Ultrastructural demonstration of hepatitis B virus production in a mouse model produced by hydrodynamic transfection

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Abstract. A mouse model of hepatitis B virus (HBV) infection produced by hydrodynamic injection of HBV DNA has been recently established. However, the ultrastructural demonstration of HBV particles in this mouse model has not as yet been reported. In our study, plasmid DNA containing wild-type HBV DNA was rapidly injected into 8-week-old female SCID mice via the tail vein. Serum levels of HBsAg were measured by ELISA kit. Intrahepatic HBV protein expression was detected by immunohistochemistry of HBcAg. Ultrastructural study of the serum samples was performed by transmission electron microscopy and immunogold electron microscopy. Serum HBsAg and intrahepatic HBcAg were detected in HBV DNA-injected mice for at least 14 days. Spherical and filamentous particles 22 nm in diameter and double-shelled Dane-like particles 42 nm in diameter were detected in the sera of mice. The ultrastructural features of these particles were identical to HBV particles observed in serum from chronic hepatitis B patients. These particles were confirmed to be HBV particles by immunogold electron microscopy. We conclude that our present HBV mouse model using hydrodynamic transfection of HBV DNA is appropriate for production of HBV virions including Dane particles. This mouse model may be useful for screening *in vivo* the efficacy of antiviral drugs.

Introduction

Hepatitis B virus (HBV) belongs to the family of hepadnaviruses that causes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (1). There are ~350 million chronic carriers of HBV worldwide (2). Interferon (IFN)-α or nucleotide analogues have been approved for the treatment of chronic HBV infection. However, the efficacy of this therapy is still limited and viral reactivation frequently occurs after cessation of therapy (3). The slow progress in the development of novel therapies for HBV is due to the lack of convenient small animal models. Several small animal models for the study of HBV replication are currently available (4-8), but the preparation of these models is relatively complicated and difficult.

Simple hydrodynamic-based naked DNA injection into the tail veins of mice can induce efficient transfection of liver cells *in vivo* (9,10). HBV gene expression and viral replication by this technique have been recently reported (11-14). However, the ultrastructural demonstration of HBV particles in this mouse model has not as yet been reported. The aim of this study was to clarify whether HBV particles can be secreted into the serum of our mouse model.

Materials and methods

Plasmid and mice. Plasmid pHBVadwHTD containing a head-to-tail dimer of wild-type HBV (subtype adw) was described previously (15). This plasmid was prepared using a plasmid purification kit (HiSpeed plasmid kit, Qiagen, Germany). Hydrodynamic injections of this plasmid into SCID mice were recently reported to express HBsAg, HBeAg, HBcAg and HBV DNA for a minimum of 30 days (14). We used 6- to 8-week-old female SCID mice (CLEA Japan, Japan). They were fed standard laboratory chow and water *ad libitum* in the animal facility. The animals were raised following the Mie University's guidelines for animal investigation.

Hydrodynamic transfection and sample collection. Fifty micrograms of the plasmid, dissolved in 1.5 ml PBS, was injected into the tail veins of mice. The injection speed was 0.3 ml/sec through a 27-gauge needle. Under light anesthesia using diethyl ether, serum samples were taken from the retro-orbital vessels after 2, 8 and 14 days of DNA injection. Mice were sacrificed on day 14 and liver samples were collected.

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Key words: hepatitis B virus, hydrodynamic transfection, animal model, electron microscopy, Dane particle

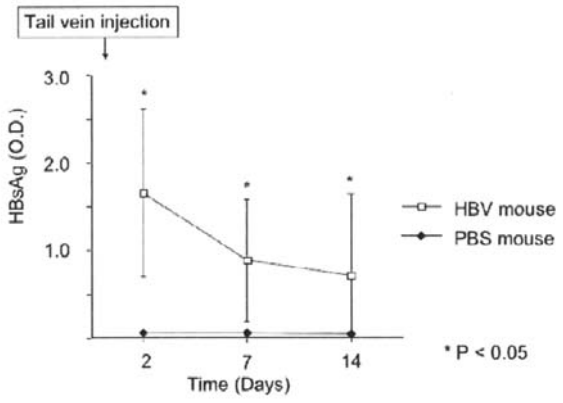

Figure 1. Time course of serum HBsAg levels after HBV DNA injection. Serum HBsAg levels are expressed as the mean ± SD from three treated mice. O.D., optical density.

Mice injected with phosphate-buffered saline (PBS) alone were used as controls. HBV-positive serum was drawn from a chronic hepatitis B patient positive for HBsAg and HBeAg and with a high level of ALT.

Detection of HBsAg in mice sera. Serum HBsAg was detected using HBsAg enzyme-linked immunosorbent assay (ELISA) kit (Hope Lab, USA) following the manufacturer's instructions.

Histology and immunohistochemistry. Liver tissues were fixed with 10% neutral-buffered formalin and embedded in paraffin. For immunohistochemical analysis of HBcAg, paraffin sections were incubated with rabbit polyclonal antibody against core antigen (Dako Japan, Japan) and followed by incubation with FITC-conjugated donkey anti-rabbit IgG (Chemicon, USA). Fluorescent signals were detected by fluorescence microscopy (Olympus, Japan). Tissue sections were also stained with hematoxylin and eosin Y for histological examination.

Conventional electron microscopy. Five hundred microliters of mouse or human sera was mixed with 12 ml PBS. The mixtures were then centrifuged at 37500 rpm for 3 h at 4˚C using a CP60E ultracentrifuge (Hitachi, Japan). The pellets were then dissolved in 50 μl PBS. Three microliters of the viral sample was directly mounted on a formvar-carbon-coated nickel grid (300 mesh, VECO grid, Nissin EM, Japan). The grids were negatively stained with 2% phosphotungstic acid solution at pH 6.5, and examined by H-7000 electron microscopy (Hitachi, Japan) at 75 kV.

Immunogold electron microscopy. Immunogold electron microscopy was carried out as previously described (16-18). We used goat polyclonal anti-HBs antibody (Biostride, USA) as the primary antibody, and colloidal gold-conjugated rabbit anti-goat IgG as the secondary antibody. The colloidal gold particles were 5 nm in diameter (BioCell Research Laboratory,

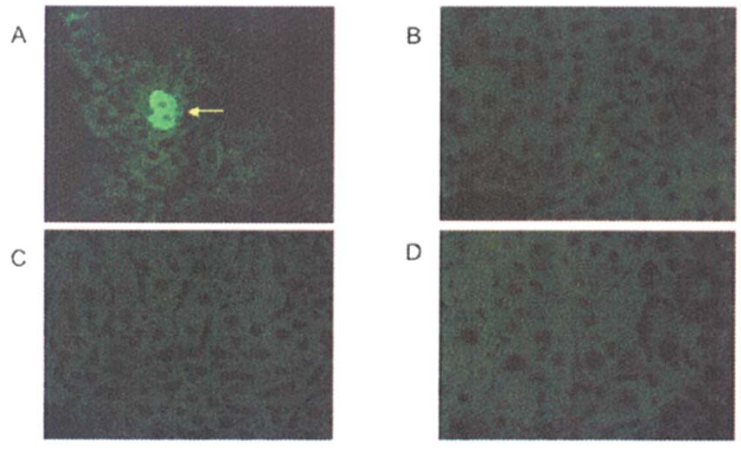

Figure 2. Immunohistochemistry for HBcAg of mouse liver at 14 days after HBV DNA injection. (A) HBV DNA-injected mouse; primary Ab^+, secondary Ab^+. (B) HBV DNA-injected mouse; primary Ab^-, secondary Ab^+. (C) HBV DNA-injected mouse; primary Ab^+, secondary Ab^-. (D) PBS-injected mouse, primary Ab^+, secondary Ab^+. Original magnification, x200. Ab^+, with antibody; Ab^-, without antibody.

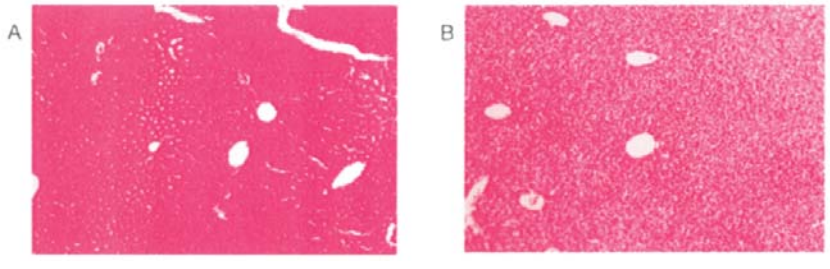

Figure 3. Hematoxylin and eosin staining of mouse liver at 14 days after HBV DNA injection. Original magnification, x100.

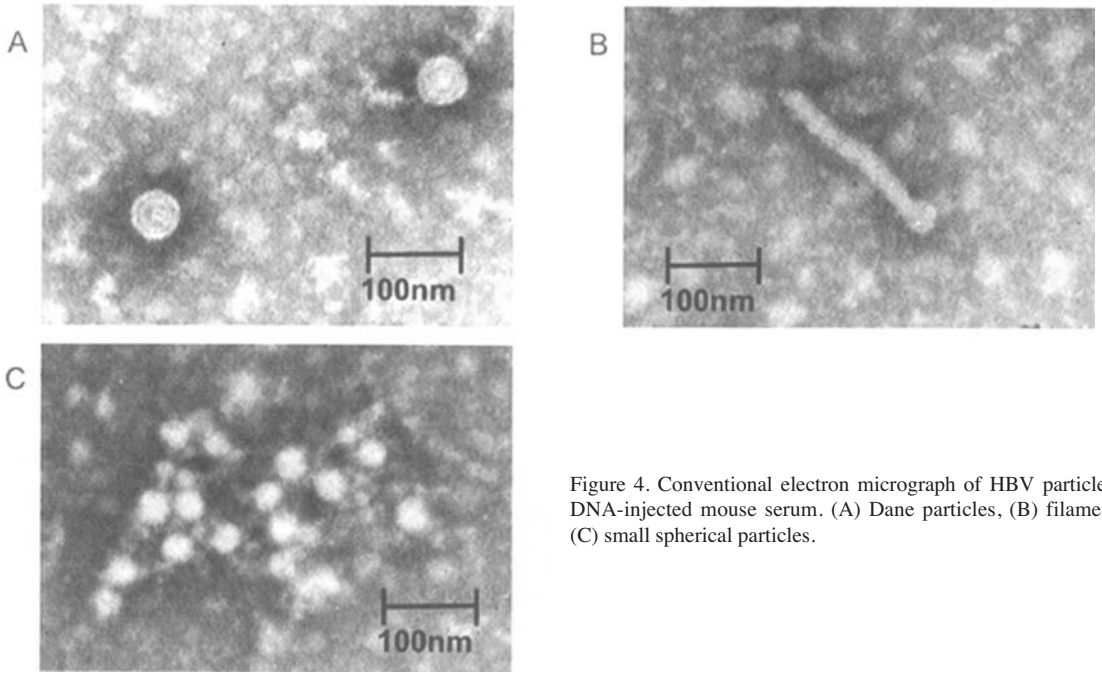

Figure 4. Conventional electron micrograph of HBV particles in the HBV DNA-injected mouse serum. (A) Dane particles, (B) filamentous particle, (C) small spherical particles.

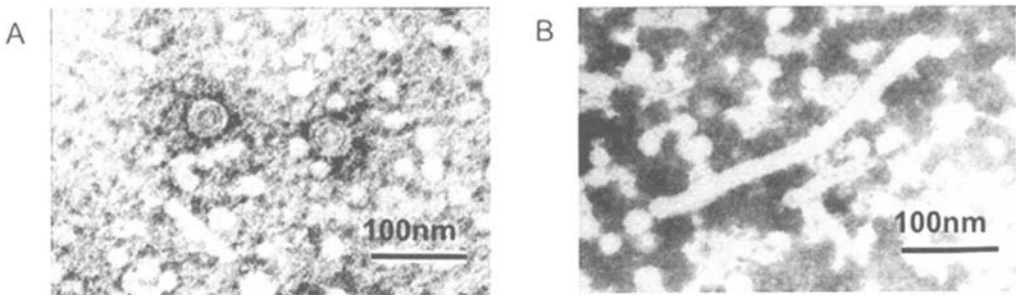

Figure 5. Conventional electron micrographs of HBV particles in the serum of a chronic hepatitis B patient. (A) Dane particles and small spherical particles, (B) filamentous particle and small spherical particles.

UK). First, 3 μl of the viral sample was adsorbed on a grid, semidried and incubated in TBS-BSA (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 2% bovine serum albumin) for 5 min in a moist chamber. The grid was then floated on TBS containing 3% gelatin for 30 min, and the excess gelatin was washed out with TBS-BSA. The grid was incubated with the primary antibody solution (diluted 1:100 in TBS-BSA) for 60 min at room temperature, and then washed three times with TBS-BSA. After incubation with the secondary antibody (diluted 1:20 in TBS-BSA) for 60 min, the grid was negatively stained with phosphotungstic acid.

Statistical analysis. The serum HBsAg levels were expressed as the mean ± SD. Statistical differences between variables were analyzed using the Mann-Whitney U test. P values <0.05 were considered as statistically significant.

Results

Serum levels of HBsAg. As shown in Fig. 1, the levels of HBsAg in three hydrodynamic HBV mice were 1.66±0.96, 0.89±0.70 and 0.72±0.92 (O.D., optical density) after 2, 8 and 14 days of DNA injection, respectively. These values were significantly higher than the levels observed in PBS-injected mice (0.06±0.03, 0.06±0.03 and 0.05±0.03 O.D.) after 2, 8 and 14 days of PBS injection, respectively ($P<0.05$). The cutoff index of this assay was 0.15 O.D.

HBcAg expression in the liver. Fig. 2 shows the immunohistochemistry for HBcAg in the mouse liver. Approximately 1% of the hepatocytes were positive for HBcAg at day 14 after HBV DNA injection (Fig. 2A). HBcAg was detected mainly in the cytoplasm of the hepatocytes. There were no positive signals in the controls. (Fig. 2B, hydrodynamic HBV mouse without primary antibody (Ab^-) and with secondary antibody (Ab^+); Fig. 2C, hydrodynamic HBV mouse with primary antibody and without secondary antibody, and Fig. 2D, PBS-treated mouse with primary and secondary antibody).

Histological analysis. Hematoxylin and eosin staining of the mouse liver 14 days after DNA injection is shown in Fig. 3. There were no significant histological changes in either the hydrodynamic HBV mouse liver (Fig. 3A) or the control PBS-treated mouse liver (Fig. 3B).

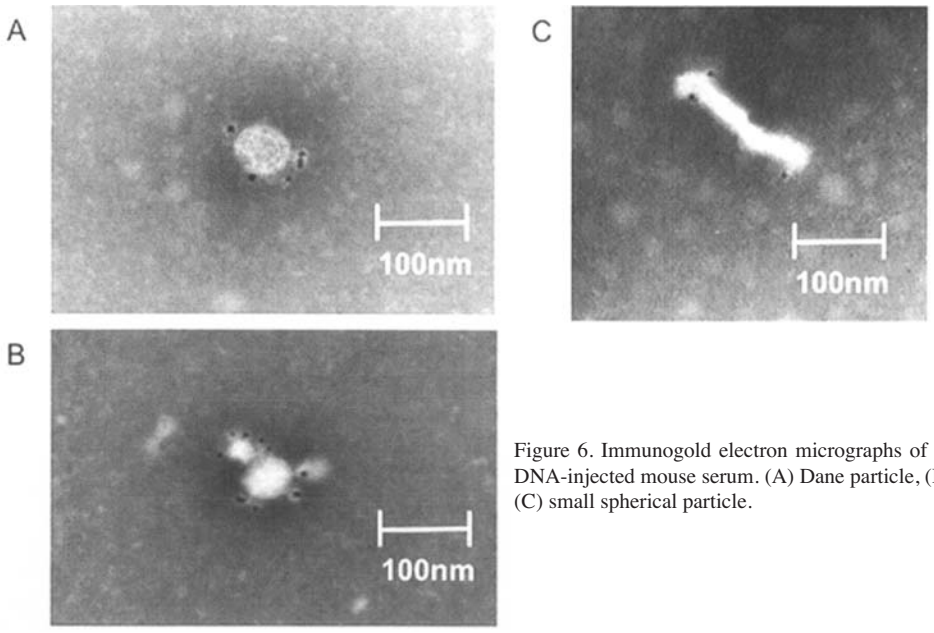

Figure 6. Immunogold electron micrographs of HBV particles in the HBV DNA-injected mouse serum. (A) Dane particle, (B) filamentous particle, and (C) small spherical particle.

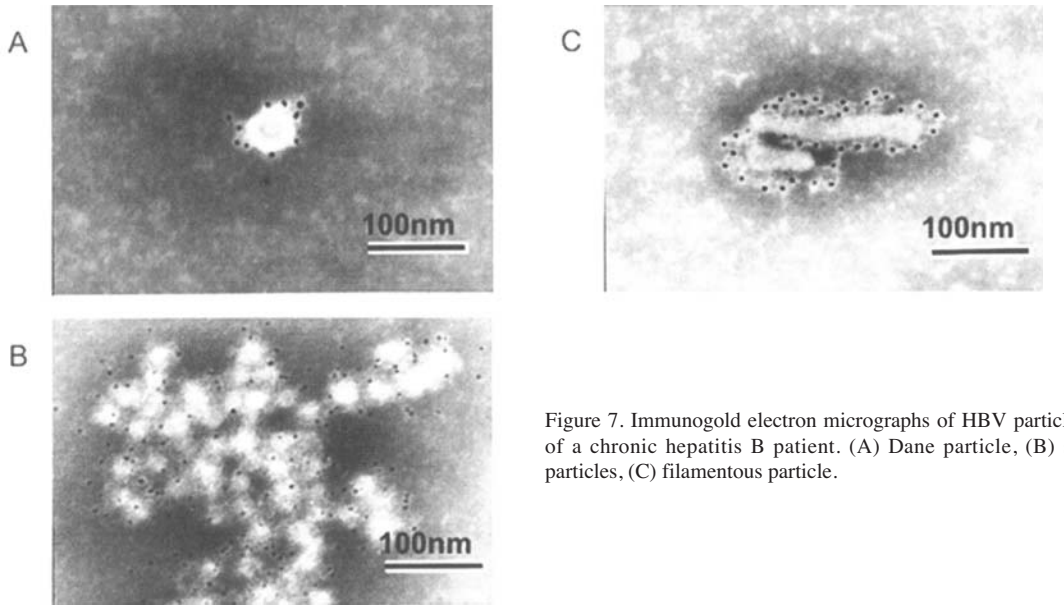

Figure 7. Immunogold electron micrographs of HBV particles in the serum of a chronic hepatitis B patient. (A) Dane particle, (B) small spherical particles, (C) filamentous particle.

Conventional electron micrographs of HBV particles. Electron microscopy was performed of the serum from the hydrodynamic HBV mice that was collected 2 days after injection. Double-shelled particles of 42 nm known as Dane particles, small spherical particles of 22 nm, and filamentous particles of 22 nm in diameter with variable length were detected in the serum (Fig. 4A-C). These ultrastructural features were identical to HBV particles found in the serum of a chronic hepatitis B patient (Fig. 5A and B). However, Dane particles, small spherical or filamentous particles were not observed in the serum from PBS-treated mice (data not shown).

Immunogold electron micrographs of HBV particles. Indirect immunogold electron microscopy was carried out to confirm that particles found in the serum from hydrodynamic HBV mice were HBV. The immunogold labeling showed specific gold labeling of Dane particles of 42 nm in diameter (Fig. 6A). Labeling with colloidal gold specific for HBsAg was also observed on the surface of small spherical particles of 22 nm in diameter (Fig. 6B) and filamentous particles (Fig. 6C). These colloidal gold-labeled particles were identical to HBV particles detected in the serum of a chronic hepatitis B patient (Fig. 7A-C).

Discussion

Several therapeutic strategies against HBV, such as interferon-α (3), nucleotide analogues (19-21), antisense DNA (22), ribozyme (23) and RNA interference (24,25), have been reported in clinical or laboratory investigations. However, to date no ideal treatment for HBV eradication has been reported. Study of HBV treatment *in vivo* is hampered by the lack of convenient animal models to produce HBV particles. A hydrodynamic HBV mouse model was reported for the first time by Yang *et al* (11). They reported long-term

HBV protein expression for at least 81 days, and persistent expression of HBV transcripts and HBV DNA replicative intermediate for 30 days after transfection in NOD/SCID mice. The presence of DNase I-resistant HBV in fractionated serum at a density of 1.21 g/ml is indirect evidence of HBV secretion into the blood of this mouse model (12). Takehara *et al* described persistent expression of covalently closed circular DNA (cccDNA), the template of viral replication in natural viral life, and long-term HBV protein expression for >12 months (13). However, the ultrastructural demonstration of HBV particle secretion into the serum has not as yet been described. In the present study, HBV particles including Dane, filamentous and small spherical particles were detected in the serum of the hydrodynamic HBV mouse model using immunogold electron microscopy. We were the first to report the presence of hepatitis C virus (HCV) and HCV core particles in human plasma from chronic hepatitis C patients by immunogold electron microscopy (26,27). This technique demonstrates more clearly the morphology of viral particles than immuno-aggregation electron microscopy (28). The particles detected in the serum of the HBV hydrodynamic mice were identical to HBV particles detected in the serum of a chronic hepatitis B patient. This finding is proof of HBV production in this mouse model.

Recently, we observed unique native HBV particles, named cobra-shaped and horn-shaped, in chronic hepatitis B patient serum without ultracentrifugation (29). These unique particles have been shown to completely disappear after ultracentrifugation. We did not observe these particles in the hydrodynamic HBV mouse serum with or without ultra-centrifugation. The lack of these particles may be due to the use of host cells different from human hepatocytes or to the use of an HBV expression plasmid containing 2 copies of HBV DNA instead of the natural HBV genome. Dane particles are thought to be infectious, and thus HBV particles produced in this animal model are also probably infectious, even in the absence of cobra-shaped or horn-shaped particles. Additional infection studies using chimeric mice (6) or chimpanzees are necessary to prove the infectivity of HBV particles produced in this animal model.

Histological examination demonstrated normal liver histology in this animal model. This finding was due to the lack of normal immunity in SCID mice. The hydrodynamic HBV mouse using an immunodeficient mouse is similar to the conditions of a human HBV healthy carrier. Although this model cannot be utilized for vaccine development, it is very useful for testing the antiviral effects of interferon, nucleotide analogues and HBV-targeted gene therapy *in vivo*. In fact, several reports have described the use of this animal model for examining the *in vivo* effect of lamivudine or HBV-targeted siRNAs (13,14,30,31).

In conclusion, in this study, we showed that hydrodynamic HBV mice produce HBV particles, including Dane, small spherical and filamentous particles as demonstrated by immunogold electron microscopy. The results of the present study together with those of previous studies that have shown the expression of HBV protein, HBV DNA, HBV cccDNA and HBV mRNA provide evidence of the usefulness of this animal model to analyze HBV replication and viral production.

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