Perinatal hepatitis B virus infection caused by antihepatitis Be positive maternal mononuclear cells

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Abstract

To investigate the infectivity of hepatitis B virus (HBV) from mothers to their newborn offspring, HBV-DNA in plasma and peripheral mononuclear cells from 28 antihepatitis Be positive, hepatitis B surface antigen positive carrier mothers was examined by a highly sensitive polymerase chain reaction/Southern hybridisation technique. HBV specific DNA was detected in three maternal mononuclear cell samples, but was absent in plasma. Two of four infants born to the three mothers with HBV-DNA positive mononuclear cells developed acute or fulminant hepatitis within three months after birth. Two infants were effectively prevented from infection with HBV by combined hepatitis B immunoglobulin/HBV vaccine administration. The 25 infants born to the HBV-DNA negative mothers were free of HBV infection within the next seven months to 3.5 years. These results suggest that latent infection with HBV in maternal mononuclear cells is responsible for perinatal HBV infection.

Since the establishment of the national project for prevention of mother to infant transmission of hepatitis B virus (HBV) with hepatitis B immunoglobulin (HBIG) and HBV vaccine, approximately 90-95% of the offspring of hepatitis Be antigen (HBeAg) positive HBV carrier mothers acquired protection from perinatal infection in Japan.^{1 2} In contrast, prophylaxis is not recommended for infants of HBeAg negative mothers, who comprise three quarters of pregnant hepatitis B carriers. About 10% of infants born to such mothers are reported to develop acute or fulminant hepatitis.³ As half of the cases of infantile fulminant hepatitis are due to perinatal HBV infection, and because most mothers of these patients are HBeAg negative, hepatitis B surface antigen (HBsAg) positive carriers,³ protective measures for these infants are urgently needed.

Although the infectivity of HBV is conventionally monitored by noting the presence of HBeAg and by measuring the activity of HBV specific DNA polymerase, recent advances in molecular hybridisation technology have provided more sensitive markers of HBV infection.^{4 5} HBV-DNA can be detected even in anti-HBe positive sera,⁶ and regardless of HBeAg status, the quantity of circulating HBV-DNA is directly related to HBV infectivity and to the activity of chronic liver disease.^{7 8} However, any mechanisms of HBV transmission from HBeAg negative, HBsAg positive

carrier mothers to their babies have never been documented.

In the present study, to explore the infectivity of anti-HBe positive, HBsAg positive carrier mothers to their offspring the expression of HBV-DNA was determined in plasma and peripheral mononuclear cells by a newly developed method, the polymerase chain reaction. Coupled with molecular hybridisation analysis the polymerase chain reaction can define attogram concentrations of HBV-DNA.⁹ The present results demonstrate the close association between the HBV-DNA expression in maternal peripheral mononuclear cells and the onset of acute or fulminant hepatitis in early infancy.

Subjects and methods

The serological markers of HBV infection were detected by reverse passive haemagglutination for HBsAg, passive haemagglutination for anti-HBs, immunoadherence haemagglutination for antibody to hepatitis B core antigen, and enzyme linked immunosorbent assay for HBeAg and its antibody (Abbott Laboratories).

We examined 28 mothers known to be anti-HBe positive, HBsAg positive carriers and 29 of their infants. The mothers were asymptomatic, without liver function abnormalities, during a one to five year follow up. All infants studied were negative for HBsAg and anti-HBs in cord blood, and serum HBsAg and anti-HBs concentrations and transaminase activities were measured every three months until age 1 year. At birth and at 2 months of age two infants were given HBIGs (pepsin digested Cohn fraction II HBIG, 200 IU/ml, provided by the Japanese Red Cross Central Blood Centre, Tokyo). They were also given HBV vaccine (HBsAg:10 µg protein/2.5 ml, Kitasato Institute, Tokyo) at 3, 4, and 6 months of age.

Samples of heparinised peripheral blood were layered onto Ficoll-Hypaque (Oncor Inc), and plasma and mononuclear cells were separated by centrifugation. Mononuclear cells were washed twice in phosphate buffered saline and then were immediately frozen at -80°C. DNA preparation, DNA amplification, and gel separation/Southern hybridisation methods have been previously described.⁹ Briefly, DNA was amplified in a 100 µl reaction mixture that contained 10 µl sample DNA, 2.5 units *Taq* DNA polymerase, 200 µmol of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, and dGTP), 1 µmol of each primer (primer 114: 5'-ACAGAGTCTAGACTCGTGGT-3', primer 586R: 5'-AAAGCCCTACGAACCACTGA-3'), 50 mmol/ml potassium chloride, 10 mmol/l

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Accepted 18 January 1991

TRIS-hydrochloric acid (pH8.3), 1.5 mmol/l magnesium chloride, and 0.01% gelatin. Amplification in a programmable thermal cycler (Techne) consisted of 25 cycles at 94°C for one minute (denaturation), 58°C for two minutes (annealing), and 72°C for three minutes (extension). Amplified DNA samples were fractionated by 1% agarose/2% Nusieve (FMC Bio-Products) gel electrophoresis, and the DNA was visualised by ultraviolet fluorescence after staining with ethidium bromide. After Southern blotting to 'Hybond N' membranes (Amersham), blots were hybridised with a cloned HBV-DNA probe labelled by random primed incorporation of digoxigenin labelled deoxyuridine triphosphate (Boehringer Mannheim). Alkaline phosphatase conjugated antibody to digoxigenin was then applied to the membrane and the colorimetric reaction was carried out on the membrane.

Results

HBV markers including HBsAg, anti-HBs, HBeAg, and anti-HBe, and transaminase activities of 28 carrier pregnant women at or shortly before delivery were determined. All sera were

HBeAg negative and anti-HBe positive, and none showed evidence of active hepatitis. During the next one to five years, serological markers and transaminase activities of these women were essentially unchanged. The HBV infection status of each father was also determined, and no evidence of infection was obtained (data not shown).

DNA was extracted from 200 µl of plasma and 10⁶ mononuclear cells of the mothers categorised as above. Each DNA was amplified by 25 cycles of the polymerase chain reaction. A sample was regarded as positive when DNA with a molecular weight expected for the amplified product (473 base pairs) was seen in agarose/Nusieve gel in three independent experiments. Southern hybridisation was used to confirm the specificity of the amplified DNA.

All of the 28 plasma samples were negative by both direct visualisation of amplified DNA and by Southern hybridisation, even when increased amounts of DNA were applied in the polymerase chain reaction mixture. In contrast, 473 base pair bands were detected in three of 28 mononuclear cell DNA samples (fig 1). The HBV specificity of these amplified DNAs was con-

Figure 1 Detection of HBV-DNA in peripheral mononuclear cells obtained from anti-HBe positive, HBsAg positive carrier mothers. Molecular weight (MW) marker, X174/Hae III digest; PC: positive control, HBV-DNA 3.2 kilobase pairs (kbp) template amplified with HBV primers; NC-1: negative control, Perkin-Elmer Cetus DNA amplification kit DNA template with HBV primers; NC-2: negative control, HBV-DNA 3.2 kbp template with Cetus kit primers. DNA in peripheral mononuclear cells was extracted as described in subjects and methods, and amplified with *Taq* enzyme in the presence of HBV primers. Amplified DNA was separated in 1% agarose/2% Nusieve gel.

Figure 2 Polymerase chain reaction and Southern hybridisation analysis of HBV-DNA in peripheral mononuclear cells obtained from anti-HBe positive, HBsAg positive carrier mothers. Molecular weight (MW) marker, PC: positive control, NC-1 and 2: negative controls. The DNA in the gel shown in fig 1 was transferred to nylon membrane and hybridised with digoxigenin-11-dUTP-labelled HBV-DNA (Dig-ELISA).

Detection of HBV-DNA by the polymerase chain reaction (PCR)

Case No	Mothers						Infants (prognosis, whether infected or not)			
	Serum					Plasma		Mononuclear cells		
	HBsAg*	HBs* antibody	HBc* antibody	Aspartate amino-transferase† (IU/l)	Alanine amino-transferase† (IU/l)	PCR + ethidium bromide staining	PCR + Southern blot hybridisation	PCR + ethidium bromide staining	PCR + Southern blot hybridisation	
1	13	—	11	23	19	—	—	++	+	Fulminant hepatitis; died Given HB1g/HBV vaccine; no infection } Sisters Given HB1g/HBV vaccine; no infection Acute hepatitis (aspartate aminotransferase 319 IU/l, alanine aminotransferase 245 IU/l)
	11	—	12	19	19	—	—	++	+	
5	9	—	14	23	7	—	—	+/-	+	
16	11	—	16	32	28	—	—	+	+	

*HBsAg by reverse passive haemagglutination (titre = 2ⁿ); HBs antibody by passive haemagglutination; and HBc antibody by immunoadherence haemagglutination (titre = 2ⁿ).
†Normal activities: aspartate aminotransferase <35 IU/l; alanine aminotransferase <30 IU/l.

firmed by Southern hybridisation (fig 2). The remaining 25 mononuclear cell DNA samples were negative by both DNA gel analysis and hybridisation (partly shown in figs 1 and 2).

The HBV markers and transaminase activities of the offspring were serially determined. Among the four infants born to the three mothers who carried HBV-DNA positive mononuclear cells, two developed acute or fulminant hepatitis within three months after birth (table). In the infant with acute hepatitis anti-HBs, and later anti-HBc, became positive, confirming HBV infection. One of the remaining two infants, who was the younger sister of the girl who died from fulminant hepatitis, was administered HBIg/HBV vaccine, and acquired protection against HBV as her anti-HBs was positive and anti-HBc negative after prophylaxis. The last one of the four was also administered HBIg/HBV vaccine because her mother seroconverted from HBeAg positive to anti-HBe positive shortly before delivery (between 29 and 36 weeks of gestation), and the infant also acquired HBV protection (table). In the other 25 infants, whose mothers were HBV-DNA negative in both plasma and mononuclear cells, no HBV markers and no increased activities of transaminases were detected in the next seven months to 3·5 years. Taken together these results strongly suggests that the association between the HBV-DNA expression in maternal peripheral mononuclear cells and the HBV infection of their infants is highly specific.

Discussion

The present study demonstrates that even asymptomatic, anti-HBe positive pregnant women may carry HBV-DNA positive mononuclear cells in the peripheral blood that can transmit infectious HBV to their offspring. This is the first report concerning the association between the presence of HBV-DNA in maternal mononuclear cells and the onset of infantile hepatitis. In Japan 1–2% of pregnant women are HBsAg positive carriers and three quarters of them are HBeAg negative. This percentage of HBV carriers seems relatively high, but the prevalence of HBV in Japan is about 20 times more than in Europe. Therefore it is important to pick up high risk infants and to find the most effective means of treatment. Our findings indicate that HBV determination in peripheral

mononuclear cells of such pregnant women will aid screening of high risk infants before delivery.

Infectivity of HBV was apparently due to mononuclear cells in maternal peripheral blood. In order to acquire the capability of viral replication, the infected mononuclear cells are probably transmitted first as intact cells to the infants, and then the HBV is activated. The possible route of mononuclear cell transmission could be transplacental passage around delivery. Previously, Desai and Creger reported that the maternal mononuclear cells definitely circulate in newborn babies.¹⁰ They labelled in vitro the maternal peripheral mononuclear cells by immunofluorescence and injected them back into the mother shortly before delivery. They detected the labelled maternal mononuclear cells in the infant's circulation. Very recently the transfer of mononuclear cells from fetus to mother was determined by the polymerase chain reaction. Y chromosome specific DNA sequences were detected in pregnant women carrying male fetuses.¹¹ These findings strongly suggested that bidirectional mononuclear cell traffic between mother and fetus is not uncommon. Therefore, it is most likely that HBV carrying maternal mononuclear cells traverse the placenta intact. But other routes of HBV transmission, for example horizontal infection after birth, are possible. Further studies are needed.

It was surprising that, though the women were asymptomatic, acute or fulminant hepatitis could occur in the infants. The HBs antigenaemia in these anti-HBe positive women might at least indicate active translation of viral DNA, which is the final step of HBV expression, suggesting that an immunological inactivation process is operating in the women. Such a mechanism might be too immature and thus unable to protect the infants from viral activation. The direct link between HBV and lymphoid cells was very recently established.¹² The evidence for the replication of HBV in peripheral mononuclear cells derives from the demonstration that a 3·4–3·5 kb RNA transcript was detected by northern blot analysis in mononuclear cells that expressed HBsAg on their surface,¹³ and that 1–10% lymphocytes obtained from HBsAg positive, HBeAg negative chronic carriers contained viral RNA by in situ hybridisation.¹⁴

Our findings will also be relevant to the

prevention of infantile fulminant hepatitis. Although one child of a HBV-DNA positive mother died at 3 months from fulminant hepatitis, HBV infection was averted in the sister by the combined administration of HBIg and HBV vaccine. There must be an argument that without the combined treatment no one can predict anything about the infant's prognosis. This seems to be reasonable, but a recent trial of the prophylactic HBIg single injection to infants born to HBeAg negative, HBsAg positive carrier mothers proved effective in reducing the rate of onset of infantile hepatitis to about 20–30% of the non-treated children.¹⁵ It is still difficult to predict the risk of fulminant hepatitis, so it would seem prudent that infants of HBV-DNA mononuclear cell positive mothers receive treatment with both HBIg and HBV vaccine.

We thank Dr Philip L Cohen, Dr Kazufumi Tsubaki, and Dr Tetsuo Mori for helpful comments; the medical and nursing staff of Yokohama City Maternity Hospital (Aiji Centre) for their cooperation; and Shigeko Yamaguchi for laboratory assistance. This study was supported by grants from a local specialised studies subsidy from Yokohama City.

- 1 Tada H, Yanagida M, Mishina J, *et al.* Combined passive and active immunization for preventing perinatal transmission of hepatitis B virus carrier state. *Pediatrics* 1982;70:613–9.
- 2 Kanai K, Takehiro A, Noto H, *et al.* Prevention of perinatal transmission of hepatitis B virus (HBV) to children of e antigen-positive HBV carrier mothers by hepatitis B immune globulin and HBV vaccine. *J Infect Dis* 1985;151:287–90.
- 3 Shiraki K, Tanimoto K, Yamada K, *et al.* Hepatitis B virus infection in the infants born to HBe antigen negative carrier mothers: a preliminary report. *Acta Paediatrica Japonica* 1986;28:338–41.
- 4 Bonino F, Hoyer B, Nelson J, Engle R, Verme G, Gerin J. Hepatitis B virus DNA in the sera of HBsAg carriers: a marker of active hepatitis B virus replication in the liver. *Hepatology* 1981;1:386–91.
- 5 Scotto J, Hadchouel M, Hery C, Yvart J, Tiollais P, Brechot C. Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique: comparison with results for other viral markers. *Hepatology* 1983;3:279–84.
- 6 Negro F, Chiaberge E, Oliviero S, *et al.* Hepatitis B virus DNA (HBV-DNA) in anti-HBe positive sera. *Liver* 1984;4:177–83.
- 7 Carloni G, Delfini C, Colloca S, Alfani E, Tallani G, DeBac C. Incidence of hepatitis B virus DNA in DNA-polymerase in sera of Italian asymptomatic carriers with the serological markers of HBV. *Arch Virol* 1986;87:97–105.
- 8 Kam W, Rall LB, Smuckler EA, Schmid R, Rutter WJ. Hepatitis B viral DNA in liver and serum of asymptomatic carriers. *Proc Natl Acad Sci USA* 1982;79:7522–6.
- 9 Mitsuda T, Yokota S, Mori T, *et al.* Demonstration of mother-to-infant transmission of hepatitis B virus by means of polymerase chain reaction. *Lancet* 1989;ii:886–8.
- 10 Desai RG, Creger WP. Maternofetal passage of leukocytes and platelets in man. *Blood* 1963;21:665–73.
- 11 Lo YMD, Patel P, Wainscoat JS, Sampietro M, Gillmer MDG, Fleming KA. Prenatal sex determination by DNA amplification from maternal peripheral blood. *Lancet* 1989;ii:1363–5.
- 12 Lamelin JP, Trepo C. The hepatitis B virus and the peripheral blood mononuclear cells: a brief review. *J Hepatol* 1990;10:120–4.
- 13 Colucci G, Jacobson I, Lalatta F, Vernance S, Waksal SD, Weksler ME. Detection of hepatitis B virus DNA, RNA and surface antigen in mononuclear cells from patients with type B hepatitis. *Clin Res* 1986;34:648A.
- 14 Hadchouel M, Pasquinelli C, Fournier JG, *et al.* Detection of mononuclear cells expressing hepatitis B virus in peripheral blood from HBsAg positive and negative patients by in situ hybridization. *J Med Virol* 1988;24:27–32.
- 15 Matsumoto S, Shiraki K, Morizuka T, *et al.* Prophylaxis of HB virus infection by single dose injection of HBIg in babies born to HBe-antigen negative HBV carrier mothers. *Journal of Japan Pediatric Sciences* 1989;93:1374–82.

Urinary infection: what little boys do in the bath

A recent paper from Quebec (J Labbé, *Pediatrics* 1990;86:703–6) raises an interesting new possibility as regards the aetiology of urinary infection in boys. A prospective consecutive series of 22 boys aged between 5 and 15 years who attended a urinary infection clinic at a university medical centre, together with nine others detected retrospectively, were questioned about penile self instrumentation in the bath or shower. Ten of the 31 admitted injecting bath water into the urethra in the few days before the onset of symptoms. The instruments used were a syringe (n=4), a rubber bulb (n=3), a plastic bottle (n=2), and a 'hand-held shower massager' (n=1). Lower abdominal pain, dysuria, and gross haematuria were more common in the 10 boys who admitted self instrumentation (group A) than in the 21 who didn't (group B). Urine culture in group A produced *Escherichia coli* in five and *Staphylococcus saprophyticus* in the other five. Only one child in group B was infected with the latter organism. Imaging of the renal tracts showed no abnormalities in group A but was abnormal in a third of the boys in group B.

Is this study representative? I imagine, but don't know, that such things as syringes and rubber bulbs may be less available in British bathrooms. The frequency of this behaviour in normal boys is unknown so it is difficult to assess the size of its contribution, if any, to the problem of urinary infection in boys, but it's an idea worth pursuing. No doubt we shall hear more on this subject.