAg (+)             | 252 (100)       |                             |
| anti-HBs (+)          | 92 (36.5)       |                             |
| anti-HBe (+)          | 0               |                             |
| anti-HBc (+)          | 136 (54.0)      |                             |
| HBV DNA (+)           | 224 (88.9)      |                             |
| HBV DNA level (log10 copies/ml) | 6.731 ± 1.84 |                             |

a For amniocentesis, n = 197; for cordocentesis, n = 55.

TABLE 2. HBVM and DNA levels at different gestational times

| Marker or DNA assayed (result) or parameter | No. (%) of positive samples or mean ± SD |
|--------------------------------------------|-----------------------------------------|
| Amniotic fluid (16–23 wks)                |                                         |
| Prenatalb                                |                                         |
| PUBS blood (24–32 wks)                    |                                         |
| Postnatal, peripheral blood (>32 wks)c    |                                         |
| HBV marker                                |                                         |
| HBsAg (+)                                 | 15 (8)                                  |
| Anti-HBs (+)                              | 51 (26)                                 |
| Anti-HBe (+)                              | 23 (12)                                 |
| All HBV markers (-)                      | 59 (30)                                 |
| HBV DNA (+)                               | 12 (6)                                  |
| HBV DNA level (log10 copies/ml)           | 3.052 ± 0.35  2.985 ± 0.33  4.416 ± 1.02* |

a, P < 0.05.
b For amniotic fluid, n = 197; for PUBS blood, n = 55.
c n = 234.

RESULTS

Patient profile. Invasive procedures were performed on 3,383 pregnant women. A total of 276 (8.16%) were found to be HBsAg carriers. Fourteen of them refused additional testing for intrauterine HBV infection. Consequently, 252 women with singleton pregnancies were enrolled in the study. Among them, 92 (36.5%) were HBeAg carriers and 147 (58.3%) were positive for HBV DNA. The maternal data are shown in Table 1. Amniocentesis was performed in 197 pregnant women between the 16th and 23rd weeks of gestation and in another 55 pregnant women, who underwent PUBS, between the 24th and 32nd weeks. For all women, fetal samples were successfully collected by a single puncture. Indications for intrauterine diagnosis included karyotyping (234 cases; 92.9%), molecular genetics testing (20 cases; 7.9%), and fetal blood biochemical parameters analysis (7 cases; 2.8%). Pregnancy was terminated in 5 cases because of either chromosomal anomaly (3 cases) or serious malformations (2 cases). During the autopsy procedure, blood samples were obtained from heart chambers of the terminated fetuses for HBV analysis. Two hundred twenty-six genetically normal infants were delivered at term and 3 with prematurity labor because of membrane rupture before 36 weeks. Peripheral blood was taken from all neonates for HBV testing. The other 18 women were lost to follow-up after delivery.

HBV infection in fetuses and neonates. During prenatal procedures, intrauterine specimens were collected for HBV test for all 252 fetuses. Low levels of HBV DNA were found in 12 amniotic fluid samples and 4 PUBS samples. The titer of fetal HBV DNA ranged from 320 to 6,000 copies per milliliter. HBV DNA-positive fetuses were limited to HBeAg-positive mothers with viral loads higher than 1,000,000 copies/ml. No difference in intrauterine HBV DNA levels was found between the middle trimester (in amniotic fluid) and the late trimester (in PUBS blood) (Table 2). Postpartum, the positivity rate and titers of HBV DNA increased significantly (Fig. 1; see also Fig. 4). Intrauterine HBsAg was found to be positive for 22 fetuses, while HBeAg was positive for 59 fetuses. Again HBsAg/
HBcAg-positive fetuses were observed only in HBcAg-positive mothers. Both HBsAg and HBV DNA were not commonly found in the prepartum period, but they were frequently detected in neonatal peripheral blood. However, such a tendency was not seen in term of HBcAg and most HBV antibodies. Similar to HBV DNA expression, the titers of HBsAg in the uterus were significantly lower than those in maternal serum. On the contrary, intrauterine HBcAg levels were more close to their mothers' (Fig. 2 and 3). Anti-HBc was the most common antibody to HBV present in the uterus, found in 59.9% of the prenatal samples. However, it seems that fetal immune systems cannot produce antibodies on their own, and all HBVM have a maternal origin. A comparison of HBV statuses of fetuses and neonates is shown in Fig. 4.

Follow-up and HBV infection in infants. All neonates were vaccinated against HBV at birth. One hundred ninety eight of them (84.6%) were visited and subjected to postvaccination testing for HBsAg and anti-HBs 1 year later. The overall re-
Response rate for the vaccine was 95%, with four negative results both for HBsAg and anti-HBs and six children persistently positive for HBsAg, which might be caused by postnatal immunoprophylaxis failure. All of the six persistently HBsAg-positive children were born to HBeAg-positive mothers with high serous HBV DNA levels. Table 3 shows the maternal and intrauterine HBV statuses for these children. All six children were found to have high HBV DNA loads in postnatal peripheral blood. It seems that the prenatal HBV status is not strongly associated with postnatal vaccination failure.

**DISCUSSION**

This study first presented whole HBV serologic parameters for prenatal fetal samples. However, we have not found a strong association between intrauterine infection and vaccination failure. Furthermore, the prenatal HBV infection rate was low in our study. In order to eliminate maternal blood contamination, modern prenatal diagnostic techniques were applied for fetal sample collection. Maternal blood contamination was effectively prevented, which was supported by the fact that HBV DNA and HBV antigens were not simultaneously found in the fetus.

**Prenatal diagnosis of intrauterine HBV infection.** The criteria to confirm prenatal HBV infection were not consistent among the previous studies (18). Since an incompetent fetal

| Case no. | Maternal DNA load (log_{10} copies/ml) | Prenatal | Postnatal |
|----------|--------------------------------------|----------|-----------|
|          | HBsAg | HBeAg | HBV-DNA | HBsAg | HBeAg | HBV-DNA |
| 1        | 10.801 | +     | +       | +     | +     | 5.705   |
| 2        | 8.665  | -     | +       | -     | +     | 5.732   |
| 3        | 8.663  | -     | +       | +     | +     | 6.209   |
| 4        | 9.982  | -     | -       | +     | +     | 6.255   |
| 5        | 11.079 | -     | -       | +     | +     | 6.321   |
| 6        | 9.672  | -     | -       | 2.929 | +     | 6.844   |

* All mothers of children for whom vaccination failed were doubly positive for HBsAg and HBeAg.
immune response hardly produce antibodies, the intrauterine antibodies detected reflected the maternal serologic status instead of the fetal status. Therefore, the presence of intrauterine antibody is not a suitable criterion for diagnosis of fetal HBV infection. It was assumed that HBeAg, like antibodies, can penetrate the placenta independently because of its small size and solubility. This hypothesis was confirmed in our investigation: 64 fetal samples from 89 HBeAg-positive mothers carried HBeAg, and 61% of them showed isolated intrauterine expression without corresponding to HBV DNA. Furthermore, the positive rates for HBV antibody and HBeAg were found to be similar between the prenatal and postpartum samples, which means that the soluble markers can easily pass through the placenta even without strong uterine contractions (in labor). Therefore, HBeAg is not suitable for prenatal HBV infection diagnosis because it probably acted as a tolerogen rather than an intrauterine immunogen. For the reasons mentioned above, in studies of the samples derived from prenatal diagnosis, HBSAg and HBV DNA were often used to indicate fetal antepartum HBV infection (18). However, few studies have detected all five HBV serologic markers and HBV DNA levels in the same samples.

In the present study, 22 fetuses were found to be HBsAg positive, and 16 fetuses carried HBV DNA. Both S antigen and DNA were present in the uterus at low titers. The mechanisms of fetal hepatocyte maturation demonstrated that HBV would not replicate in bulk during the early gestational weeks, which might help in interpreting our results (13). The fetus does not begin to develop a liver until 12 weeks of gestation. It is not known at what stage the development of the hepatocyte can allow HBV to infect. In a study of the in vitro system for HBV infection using human fetal hepatocytes (17), early-stage hepatocytes (10 to 12 gestational weeks) cannot be infected by HBV in vitro, while late-stage hepatocytes (>22 gestational weeks) demonstrated susceptibility to the virus without induced maturation. At the early stage of gestation, immature placenta might allow small quantities of soluble HBSag and free HBV DNA leakage into the uterus. Because full hepatocyte maturation might occur only in the late weeks of gestation and immature hepatocytes do not support whole HBV replication, postnatal vaccines combined with HBIG were still valid for that small quantity of HBV present in the uterus. This can also explain why fetal infection was rarely caused by invasive prenatal puncture in the middle trimester. On the other hand, the maternal antibodies can easily transverse the placenta and might play an important role in limiting intrauterine viral replication. This can explain the fact that HBSag and HBV DNA were detected only in the uterus of HBeAg-positive mothers whose serous antibodies levels were insufficient (2).

We have detected a significantly increased level of HBV DNA expression in neonatal blood. One possible reason is that the incubative virus replicated in bulk with fetal liver maturation during later gestation. However, we have not harvested the samples for HBV analysis at this stage due to ethical considerations. But in the Towers study (18), the researchers found that infection with HBV is also scarce even just before labor (>37 gestational weeks). Because the titer of HBV in neonatal blood is closely associated with labor time (22) and Cesarean section can obviously lower the viral level in newborns (9, 26), transplacental leakage might be the main route of HBV transmission, especially at the time of uterine contractions, as in labor.

**Mechanism of immunoprophylaxis failure.** The protection rate for postnatal vaccines in our study was 95%, which is similar to the finding of 93.1% in a previous report (14). However, this rate may fluctuate with maternal HBeAg status. Little is known about the mechanism of postnatal immunoprophylaxis failure. In retrospective research on neonates, the failure of postnatal immunoprophylaxis was significantly associated with maternal HBV DNA loads (16). Since most of the immunization escape occurred in children of HBeAg-positive mothers, exposure to E-antigen in the uterus during early pregnancy might allow it to act as a tolerogen, causing vaccine failure (11, 12, 19). In our study, half (3/6) of children with vaccine failure had been found to be intrauterine HBeAg positive. The sample is too small to confirm the theory, and more studies are needed in the future. In addition, immunization escape can be caused by viral mutations, usually on the S gene (7, 26, 27, 28).

Since combined postexposure immunoprophylaxis is very effective for HBV prevention in adults, some authors have supposed that vaccine failure originated with intrauterine HBV infection. However, in this study, only one of six vaccination-failed children was found to be HBV positive in the uterus. Differing from maternal serologic statuses, none of the fetuses were “double positive” with HBV antigens and HBV DNA in the prenatal specimens. Based on the evidence we discovered, our study does not support the hypothesis that prenatal infection is the cause of passive–active immunoprophylaxis failure.

However, we did not detect integrated HBV DNA in the prenatal samples. Some authors have assumed that DNA integration might be the reason for postnatal immunoprophylaxis failure (23). In a study of children with serious liver disorders, HBV DNA sequences were found integrated into liver cells alone, without HBV antigens and HBV DNA being present in the serum (15). Whether this kind of silent infection originates from transmission via the ovum or the early embryo will be difficult to confirm in humans due to ethical considerations. Nevertheless, in our study, no acute or fulminant hepatitis occurred in any infant during the 1-year follow-up. Most of the children born to HBsAg/HBeAg-positive mothers were more responsive to the vaccine. The incidence of such viral integration seems very low. However, HBV genotypes of this study population were mainly B and C, which may take different transmission routes from those in previous studies. In order to verify this hypothesis, longer follow-up will be needed.

In conclusion, prenatal intrauterine expression of HBV antigens and HBV DNA does not necessarily indicate postnatal HBV infection and vaccine failure. Similar to case with infection from exposure at birth, most of the seropositive fetuses showed successful seroconversion after postnatal prophylaxis.

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