劇症肝炎患者由来Ｂ型肝炎ウイルスの生物学的特性の解析

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研究発表

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Abstract

We established a mouse model of hepatitis B involving liposome-mediated gene transfer, aiming to compare the antibody response to viral DNA from a patient with fulminant hepatitis with the response to wild-type viral DNA. A mixture of liposomes and full-length viral DNA was injected intrahepatically. Three days after transfection, viral transcript and antigen were detected in the liver by reverse transcription-polymerase chain reaction and immunohistochemistry. Also on day 3, hepatitis B surface antigen became detectable in the circulation. At 7 days mice of an initially nonresponding strain were reinjected, and they developed demonstrable serum antibody against viral surface antigen 5 days later. The mice transfected with the fulminant hepatitis-associated DNA produced six times the amount of antibody produced by mice transfected wild-type viral DNA. No difference was observed in amount of viral transcript or antigen in the liver or amount of in circulating hepatitis B surface antigen, suggesting that the higher antibody production in mice transfected with fulminant hepatitis-associated viral DNA resulted from increased antigenicity rather than antigen quantity. Previous DNA sequence analysis has identified mutations producing four amino acid substitutions in the envelope region compared to the wild-type virus. One or more of these amino acid changes presumably incited the hyperimmune response in mice and possibly also the patient's fulminant clinical course.

Introduction

The pathogenesis of hepatitis B virus (HBV)-induced liver disease is still under investigation. A recent *in vitro* study demonstrated that injury to the inflamed liver was caused by attack upon infected hepatocytes by cytotoxic lymphocytes (CTL) [1]. The deduced target epitope was located within the viral core region,[2,3] while the severity of liver injury was dependent upon the peptide sequence of the HLA class I motif.[4] However these investigations did not provide direct evidence on how liver damage is induced in patients, and studies in experimental animals were largely precluded by the narrow host range of HBV. Transgenic mice with complete or partial HBV genomes have been produced,[5] but neither liver injury nor an immune response is observed in these mice because the hosts are tolerant to HBV-related antigens. However, injection of spleen cells primed with hepatitis B surface antigen (HBsAg) into these transgenic mice induced biochemically evident liver injury,[6] and other investigators induced liver injury in rats by injecting HBV-Sendai virus into the liver.[7]

Both humoral and cellular immune responses to HBV are enhanced in patients with fulminant hepatitis.[8] In HBV DNA obtained from patients with fulminant hepatitis, numerous mutations have been found throughout the entire HBV genome.[9, 10] However, whether the viral mutation or host immune background is responsible for the enhanced host reaction to HBV is unclear. To analyze liver injury in terms of immune response to HBV, interactions between the mutant HBV strain and host major histocompatibility (MHC) background must be studied.

We established a murine model of HBV acquired in adulthood via liposome-mediated transfection into the liver, so antigen production, antibody response, and viral DNA could be studied in combination.

Materials and Methods

Constructs. Replication-competent constructs of wild-type strains (adwR9, aywR9) and a fulminant hepatitis-associated HBV strain (FHR9) have been described prevously.[9] These constructs contained 1.2 times the genomic length of HBV.

Tissue culture and in vitro transfection. Human hepatoma HuH-7 cells were maintained in DME (Gibco, Grand Island NY) with addition of 10% fetal calf serum (FCS; Sigma Chemical, St. Louis, MO) at 37°C in an atmosphere including 5% CO_2. The HuH-7 cells were grown to 70% confluence in plastic tissue culture wells 16mm in diameter and then transfected with DNA. Lipofectin (GIBCO-BRL, Gaithersburg, MD) diluted in serum-free medium (OPTI-MEM; GIBCO-BRL) was used according to the manufacturer's protocol to transfect the cells. Briefly, for each transfection 2 μg of DNA was mixed with 100 μL of OPTI-MEM, and 20 μL of Lipofectin was mixed with another 100 μL of OPTI-MEM. The two solutions then were gently mixed and kept at room temperature for 15 min, after which another 800 μL of OPTI-MEM was added. This transfection medium was overlaid upon the cells, which were incubated for 18 hr at 37°C in a CO_2 incubator. The DNA-containing medium was replaced with 1 mL of normal growth medium after 18 hr, and culture was continued at 37°C in the CO_2 incubator until 7 days after transfection.

Mice and in vivo transfection. Purebred 4-weeks-old mice, BALB/c (H-2d), C57BL6 (H-2b), and C3H (H-2k), were purchased from Nihon Crea (Tokyo, Japan). A complex containing 100 μg of DNA and 400 μL of Lipofectin-containing OPTI-MEM was incubated following the procedure above. The mice were anesthetized with ether and

injected with DNA-Lipofectin complex solution in the subcapsular region of the liver. This procedure was approved by the institutional review board for animal experimentation at Tokyo Women's Medical University.

Assays for HBs antigen and antibody. For *in vitro* experiments, 200 μL of culture medium was removed every day after the transfection. For *in vivo* experiments, blood was sampled from an orbital vein each day following transfection. Samples were assayed for hepatitis B surface antigen and for antibody against hepatitis B surface antigen (anti-HBs antibody) using a commercial enzyme immunoassay kits (Abbot, North Chicago, IL).

Detection of HBs and HBcore-antigen in hepatocytes. Three days after transfection the mouse liver was perfused with a 4% paraformaldehyde solution containing 0.1 M phosphate buffer at pH 7.4. After fixation, the tissue was sectioned at a 5-mm thickness using a cryostat. Sections were stained with polyclonal antibodies to HBsAg and hepatitis B core antigen (HBcAg; Dako, Carpinteria, CA) using an LSAB kit (Dako) according to the manufacturer's recommendations.

Detection of HBV transcript in liver. HBV transcripts were detected in the liver by reverse transcription of total RNA followed by polymerase chain reaction analysis (RT-PCR). Total RNA extracted from the livers of mice transfected with the HBV transgene[11] was reverse transcribed using avian myoblastoma virus-reverse transcriptase (20 U/mL; Boehringer Ingelheim, Heidelberg, Germany). The resulting complementary DNA (cDNA) was amplified by PCR. Briefly, 1 μg of RNA was denatured by heating for 10 min at 70°C and then added to a mixture containing 2 μL of 10 mM antisense primer in the envelope region (HBV nt 882 to nt 861, 5'-CCATGAAGTTAAGGGAGTAGCC-3'), 10 μL of 5X concentrated AMV buffer

(Boehringer Ingelheim), 3 μL of dithiothreitol (DTT; 0.1 mol/L), 8 μL of deoxynucleoside triphosphate (dNTP; each 1.25 mmol/L), 1.5 μL of a ribonuclease inhibitor (RNAsin, 40 U/mL; Boehringer Ingelheim), and 2 μL of reverse transcriptase. Each cDNA synthesis was performed for 1 hr at 37°C and stopped by incubation for 10 min at 95°C. For PCR detection of HBV transcripts, two sets of primers were used (HBV core sense, nt 1700 to nt 1727, 5'-GAGGCATGCTTCAAAGACTGTGTGTTTA-3'; HBV core antisense, nt 2464 to nt 2440, 5'-CCAAGGGATACTAACATTGAGATTC; HBV envelope region sense, nt 2819 to nt 2847, 5'-TGGAGCTCACCATATTCTTGGGAACAAGA-3'; HBV envelope region antisense, nt 242 to nt 223, 5'-GGTATTGTGAGGATTCTTGT). PCR of the cDNA was performed in the presence of 3 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxyribonucleoside triphosphate, 25 pmol of each primer, and 1.25 U of AmpliTaq (Perkin Elmer-Cetus, Nowalk, CT) in a total volume of 50 μL. After an initial denaturation step for 5 min at 94°C, amplification involved the following three-step cycle: 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, and 60 sec of extension at 75°C. Forty cycles were followed by a final incubation for 10 min at 74°C. All PCR products were subjected to agarose gel electrophoresis (low melting temperature agarose; GIBCO-BRL) and visualized by ethidium bromide staining. To ensure that contaminating DNA had not been amplified, PCR also was performed on an aliquot of each RNA sample that had not been reverse transcribed.

Results

HuH-7 cells transfected with HBV DNA secrete recombinant HBsAg *in vitro*. The time course of HBsAg secretion was analyzed *in vitro* after transfection of

HBV DNA into HuH-7 cells. Detectable amounts of HBsAg were secreted into the supernatant of transfected cultured cells beginning 1 day after transfection. Secretion increased gradually for up to 7 days (Fig. 1). We initially tested two wild-type HBV virus strains, adw and ayw. Since we could not detect any difference between adw and ayw, we used adw type HBV DNA for the remaining experiments.

Mouse production of antibodies to HBsAg after injection with HBV-Lipofectin conjugate. Blood was sampled daily from mice after injection with HBV DNA. HBsAg concentrations peaked 2 days after injection and then gradually decreased (Fig. 2). Anti-HBs antibody could be detected 10 days after injection and remained continuously positive for at least 16 days after infection. No mice simultaneously expressed both HBsAg and anti-HBs antibody.

HBV transcript and HBV-related antigen in murine hepatocytes. HBV transcripts in hepatocytes were detected by RT-PCR. The primers used for RT-PCR spanned both core and envelope regions. Reverse transcription was followed by amplification of cDNAs of the predicted size (Fig. 3). Next, HBsAg and HBcAg were stained using anti-HBs and anti-HBc antibodies, respectively. HBsAg was localized mainly to the cytoplasm, while HBcAg was predominantly nuclear (Fig. 4). Both HBsAg- and HBcAg-positive hepatocytes were diffusely distributed throughout the lobular architecture.

Differences in anti-HBs antibody production between murine strains. We injected wild-type HBV DNA (adwR9) constructs into 10 mice from each of strains with different major histocompatibility complex (MHC) backgrounds. BALB/c and C57BL6 mice began to produce anti-HBs antibody 9 days after injection and 15 days after injection, respectively, and 90% of C57BL6 and 70% of BALB/c mice produced anti-HBs antibody (Fig. 5). No mice from the C3H strain produced anti-HBs antibody during

the observation period.

Production of anti-HBs antibody in response to fulminant hepatitis-associated mutant HBV in a "nonresponder" murine strain. Next, we transfected wild-type (adwR9) and mutant (FHR9) HBV DNA into the livers of C3H mice, and compared the production of anti-HBs antibody (Table). We found no difference in amount of HBsAg in mouse serum between the two types of DNA, nor was any difference in evident amount of HBsAg secreted into cell culture media. Five days after injection, no antibodies to HBsAg were detected in the mice injected with adwR9 and FHR9. We reinjected 100 μg of construct 7 days after the first injection and assayed anti-HBs antibody production 5 days later. In mice injected with FHR9, concentrations of anti-HBs antibody as high as 18.5 ± 6.8 mIU/mL were detected, about six times higher than concentrations of anti-HBs antibody in mice injected with adwR9.

Discussion

We established an adult murine model expressing two HBV-related antigens, HBcAg and HBsAg, in the liver. Furthermore, HBV transcripts were found in liver tissue by RT-PCR. In our study, HBcAg was localized preferentially in the liver cell nucleus and to a lesser extent in the cytoplasm.[12] These observations confirmed replication of injected HBV constructs in our model. HBsAg was observed in the cytoplasm, although the accumulation of HBsAg may have been slight, because the characteristic "ground-glass" cytoplasmic appearance was not present in all specimens.[13]

Circulating HBsAg was detected 2 days after injection, and then promptly disappeared. In contrast, Hepatitis B e antigen (HBeAg) could not be detected in serum by a commercially available assay (data not shown). HBeAg, a soluble low-molecular weight component of the nucleocapsid, has been identified as an HBV core-associated

antigen encoded by the precore to core region and processed by protease prior to secretion.[14] In our murine model, expression of the precore protein, processing of the precore/core protein, or secretion of HBeAg may be disturbed, since HBcAg was detectable in hepatocytes.

A previous report indicated that transfection can cause parenchymal lymphoid infiltration and focal necrosis of hepatocytes,[7] and we presently observed similar microscopic findings. However, the possibility of an artifact from direct injection of HBV DNA into the liver cannot be eliminated, considering that serum aminotransferase concentrations was not elevated (data not shown). In our model, production of HBsAg diminished within 1 week. To produce massive liver injury, continuous expression of HBsAg for a prolonged period may be needed.

Seroconversion from HBsAg to anti-HBs antibody occurred about 10 days after injection with HBV DNA. A construct encoding HBsAg under the control of the CMV promoter has been injected intramuscularly into mice as an experimental vaccine.[15-17] In these studies, antibody titers were maintained for at least 6 months without further DNA injections. In our study, anti-HBs antibody titers were maintained without a decrease for at least 2 months (data not shown). One possible advantage of a DNA-based vaccine is that *in situ* production of the expressed protein(s) may allow presentation of antigens by MHC class I molecules through an endogenous pathway to permit their recognition by CTL.[18]

Injection of HBV DNA elicited anti-HBs antibody production in BALB/c (H-2d) and C57BL6 (H-2b) mice to a similar extent. In contrast, no anti-HBs antibody production could be detected in C3H (H-2k) mice after a single injection of HBV DNA. Differences of responsiveness to HBsAg between different strains of inbred mice have been reported.[19] These differences may result from different T-cell subsets responding to HBsAg among murine strains. HBsAg reportedly induces T-helper (Th) 2 cells in BALB/c mice and both Th1 and Th2 cells in SWR/J mice.[20] Our finding was consistent with a previous report by Milich et al., in which the B10.BR (H-2k) strain exhibited a

lower response to HBsAg than B10.D2 (H-2d) or B10 (H-2b) strains.[21] After a second injection 7 days following the first, weak production (3.3 mIU/mL) of anti-HBs antibody could be detected in the C3H strain of mice. On the other hand, FHR9, an HBV virus from a patient with fulminant hepatitis, elicited a high titer (18.8 mIU/mL) of anti-HBs antibody in this low-responder strain. Enhancement of immune response to the HBsAg of FHR9 in C3H mice may have been caused by an altered antigenicity of HBsAg from mutations in the envelope region of FHR9, since no difference was seen in serum titers of the two respective HBsAgs. Rapid seroconversion from HBsAg to anti-HBs antibody usually is seen clinically, and our results appear consistent with this clinical observation. We previously demonstrated that FHR9 has a 4 amino acid substitutions in the envelope region.[9] Further studies will be needed to determine which mutations are responsible for the enhanced immune response.

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Table. Anti-HBs production in response to wild-type and mutant HBV in transfected mice of a poorly responding strain

		5 days after first injection	5 days after second injection
HBsAg	wild-type	4.5±1.6	0.7±0.2
	mutant	5.4±1.4	0.6±0.8
anti-HBs	wild-type	0	3.3±1.9
	mutant	0	18.5±6.8

Wild-type (adwR9) and fulminant hepatitis-associated mutant (FHR9) hepatitis B virus (HBV) DNA were reinjected into the livers of 10 C3H mice 7 days after first injection. Serum was sampled 5 days after each injection and hepatitis B surface antigen (HBsAg) and anti-HBs antibody were assayed. Units: HBsAg, signal/noise ratio (S/N); anti-HBs antibody, mIU/mL.

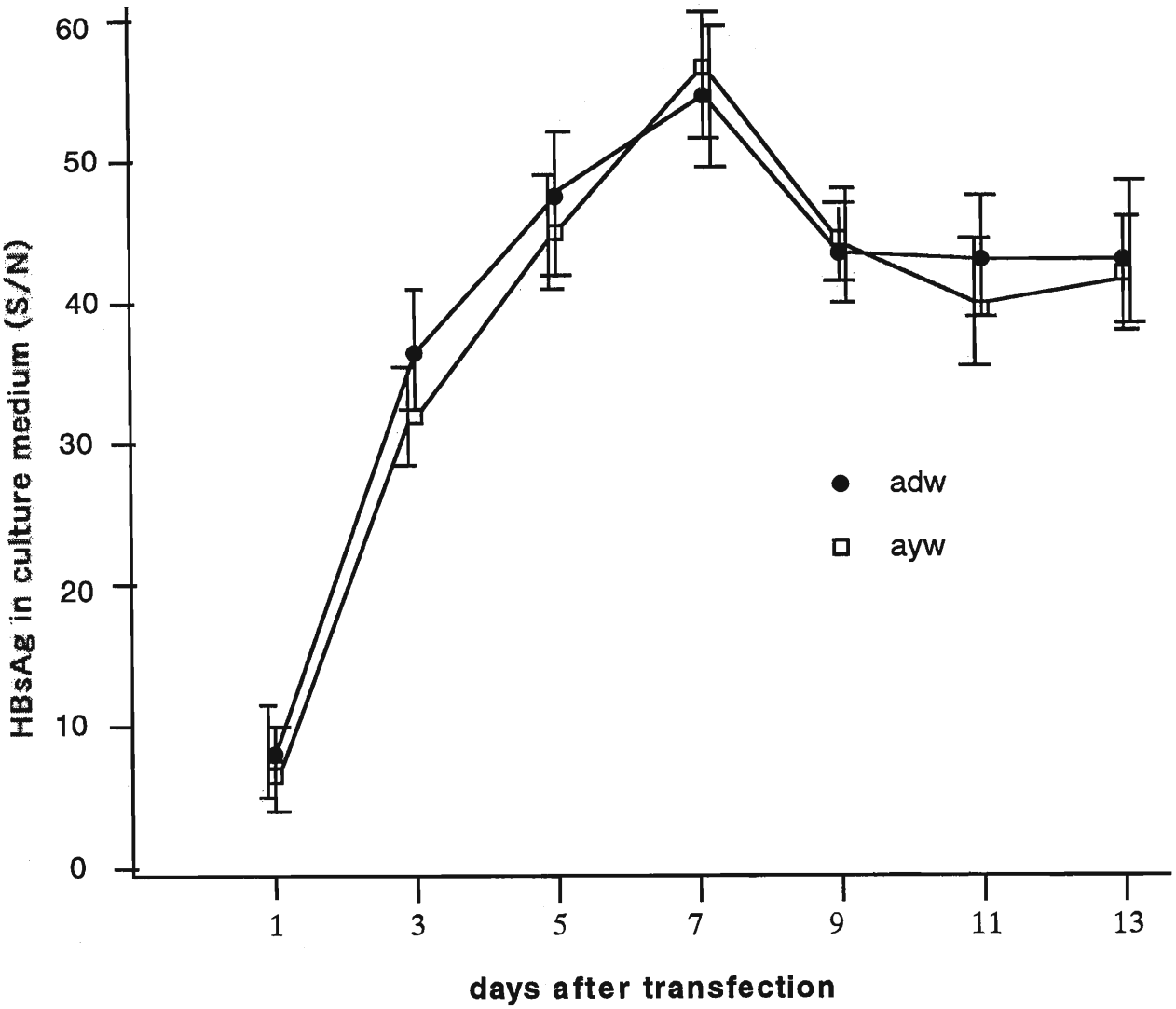

Fig. 1. Expression of HBsAg after transient transfection in cell culture. HuH-7 cells were maintained and transfected with various HBV constructs. Culture media were harvested for detection of HBsAg as described in Materials and Methods. Individual values from EIA for HBsAg are shown, with a signal/noise ratio (S/N) of greater than 2 considered positive. Results are representative of at least three separate experiments. HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; EIA, enzyme immunoassay.

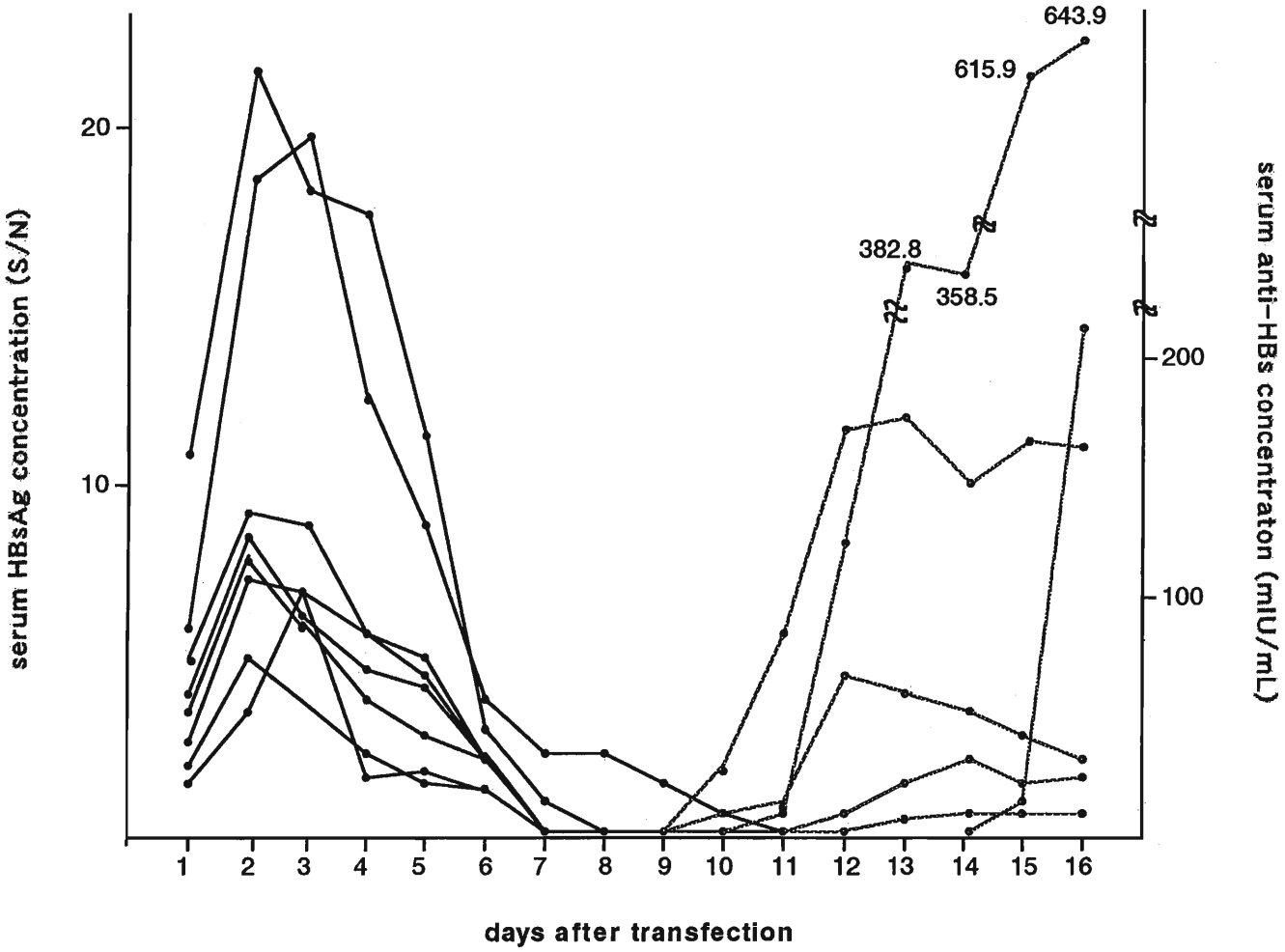

Fig. 2. Secretion of HBsAg and seroconversion to anti-HBs antibody in mice. A conjugate of HBV DNA (wild type) and Lipofectin was injected into the livers of eight C57BL6 mice. HBsAg (solid line) and anti-HBs antibody (broken line) in serum sampled daily after transfection were measured by EIA. HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; EIA, enzyme immunoassay; S/N, signal/noise ratio.

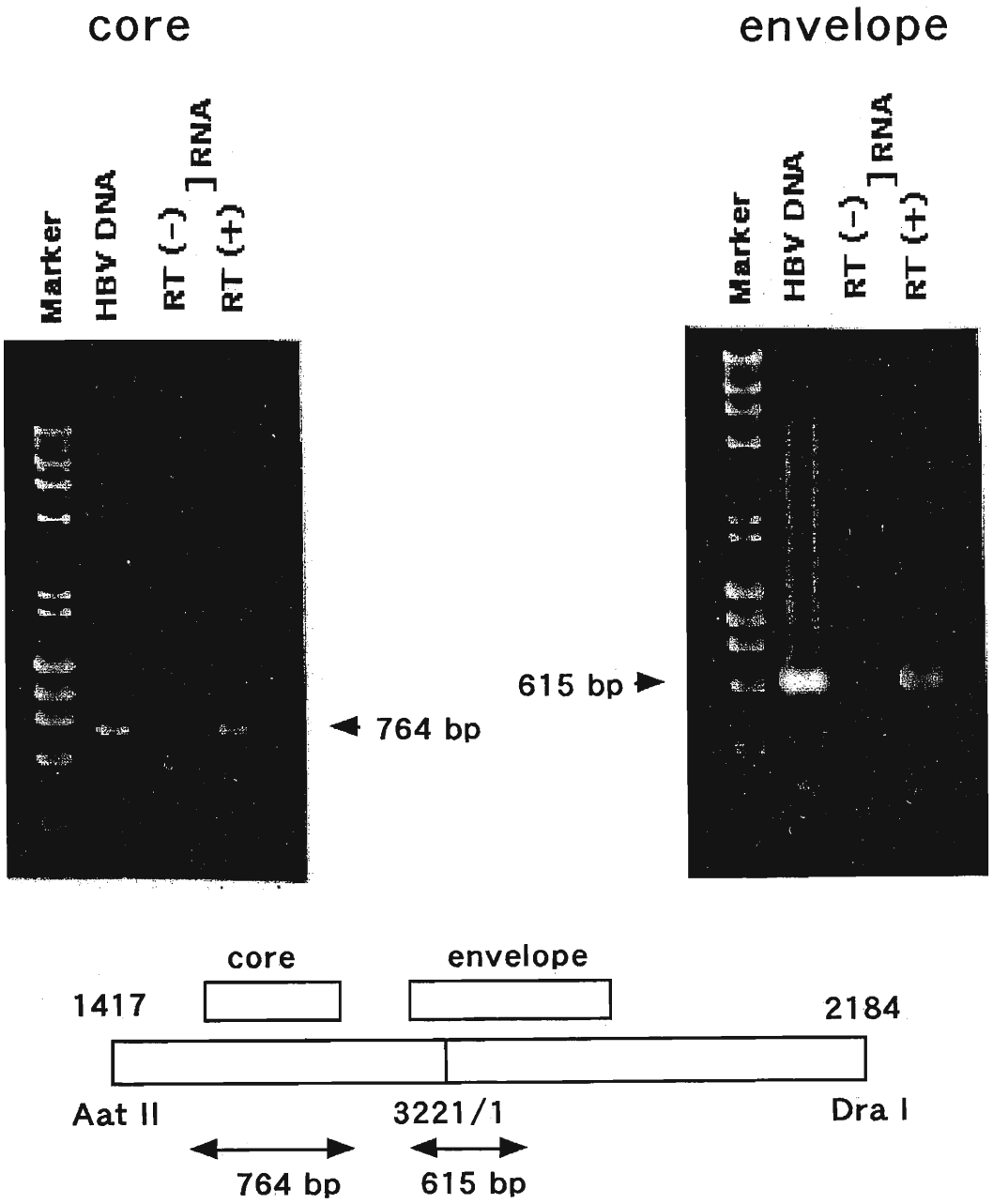

Fig. 3. Detection of HBV transcripts in livers of mice injected with adwR9 HBV DNA. Upper: RT-PCR of RNA extracted from liver tissue resulted in amplification of products of the predicted size for the core region (left panel) and envelope region (right panel) when RT was added (+) but not when it was omitted (RT-). PCR amplification of cloned wild-type adw HBV DNA served as a positive control. Lower: The position and predicted sizes of the PCR products in the core and envelope regions. The sdwR9 construct shown has been described previously [9]. HBV, hepatitis B virus; RT, reverse transcriptase; PCR, polymerase chain reaction.

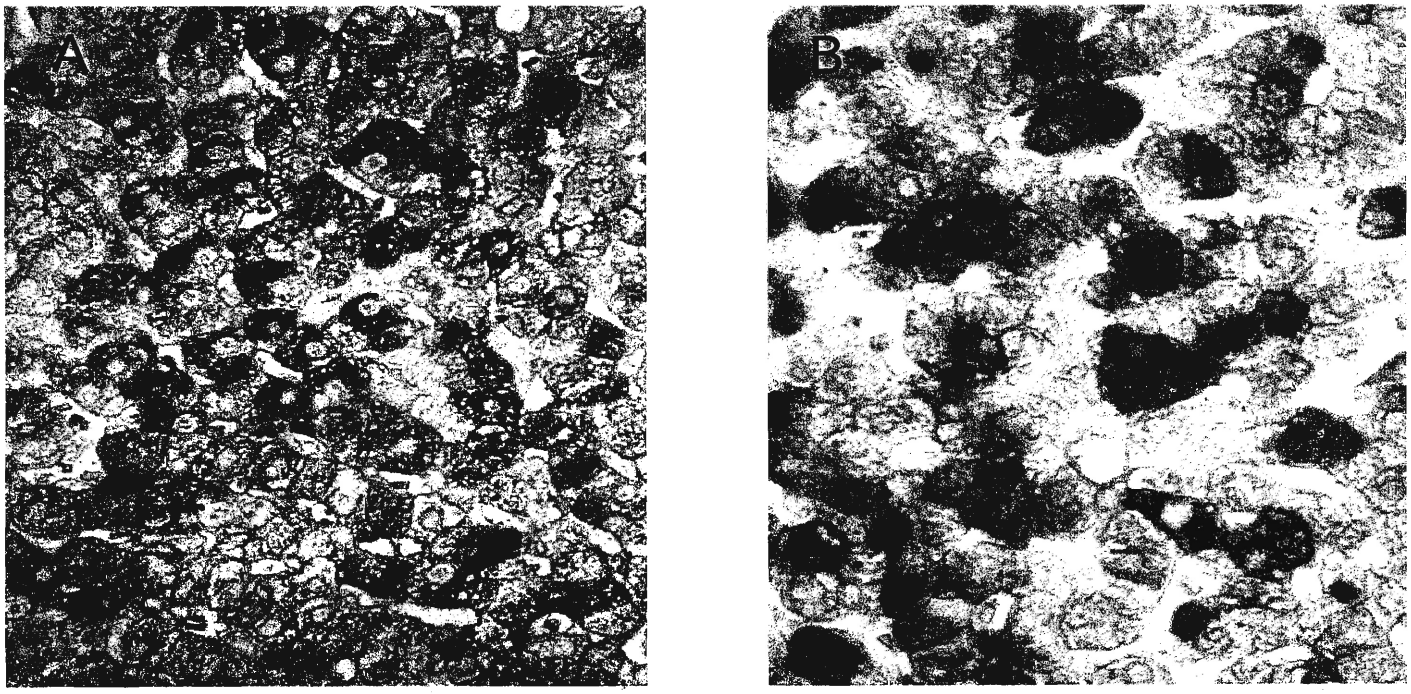

Fig. 4. Expression of HBsAg and HBcAg in livers of mice injected with HBV DNA. Three days after injection of adwR9 (wild type) into the livers of C57BL6 mice, expression of HBsAg and HBcAg in hepatocytes was examined after studied by immunofluorescent staining. A, HBsAg in hepatocytes stained with anti-HBs antibody. B, HBcAg in hepatocytes stained with anti-HBc antibody. HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus. Original magnification, x 100.

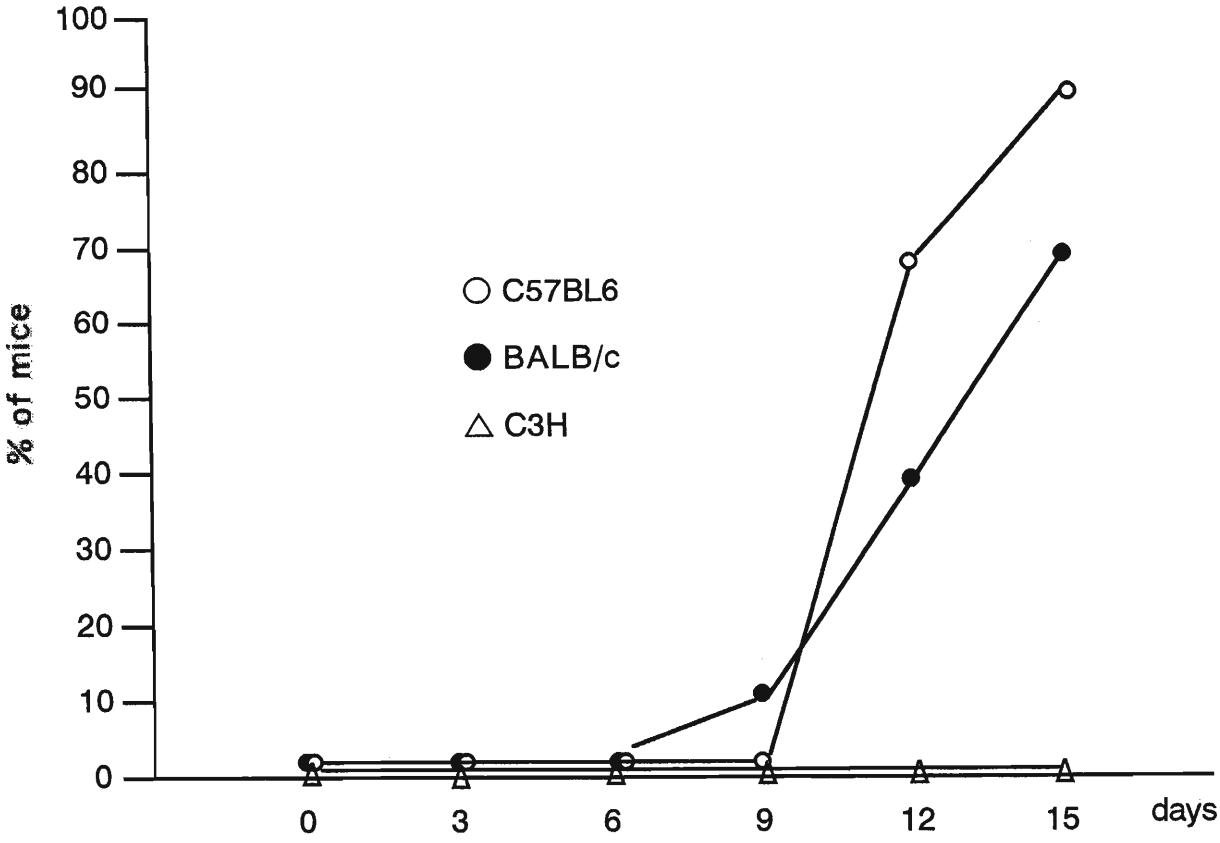

Fig. 5. Differences in production of anti-HBsAb between three mouse strains. Ten mice from each of three strains were injected with adwR9 HBV DNA, and production of anti-HBs antibody was measured daily for 15 days. The data are presented as the percentage of mice from each strain showing detectable concentrations (>5mIU/mL) of circulating anti-HBs antibody. (○): C57BL/6; (●): BALB/c; (△): C3H. HBsAb, hepatitis B surface antibody; HBV, hepatitis B virus.