IS THERE EVIDENCE FOR INTRAUTERINE HBV INFECTION IN NEWBORNS OF HEPATITIS B CARRIER MOTHERS ?

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Abstract. It has been proposed that some neonates infected with hepatitis B virus (HBV), acquire their infections *in utero* as demonstrated by HBV seromarkers in venous blood samples at birth. In this study, paired blood samples from 13 HBsAg-positive, 19 HBsAg-and HBeAg-positive, 2 HBsAg-negative mothers and 34 of their neonates, were drawn 24-72 hours after birth and tested for HBV-DNA in their peripheral blood mononuclear cells (PBMC). The presence of HBV-DNA in PBMC was detected in 69.2% (9/13) of HBsAg-positive mothers, 94.7% (18/19) of HBsAg- and HBeAg-positive mothers, and in none of their neonates. The conclusion from these results is that the evidence for hepatitis B infections occurring in neonates of hepatitis B carrier mothers *in utero* is uncommon.

INTRODUCTION

Hepatitis B virus (HBV) infection represents a major public health problem world-wide especially in East and Southeast Asia and in Africa. It is estimated that there are approximately 300 million chronic carriers of the virus world-wide and that 25% to 30% of carriers who acquire their infection in childhood will eventually die of chronic liver disease or liver cancer. About 1 million to 1.5 million HBV carriers die annually as a direct result of their infection. This makes HBV comparable to measles as a cause of death, and a more common cause of death than pertussis or tetanus, and of paralysis caused by poliomyelitis prior to the introduction of polio vaccination (World Health Organization, 1991). Perinatal infection is a significant route of transmission from carrier mothers to their babies (Stevens *et al*, 1975; Ghendon, 1987). Infection in infancy with hepatitis B virus usually leads to the chronic carrier state. Most infected infants appear healthy, but gradually develop chronic hepatitis as they grow up.

At present, no therapeutic measures have proved beneficial to the disease process after onset of carrier status or acute hepatitis. HBV infection can be prevented by an effective recombinant DNA hepatitis B vaccine (Andre, 1990). This vaccine is currently being used to prevent HBV infections in newborns. However, there have been reports of a very low number of high-risk infants of HBeAg-positive mothers with early hepatitis B immunization subsequently being infected and becoming hepatitis B chronic carriers (Stevens *et al*, 1987; Poovorawan *et al*, 1989; 1990).

Many hypotheses have been proposed for vaccine failure in newborns. The first was the emergence of a vaccine escape mutant strain of HBV (Harrison *et al*, 1991; Waters *et al*, 1992; Hino *et al*, 1995). The second was intrauterine infection or infection before vaccine immunization. To prove intrauterine infection, HBV-DNA must be demonstrable in the infected tissue of the fetus or newborn. However the liver tissue of neonates cannot be used to detect HBV-DNA for proving the cause of vaccine failure. HBV-DNA in peripheral blood mononuclear cells (PBMC) is an alternative way for determining the status of HBV infection (Pontisso *et al*, 1984; Wong *et al*, 1993). The purpose of our study was to assay for HBV-DNA in the plasma and PBMC of hepatitis B carrier mothers and their newborns for evidence of intrauterine infection.

MATERIALS AND METHODS

Population study

Thirty-four mothers positive for HBsAg with or without HBeAg at routine screening during antenatal care were selected into the study. After communicating the purpose of the study, written informed consent was obtained from all participating mothers. Blood samples were taken from mothers and newborns 24-72 hours after birth. The plasma and PBMC were separated from the heparinized blood and kept at-70°C until analyzed for the study.

Laboratory test

HBsAg was measured by using a commercially available ELISA test (Auszyme II, Abbott Laboratory, North Chicago, III). HBeAg was only tested in blood samples positive for HBsAg (IMx HBe, Abbott Laboratories technique).

Peripheral blood mononuclear cells (PBMC)

PBMC were separated from heparinized blood by density gradient centrifugation in Ficoll-paque (Phamacia Biotect). The PBMC were washed 3 times with PBS. A microscope was then used to make sure that there were adequate PBMC in the sample. The last PBS washing supernatant was kept for detecting any HBV-DNA contamination between plasma and PBMC.

DNA extraction

DNA extraction was performed by using the NaOH extraction method (Kaneko *et al*, 1989), a 10 µl aliquot of plasma was pipetted into a 0.5 ml microcentrifuge tube and incubated with NaOH at a final concentration of 0.1 M at 37°C for 60 minutes. The solution was next centrifuged for 15 seconds in a microfuge and neutralized with HCl at a final concentration of 0.1 M.

HBV-DNA detection

HBV-DNA was detected by an amplification method (nested PCR). The treated DNA sample was amplified in a 100 µl reaction volume containing 2.5 U of Taq polymerase (Perkin Elmer Cetus), and each of four deoxynucleotide triphosphates at a concentration of 200 µM, primer pair of X-101 (A), X-102 (S) and MD-26, X-101 (A) 1 µM each, 10 mM Tris, 1.5 mM $MgCl_2$. The X primer region in the first round was: X-101 (A) (5′ TCT GTG CCT TCT CAT CTG 3′), X-102 (S) (5′ ACC TTT AAC CTA ATC TCC 3′), and the second round primer was: MD-26 (5′ GTT CAC GGT GGT CTC CAT 3′) X-101 (5′TCT GTG CCT TCT CAT CTG 3′). The reaction was then performed for 30 cycles using primer X-101 (A) and X-102 (S), each consisting of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute. Then 2 µl of the PCR product was used for second round with primer (MD-26, X-101) for 30 cycles. The amplified DNA reaction mixture was then spun for 1 minute at 10,000 rpm, and the 10 µl of amplified DNA fractionated by 2% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide. The product band will show at 213 base pairs for the first round and at 74 base pairs for the second round. The gels were photographed on a UV light box (Fig 1).

RESULTS

A total of 34 pairs of blood specimens from newborns and mothers were used in this study. Two of the repeated maternal blood samples at 24-72 hours after birth were negative for HBsAg. Of the 32 specimens who were positive for HBsAg, 19 (59.4%) were also HBeAg positive. Three neonatal plasma specimens were positive for HBsAg (Table1).

HBV-DNA was detected in 87.5% (28/32) plasma samples positive for HBsAg. All HBeAg-positive women had measurable HBV-DNA in their plasma while 94.7% (18/19) specimens were positive for HBV-DNA in their PBMC. In HBeAg-

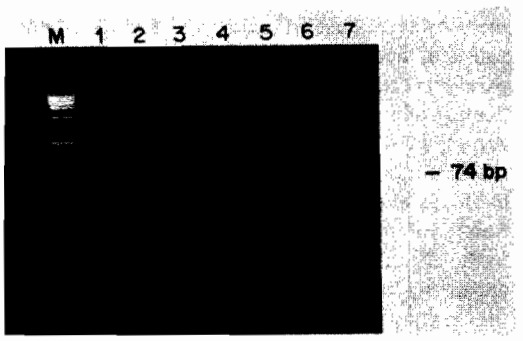

Fig 1–Agarose gel electrophoretic analysis of PCR products (74 base pairs) from plasma and PBMC were shown in lanes 1, 2, 4, 6,7. Molecular size marker (M) is Phi X 174 HaeIII.

Table 1

HBsAg, HBeAg, plasma and PBMC HBV-DNA among the 34 mothers and their neonates.

	ELISA		HBV-DNA		No.
	HBsAg	HBeAg	Plasma	PBMC	
M	+	+	+	+	18
N	-	nd	-	-	18
M	+	-	+	+	6
N	-	nd	-	-	6
M	+	-	-	-	4
N	-	nd	-	-	4
M	+	-	+	+	2
N	+	-	-	-	2
M	+	+	+	-	1
N	+	+	-	-	1
M	+	-	+	-	1
N	-	nd	-	-	1
M	-	nd	-	-	2
N	-	nd	-	-	2

nd=not done; M=mother; N=newborn

Table 2

The presence of HBV-DNA in the plasma and PBMC of HBsAg-positive mothers.

Mothers with HBeAg (No.)		Plasma HBV-DNA		PBMC HBV-DNA	
+ve		-ve	+ve	-ve	
Positive	(19)	19	0	18	1
Negative	(13)	9	4	8	5

negative mothers, 69.2% (9/13) had detectable HBV-DNA in their plasma and PBMC (Table 2). None of the PBS washings showed detectable HBV-DNA. We were unable to detect HBV-DNA in the plasma and PBMC of all newborns.

DISCUSSION

Vertical transmission of hepatitis B virus is an important route of transmission especially in Asia. This form of transmission may account for up to 50% of chronic carriers. The incidence of transmission is particularly high for neonates whose mothers are seropositive for both s and e antigens. Without immunoprophylaxis, 65-90% of newborns of e antigen positive mothers become chronic carriers (Wong *et al*, 1994; Beasley *et al*, 1983; Xu *et al*, 1985). Hepatitis B infections in newborns mostly happen during the perinatal period. Many studies on the interruption of vertical transmission of HBV by immunoprophylaxis report few newborns being infected and becoming chronic carriers (Stevens *et al*, 1987; Poovorawan *et al*, 1989; 1990). Iwarson *et al* (1988) have demonstrated previously that hepatitis B vaccine, when given immediately after exposure to the virus, can prevent hepatitis B virus infection in chimpanzees as effectively as post-exposure immunoprophylaxis after needlestick injuries. Potential reasons for vaccine failure in newborns may include prenatal infections, intrauterine infections, or HBsAg gene escape mutants (Harrison *et al*, 1991; Waters *et al*, 1992; Hino *et al*, 1995).

A study concerning intrauterine infections in the People's Republic of China showed about 8% of fetuses from terminated pregnancies in HBsAg positive mothers of 20 to 32 weeks gestation, had transplacental HBV infection (Li *et al*, 1986). Another study, which detected HBV-DNA in fetal liver tissue, revealed a higher infection rate (Tang and Yu, 1990), that contrasted with the extremely low vaccine failure rates in newborns vaccinated with recombinant hepatitis B vaccine starting at birth (Stevens *et al*, 1987; Poovorawan *et al*, 1990). However, detection of intrauterine infection by chorionic villi sampling (CVS) or fetal liver tissue examination is not practical. Pontisso and colleagues (1984) have demonstrated that HBV can be detected not only in infected liver tissue but also in PBMC. Yet, if HBV DNA exists in its integrated, *ie* latent form in the fetal liver during the early months of pregnancy, this might occur without detectable viral DNA in replicative form in mononuclear cells. This might in turn lead to immunological tolerance in a certain percentage of the infants and thus to poor immunization response. Furthermore Wong and colleagues (1993) have analyzed the results of HBV-DNA assays in the PBMC of patients with chronic hepatitis B virus infection and found HBV-DNA in 45.7% by Southern blot analysis. Moreover, HBV-DNA was found in both T and B lymphocytes, but not in macrophages. In the present study on HBV-DNA in HBeAg+ve mothers, it was found in all plasma samples and in 94.7% (18/19) of PBMC.In HBeAg-ve mothers it was found in 69.2% (9/13) of plasma samples and PBMC. In this study, HBV-DNA was not detected in plasma and PBMC from 32 newborns with HBV carrier mothers. In addition, in 3 of the newborns who were HBsAg positive, HBV-DNA was found neither in plasma nor in PBMC. Thus, HBV intrauterine infection should be an uncommon event. The presence of HBsAg in the plasma of newborns is probably more from the leakage of, or contamination from, their mother's blood, rather than from actual intrauterine infections in the newborns themselves.

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