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Production and Effect of Infectious Dane Particles in Transgenic Mice

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We have demonstrated by immunoelectron microscopy that 42-nm particles with double-shelled structures characteristic of Dane particles are present in the serum of transgenic mice, 1.2HB-BS 10, carrying partly duplicated hepatitis B virus (HBV) genome. Furthermore, these particles were shown to infect primary human fetal hepatocytes as demonstrated by the elevation of HBV surface antigen (HBsAg) in the culture medium. HBV DNA is known to be expressed in a liver- and kidney-specific manner in the adult mouse, so we examined the developmental expression of viral antigens. In the liver, viral antigens (HBsAg and HBV e antigen) began to be expressed before birth and the level of expression showed a sharp rise after birth. On the other hand, in the kidney, viral antigens began to be expressed after birth. Serum levels of viral antigens were roughly proportional to the levels of expression in the liver, suggesting that the liver is the main source for viral antigens in the serum. None of these transgenic mice produced anti-HBs or anti-HBV core response or showed biochemical or pathological change up to at least 24 months of age. All these results suggest that infectious viral particles can be produced in transgenic mice, and that expression and replication of HBV DNA are not toxic *in vivo*.

Key words: Transgenic mouse — Dane particle — Developmental expression

Hepatitis B virus (HBV) is a causative agent of hepatitis. The pathological consequences of HBV infection are unpredictable and range from unapparent forms to acute hepatitis and chronic liver disease. Carriers with severe liver disease often progress to cirrhosis and, after three to four decades, to hepatocellular carcinoma (HCC). The relative risk of HCC among HBV surface antigen (HBsAg) carriers compared to noncarriers is nearly one hundred.[1)] Although much is known about the molecular biology of HBV (for reviews, see 2, 3), many pathological aspects of HBV infection and malignant transformation are not well understood. The main reason is that HBV infects only human and chimpanzee livers.

To overcome the species-specificity of HBV infection, we produced HBV transgenic mice, 1.2HB-BS 10, by the introduction of a partly duplicated HBV genome.[4)] The 1.2HB-BS 10 mice are positive for both HBsAg and HBV e antigen (HBeAg) in their sera. The HBV DNA is expressed in a liver- and kidney-specific manner, and the normal process of HBV replication, including the packaging of the 3.5-kb pregenome RNA into a nucleocapsid, the reverse-transcription of this pregenome RNA to the complete minus strand DNA, and the synthesis of the plus strand, has been shown to occur in these transgenic mice.

We also showed previously that there were materials with a density of 1.25 g/ml, containing HBV DNA, in the serum.[4)] In the present study, we tried to obtain direct evidence of the existence of Dane particles by immunoelectron microscopy. We collected about 50 ml of serum from 1.2HB-BS 10. The serum was concentrated by pelleting through a sucrose gradient, and then was partially purified by isopycnic centrifugation.[5)] The fraction with the density of 1.25 g/ml was further concentrated and examined by immunoelectron microscopy. As shown in Fig. 1, particles of about 42 nm with a double-shelled structure as well as small HBsAg particles were observed. This suggests that the normal process of HBV replication and the release of Dane particles into the serum can occur in this transgenic mouse from the integrated HBV DNA, as in a cell culture system transfected by HBV DNA.[6–9)]

To examine the infectivity of this virus, we performed an *in vitro* infection experiment according to the method of Ochiya *et al.*[10)] Dane particles were concentrated from 50 ml of 1.2HB-BS 10 mice serum by sucrose gradient centrifugation, and suspended in Dulbecco's modified Eagle's medium (DMEM). This suspension was expected to include about 10^5 virons. Human fetal hepatocytes (1×10^6 cells per well) were cultured in the presence of the concentrated Dane particles from serum for 6 h. Then, the hepatocytes were washed three times with 10% fetal calf serum/DMEM (10% FCS/DMEM) and further incubated in 3 ml of 10% FCS/DMEM (time zero).

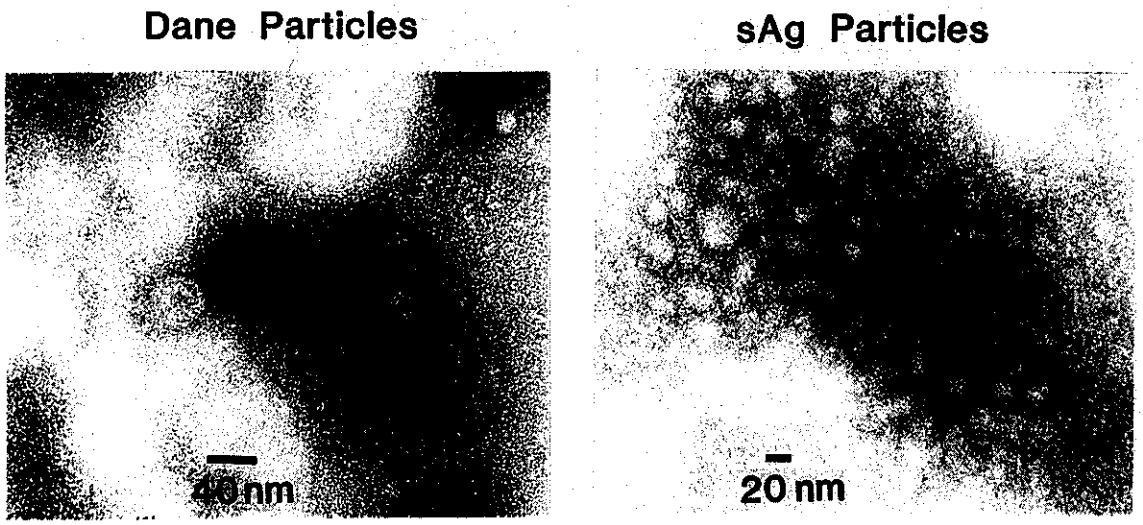

Fig. 1. Immunoelectron microscopy of particles from the serum of 1.2HB-BS 10. The serum (about 50 ml) was centrifuged in 10%–20% (w/w) discontinuous sucrose gradients at 24,000 rpm for 24 h in a Beckman SW27 rotor. The supernatant was then discarded, and the virus and HBsAg particles pellet was suspended in PBS (150 m*M* NaCl/10 m*M* Na-K phosphate pH 7.2). Isopicnic centrifugation of pelleted virus was done in 30%–60% (w/w) discontinuous sucrose gradients at 38,000 rpm for 40 h in a Hitachi RPS40 rotor. Fractions were collected from the bottom, and the fraction with a density of 1.25 g/ml was concentrated using a Centricon TM20 (Amicon). The samples were incubated with an equal volume of rabbit anti-HBs serum for 12 h at 4°C, then centrifuged at 12,000*g* for 30 min, and the pellet was stained with uranyl acetate for electronmicroscopic examination.

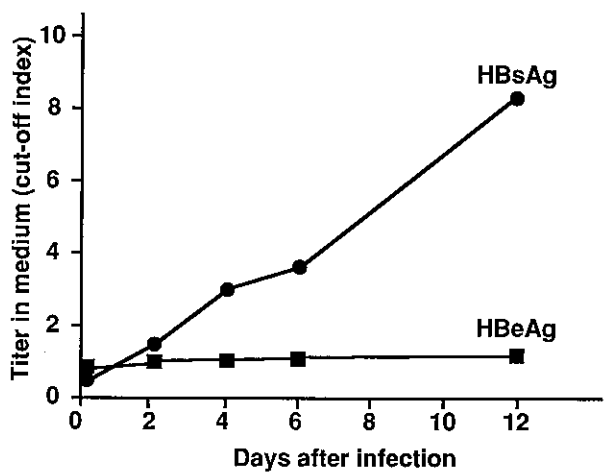

Fig. 2. Production of HBsAg and HBeAg in cells infected with HBV produced in 1.2HB-BS 10. Human fetal hepatocytes were cultured for 7 days to make confluent monolayers. They were then incubated with concentrated HBV fraction of 1.2HB-BS 10 serum for 6 h for infection. Then, they were washed and incubated again (time zero) without change of medium. Aliquots of culture medium were taken at intervals and assayed for HBsAg (●) and HBeAg (○) by using commercial enzyme immunoassay kits (Abbott). Titers were expressed as the ratios against cut-off values (A492 of negative control +0.06 for HBsAg and +0.05 for HBeAg).

Aliquots of the culture medium were collected every two days during the 12-day incubation and HBsAg and HBeAg were assayed. As shown in Fig. 2, the HBsAg was first detected after 2 days and the titer increased gradually during the 12 days of incubation. The HBsAg is considered to be synthesized *de novo,* because the HBsAg appeared again after the cells were washed on the 12th day (data not shown). HBeAg could not be detected in this experiment, probably due to the low titer of Dane particles. We tried to detect HBV RNA in these infected cells, but without success, probably for the same reason. Ochiya *et al.*[10] detected HBeAg as well as mRNA by using 1×10^7 virons per well, which was 100 times as much as in this experiment. Thus, the number of infected cells may be very low and both the HBeAg titer and the signal of HBV RNA were thought to be below the limit of detection.

In any case, the increase of HBsAg titer clearly suggests that human fetal hepatocytes were infected with Dane particles produced in the transgenic mice, 1.2-HB-BS 10. This is the first evidence that infectious HBV can be produced in an animal other than human and chimpanzee. Thus, these 1.2HB-BS 10 mice are the only laboratory animal in which HBV is constantly produced, and should be very useful for *in vivo* experiments concerning HBV proliferation and HBV-related diseases.

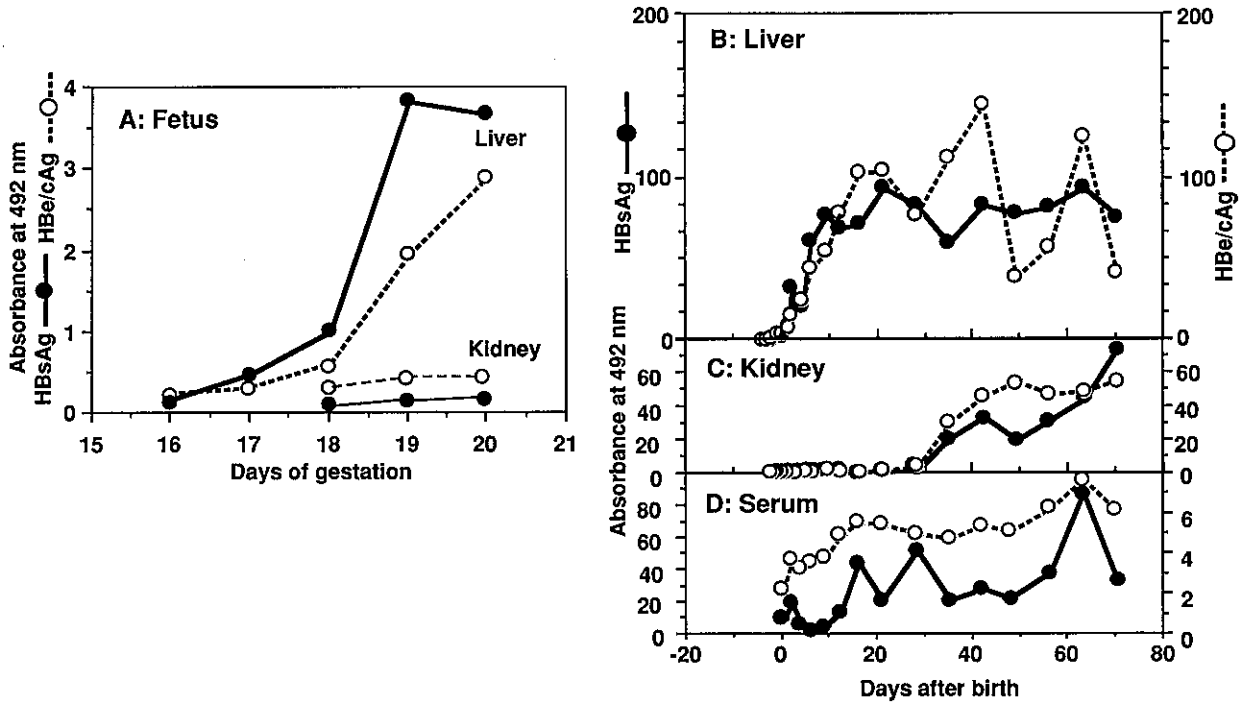

Fig. 3. Developmental expression of HBsAg and HBe/cAg in 1.2HB-BS 10 mice. The livers or kidneys were homogenized in extraction buffer (150 m*M* NaCl, 10 m*M* Na-K phosphate pH 7.2, 0.2% Triton X-100 1 m*M* phenylmethylsulfonyl fluoride) at the rate of 0.1 g of tissue/300 μl of extraction buffer. Then the homogenate was centrifuged at 12,000*g* for 10 min at 4°C, and the supernatant was assayed with AUSZYMEII (Abbott) for HBsAg and HBe EIA Abbott for HBe/cAg. This kit is cross-reactive with both HBeAg and HBcAg. The titers were expressed as calculated values of absorbance at 420 nm for a 200 μl sample. A, Developmental expression in fetus. Fifty μl of sample was used for assay. Thick lines represent the results for liver and thin lines represent those for kidney. B, Change of HBsAg and HBe/cAg of the liver extracts. Samples up to 4 days of age were pooled from five mice, and samples from 6 days to 9 weeks of age were pooled from two mice which were littermates. A volume of 10–50 μl of extracts was used for assay. C, Change of HBsAg and HBe/cAg of the kidney extract. Details were as for B. D, Change of HBsAg and HBeAg in the serum after birth. Each sample was pooled from 2–3 mice.

The transgene is transmitted stably to offspring and all mice that carry the transgene always express HBV DNA and HBV-related antigens, although there are 10-fold and 2-fold variations in serum HBsAg titer and serum HBeAg titer among these offspring, respectively. In the homozygote of 1.2HB-BS 10, the titers of serum HBV-related antigens are usually much higher than those of the hemizygote.

As we had previously demonstrated the liver- and kidney-specific expression of the transgene, we examined the developmental expression of HBsAg, and HBV e and core antigen (HBe/cAg) in these organs of the transgenic mice. Tissue extracts were prepared from the liver and the kidney before and after birth and were assayed for HBsAg and HBe/cAg by the use of enzyme immunoassay kits, AUSZYMEII and ABBOTT-HBe EIA, respectively (Abbott Laboratories). As shown in Fig. 3A and B, in the liver, HBsAg and HBe/cAg started to be expressed at around 17 days of gestation, but stayed at relatively low levels, about one-twentieth of those of adult mouse, before birth. After birth, the levels of these antigens increased rapidly and reached the maximum by 3 weeks of age. On the other hand, in the kidney, neither antigen could be detected until 2 weeks of age. Then, the titers increased rapidly from 4 weeks of age and reached the adult level by 9 weeks of age (Fig. 3C). The serum titers of both antigens changed in parallel with those of liver extracts, suggesting that viral antigens in the serum are mainly derived from the liver (Fig. 3D).

The delayed expression of viral antigens in the kidney is quite interesting. Recently, several *cis*-acting elements including an enhancer sequence have been identified in HBV DNA.[11-13] Burk *et al.*[14] and we[4] demonstrated that HBV expression was liver- and kidney-specific in transgenic mice, suggesting that there are *trans*-acting factor(s) which can act on these HBV regulatory elements in the liver and the kidney. Different developmental specificity, as demonstrated in Fig. 3, suggests

that the *trans*-acting factor(s) present in the kidney is different from that in the liver. Our transgenic mice should provide useful information concerning tissue- and developmental-specific expression.

To examine whether the production of viral antigens or the replication of HBV is harmful to the liver, biochemical and pathological studies were carried out on four transgenic mice which were 2 years old. There was no elevation of serum transaminase level such as glutamic-pyruvic transaminase, and no sign of hepatitis in the liver sections. This suggests that HBV is not directly cytopathic and that the immunological responses to viral antigens play an important role in the induction of hepatitis. Actually, these transgenic mice did not produce either anti-HBs or anti-HBc antibody, suggesting that these mice are telerant to the viral antigens. This tolerance may be induced by the expression of viral antigens from the fetal stage, because the level of expression in adult transgenic mouse is high enough to induce immune response in control C57BL/6 mouse. Recently, Moriyama *et al.*[15] succeeded in inducing hepatitis by the injection of a cytotoxic T cell clone against HBsAg into transgenic mice producing HBsAg, suggesting that at least the immune response at the T cell level is important for the hepatocellular injury.

From the immunological standpoint, our transgenic mice are in a similar situation to human HBsAg carriers for the following reason. These carriers do respond to viral antigens, but usually at a low level. The low responsiveness may be due to HBV infection at the neonatal stage and, in addition, to the presence of a certain type of major histocompatibility antigen complex (MHC) class II genes that can control the immune response to viral antigens.[16] In this experiment we used an inbred strain of mouse, C57BL/6 mouse, which is an intermediate responder to HBsAg.[17] As described before, these transgenic mice express viral antigen before birth. Therefore, they do not show any immune response to the viral antigens. However, we found that they could respond to viral antigens after immunization (unpublished data), suggesting that they are tolerant, but only partially.

The elucidation of the developmental process of hepatitis is quite important not only for understanding the molecular mechanisms of HCC generation, but also for devising new modes of treatment for HBsAg carriers. These transgenic mice should be a powerful tool in such studies.

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