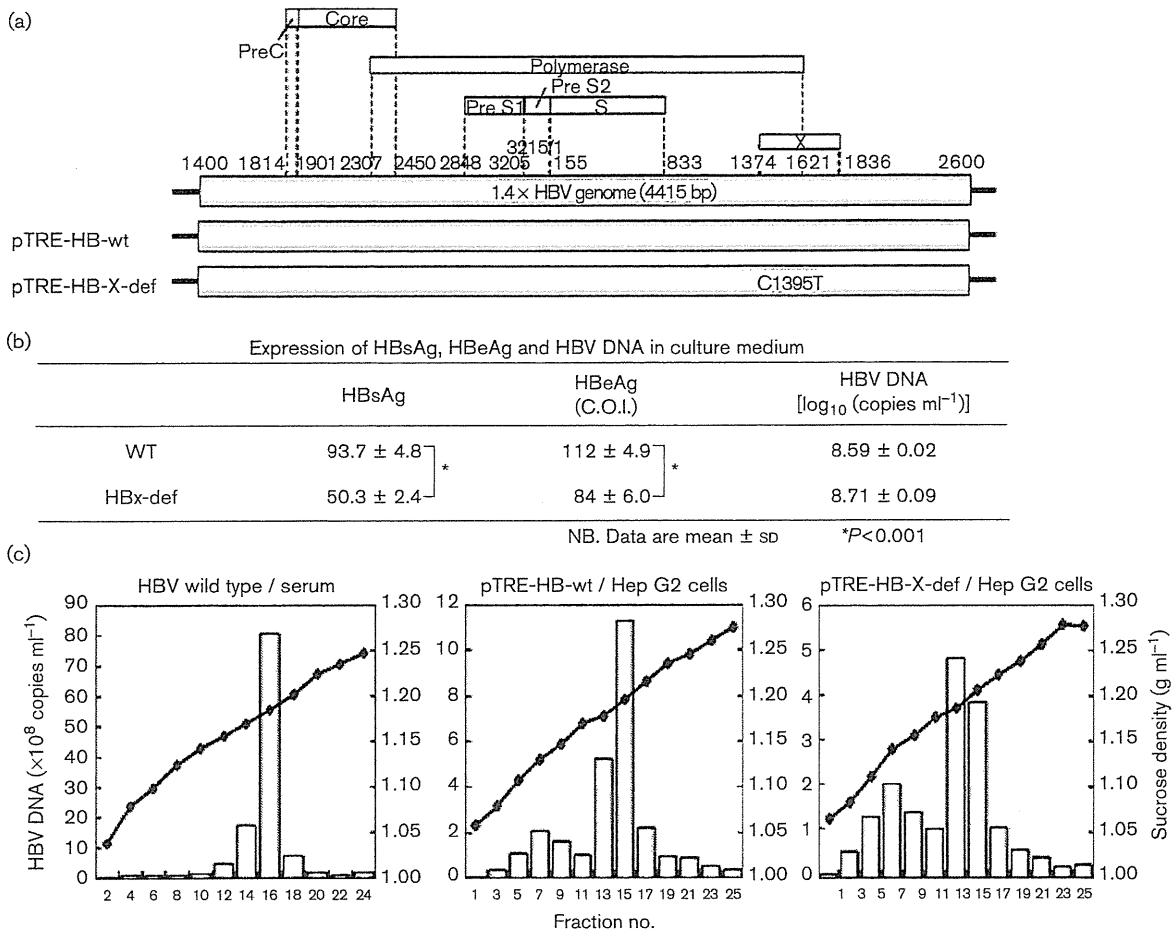
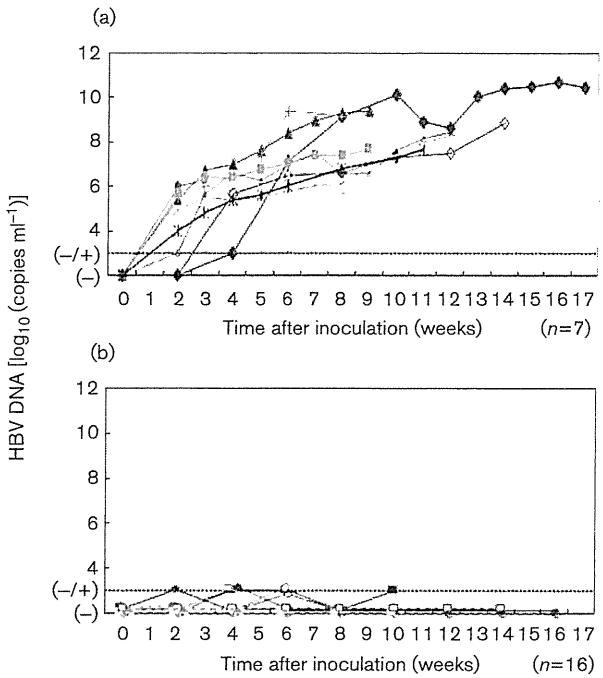


Fig. 1. Construction of HBV expression plasmids. (a) Wild type (WT) 1.4× genome length HBV was cloned into the pTRE2hyg vector (pTRE-HB-wt) and a nucleotide substitution, C1395T, was introduced to create the HBx-def mutant pTRE-HB-X-def. (b) Comparison of expression of HBsAg, HBeAg and HBV DNA in culture medium between WT and HBx-def. (c) Sucrose density gradient analysis of HBV particles (◆) and HBV DNA copies (bars) obtained from a serum sample (left) and supernatants from a cell culture transfected with WT HBV (pTRE-HB-wt, middle) and HBx-def pTRE-HB-X-def. C.O.I., cut-off index.

replication of HBV to the WT level. The effects of HBx protein were also evident on the expression of HBsAg (Fig. 3c) and HBeAg (Fig. 3d). As reported previously, the effect of the C-terminal two-thirds (aa 51–154) of the HBx protein was stronger than that of the entire protein and the N-terminal one-third (aa 1–50) (Tang *et al.*, 2005). The production of replication intermediates was increased similarly by co-transfection of the X proteins (Fig. 3e). To further study the effect of HBx expression, we analysed the levels of intracellular core protein expression. As shown in Fig. 4(a), the expression levels of the core protein were upregulated with the expression of the entire (WT) and C-terminal two-thirds (aa 51–154) of the HBx protein. Immunocytochemical analysis showed that only the cells with strong HBx protein expression were stained with the core protein (Fig. 4b). The core and HBx proteins in these cells were stained mainly in the cytoplasm.

Expression of HBx protein in mouse liver by hydrodynamic injection

Next, we expressed the HBx protein in the chimeric mouse liver with hydrodynamic injection. As shown in Fig. 5(a), a dose-dependent expression of the HBx protein with a haemagglutinin (HA) tag was confirmed by Western blot analysis. Although Henkler *et al.* (2001) showed an aggregation of HBx under the control of the human cytomegalovirus (CMV) promoter, we were able to observe expression of properly sized HBx. Immunohistochemical analysis also revealed HBx protein expression in the mouse



No. analysed mice

Weeks		2	4	6	8	10	12	14	16
HBV	(–/+)	1	3	3	0	1	0	0	0
DNA	(–)	15	10	6	9	6	5	5	3

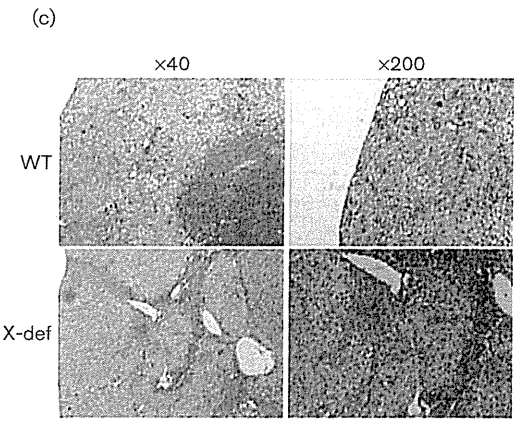


Fig. 2. Infection of human hepatocyte chimeric mice with cell-culture-derived HBV. (a, b) WT (a) and HBx-def (b) HBV particles generated from HepG2 cell lines by transfection of overlength HBV plasmid injected into chimeric mice. (c) Immunohistochemical analysis of mouse liver infected with WT (upper panel) and HBx-def (lower panel) HBV using anti-HBc antibody. HBV-infected hepatocytes were stained brown.

liver. Notably, the HBx protein staining was strong around the central vein (Fig. 5b).

Infection of HBx-def HBV particles with intrahepatic expression of the HBx protein

As the infection experiments with HBx-def HBV failed to result in measurable viraemia (Fig. 2b), we then tried to infect HBx-def HBV after expression of HBx protein by hydrodynamic injection. As shown in Fig. 6(a), six of seven mice developed measurable viraemia 2–8 weeks after inoculation. The incidence of measurable viraemia was significantly higher in mice that received hydrodynamic injection than in those without (Fig. 2b versus Fig. 6a, $P<0.0001$). Immunohistochemical analysis of the infected mice showed simultaneous staining for human serum albumin (hAlb) and HBcAg in the same portion of the liver (Fig. 6b).

Sequence analysis of inocula and the infected mouse sera

We analysed nucleotide sequences of the virus recovered from all six infected mice and compared them with those of inoculated HBx-def HBV. As shown in Fig. 7(a), direct sequencing analyses of the amplified HBV DNA products showed that all revertant viruses had T1395C (mouse MHX#1, 3, 5–7) or T1395A (mouse MHX#2) point mutations, which reverted the introduced stop codon to amino acids. We further analysed nucleotide sequences of HBV by cloning and sequencing using serum samples obtained from two mice (MHX#1, 33 clones; MHX#2, 38 clones) (Fig. 7b). Only one of 33 clones obtained from MHX#1 and none of the 38 clones from MHX#2 had the stop codon mutation that was introduced into the transfected plasmid.

DISCUSSION

In previous studies, HBx has been reported to be a multifunctional protein affecting cell growth and proliferation and activating transcription of mRNA (Arbuthnot *et al.*, 2000; Bouchard *et al.*, 2001; Klein *et al.*, 1999; Murakami, 2001) and virus replication in HCC cell lines (Bouchard *et al.*, 2001; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Tang *et al.*, 2005) and mouse hepatocytes (Keasler *et al.*, 2007; Xu *et al.*, 2002). However, these results were obtained by introduction of HBV genomes into cells using artificial methods such as transfection, gene transfer and hydrodynamic injection. Recently, we established an *in vivo* HBV infection system using human hepatocyte chimeric mice (Tsuge *et al.*, 2005). The system enabled us to perform infection experiments using HBV-containing patient sera and cell-culture medium. Using this system,

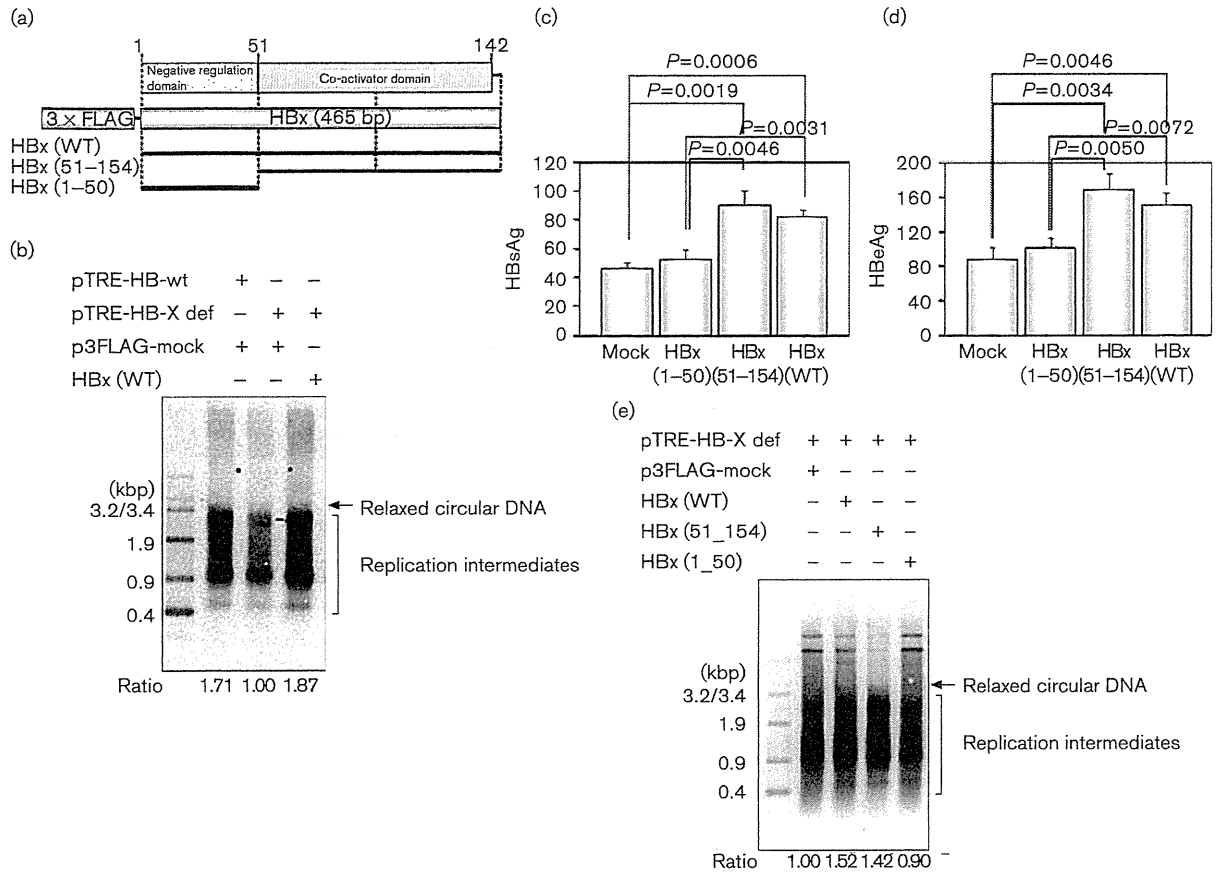


Fig. 3. Recovery of reduced formation of replication intermediate and HB antigens from HBx-def HBV by *trans*-complementation of HBx. (a) Construction of HBx expression plasmids. Full-length and deletion mutants of HBx gene were cloned into the p3FLAG-CMV10 or pcDNA3 or pcDNA3.1-3HA vector. Examples of three FLAG-tagged plasmids are shown. (b) *Trans*-complementation of HBx protein restored the reduced formation of replication intermediates of HBx-def HBV. The core-associated HBV replication intermediates were collected from HepG2 cells and detected with Southern blot hybridization with a full-length HBV probe. (c–e) Recovery of reduced production of HBsAg (c) and HBeAg (d) in culture medium and replication intermediates (e) of HBx-def HBV with *trans*-complementation of HBx expression plasmids. HBx (WT) and HBx(51–154), but not HBx(1–50), effectively enhanced the formation of HBV products. (b, e) The levels of core-associated HBV DNA are shown at the bottom of each lane. Data in (c) and (d) are mean ± SD of three experiments.

we showed previously that HBeAg is dispensable for HBV infection and active replication *in vivo* (Tsuge *et al.*, 2005). Virus replication following infection of HBV particles is quite similar to natural infection. We thus applied the system to study the function of HBx protein in this study. We also utilized hydrodynamic injection of HBx expression plasmid to *trans*-complement the defective HBx. As shown by Western blot analysis (Fig. 4a), HBx protein of the expected size was produced without development of antibody in this SCID-mouse-based model system.

This natural infection mode is quite different from previous animal studies. Virus titres of HBx-def HBV were approx. 50–99 % compared with WT HBV *in vitro* (Bouchard *et al.*, 2001; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Tang *et al.*, 2005) and *in vivo* (Keasler *et al.*, 2007; Xu *et al.*, 2002). High-level HBx-def virus production seen in these experiments may be the result of expression of HBV proteins other than HBx following forced introduction of plasmids into mouse liver cells by hydrodynamic injection or transgenes. Such introduction probably resulted in virus production that is similar to *in vitro* transfection experiments using cultured cells.

In vitro experiments in this study showed that normal-density HBV particles (Fig. 1c) were produced in the absence of HBx. Curiously, the amount of HBV DNA released from the cells into the supernatant was not different between WT and HBx-def HBV, even though the

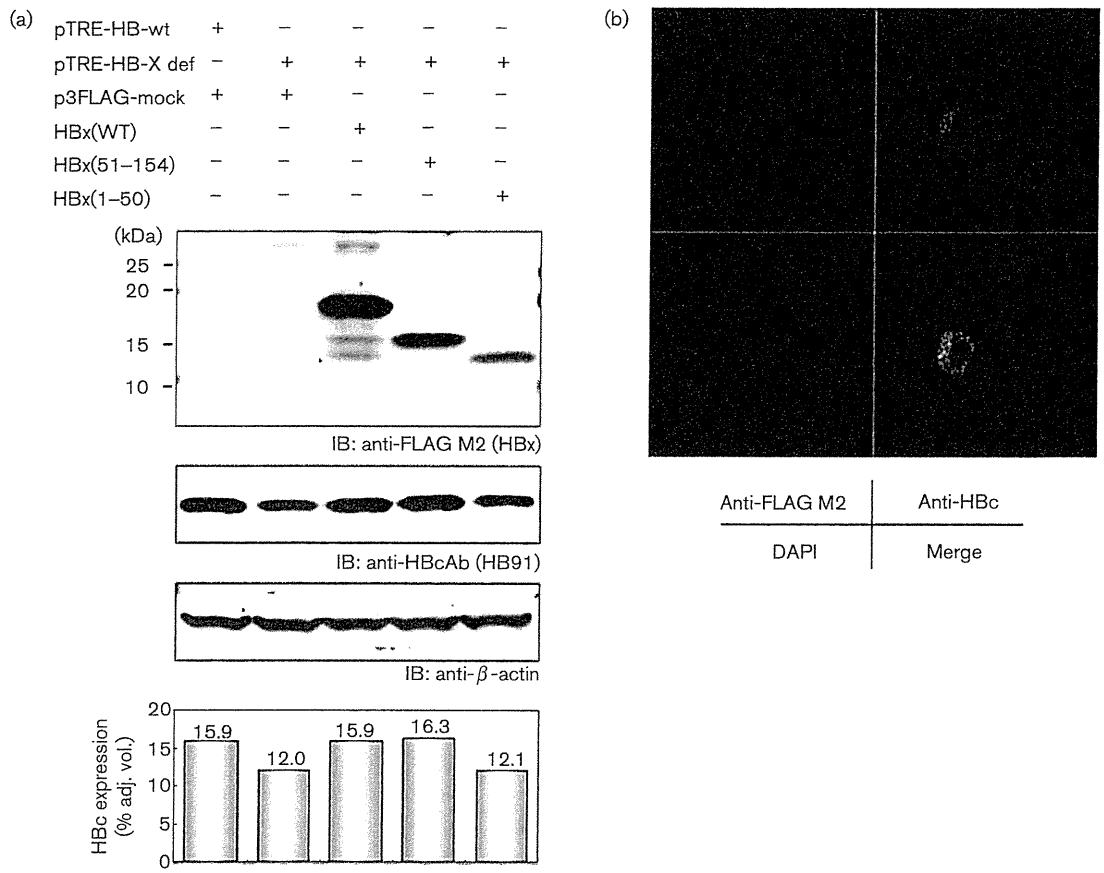


Fig. 4. Upregulation of intracellular core protein formation by *trans*-complementation of the HBx proteins. (a) Western blot analysis of intracellular proteins. Expression of the HBx proteins (percentage adjusted volume) is shown by staining the fused FLAG tag (upper panel). The membrane was also stained by the anti-HBc (middle) and anti-β-actin (lower) antibodies. Values obtained by scanning via densitometer are shown at the bottom of each lane. (b) Immunohistochemical analysis of HepG2 cells co-transfected with pTRE2-HB-X-def and p3FLAG-HBx plasmids. The expression of HBx and HBc proteins was detected by anti-FLAG (upper left) and anti-HBc (upper right) antibody, respectively. The merged image is shown in the lower right and nuclei are shown in the lower left panel. Note that only cells positive for HBx are also positive for HBc protein.

amounts of HBsAg and HBeAg as well as the amount of HBV DNA in cells were significantly greater in WT (Fig. 1b). Efficacy of release of the virus from the cells might be different between WT and HBx-def HBV. Alternatively, production of defective virus, which appeared as the second peak of HBV DNA in the sucrose gradient experiment (Fig. 1c, right panel), might be enriched in HBV DNA in the supernatant of HBx-def HBV. The reason for this discrepancy is unknown. Previous papers did not mention such production of HBV into the supernatant.

Similarly, in the absence of HBx protein *in vitro*, the formation of the replication intermediates (Fig. 3) and production of intracellular core protein (Fig. 4) continued, although their amounts were much lower. It is thus difficult to explain the inability of HBx-def HBV to infect *in vivo* simply from its transcription-activating ability, although our results confirmed that HBx has *trans*-activation ability, as reported previously (Keasler *et al.*, 2007; Tang *et al.*, 2005; Xu *et al.*, 2002). A different mode of introduction of viral nucleic acid might explain the difference seen in *in vitro* and *in vivo* experiments. In the transfection experiments, a relatively large amount of HBV DNA is introduced by transfection. In contrast, only successfully attached virus particles can introduce viral DNA into liver cells.

Strikingly, all but one (70 of 71 clones) revertant viruses had nucleotide substitutions that reversed the introduced stop codon to a coding amino acid. This is in contrast to the fact that HBV replicates in the HBx-def form in cultured cells, even though the efficacy is lower than in WT. We assumed that complemented HBx protein stimulated the replication of HBx-def HBV and increased the chance of nucleotide sequence substitutions in the HBx

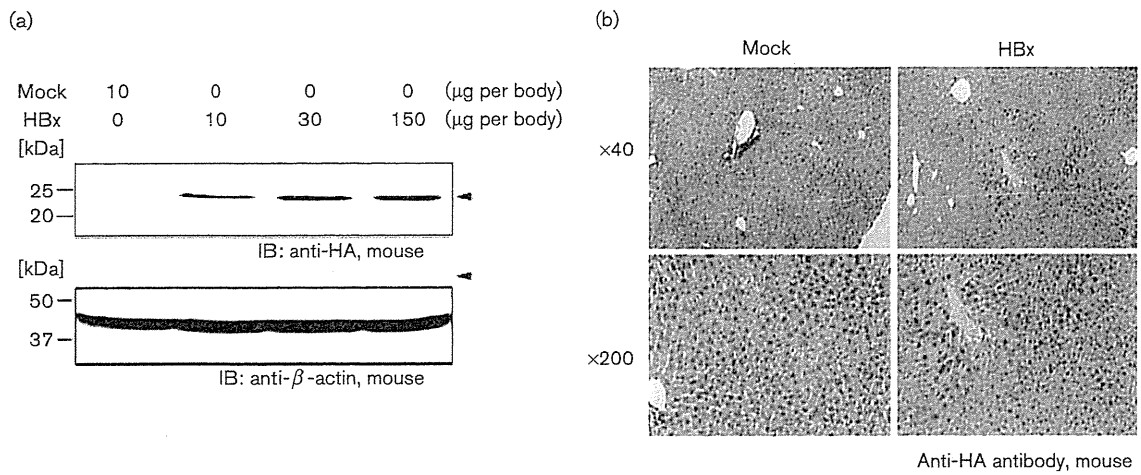


Fig. 5. Expression of HBx protein by hydrodynamic injection of HBx plasmid. (a) Liver-expressed HA-tagged HBx proteins were detected by Western blot analysis using anti-HA antibody (HA tag was used to avoid non-specific binding of anti-FLAG tag to mouse liver proteins). Dose-dependent expression of the protein was observed with different doses of the injected plasmid. (b) Immunohistochemical analysis of mouse liver using anti-HA antibody revealing expression of HBx protein. The protein was mainly expressed around the central vein.

gene, and that only revertant HBV variants predominantly increased, due to their rapid replication ability through the infection–replication cycle that only exists in the *in vivo* model. One might consider the possibility that the HBx protein works as a mutagen. However, we did not observe clear differences in the incidence of nucleotide sequence substitutions between the presence and absence of HBx (Fig. 7b and data not shown).

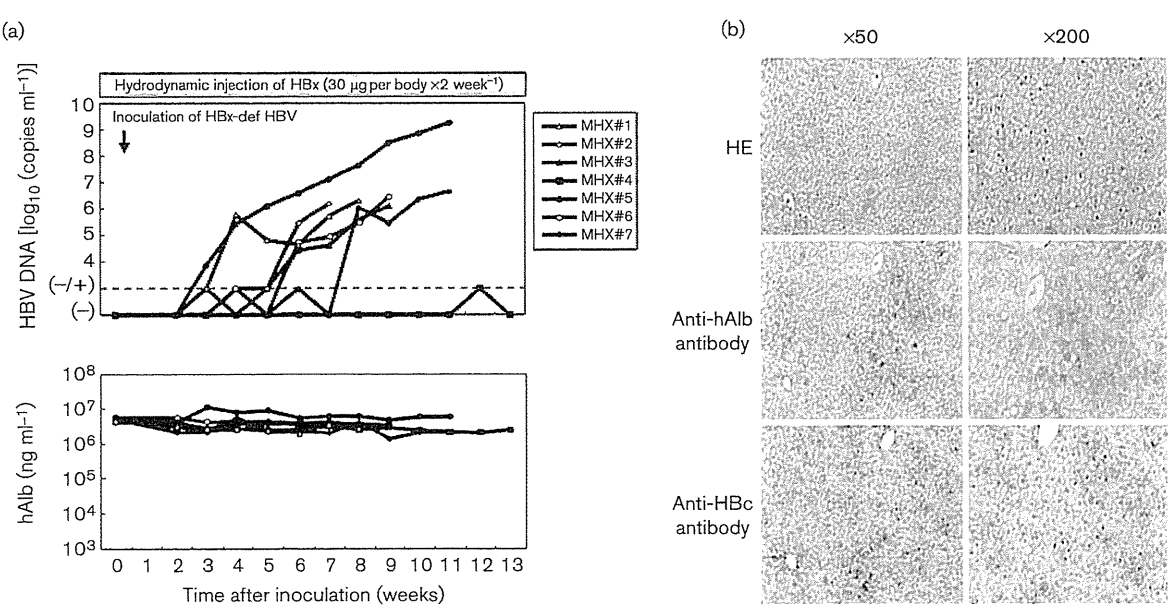


Fig. 6. Infection of HBx-def HBV particles after hydrodynamic injection of HBx expression plasmid. (a) Full-length HBx protein expression plasmid was hydrodynamically injected twice a week into human hepatocyte chimeric mice. Two weeks after the beginning of the injections, cell-culture-derived HBx-def HBV particles were injected through the tail vein. HBV DNA (upper panel) and hAlb (lower panel) were measured. (b) Immunohistochemical analysis of the infected mouse. The liver was stained with haematoxylin and eosin (HE) (upper), antibody against hAlb (middle) and anti-HBc antibody (lower).

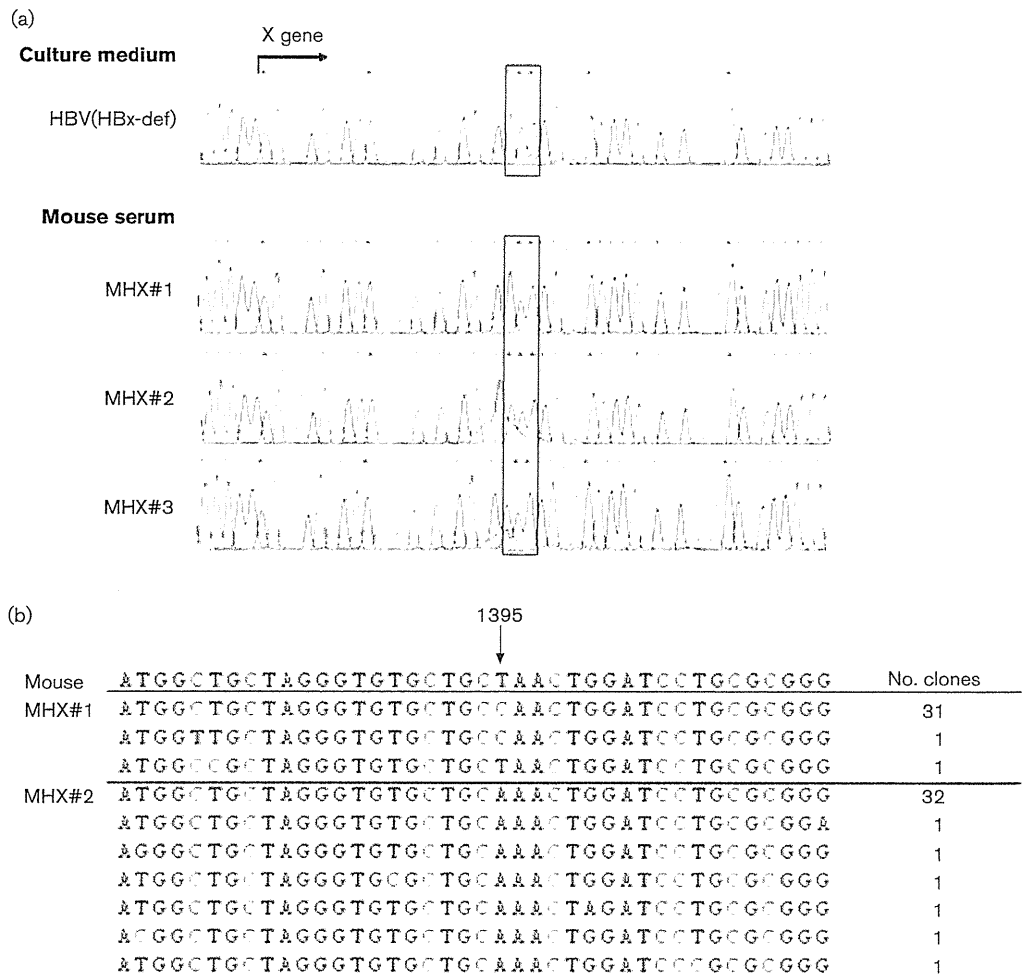


Fig. 7. Nucleotide sequence analysis of HBV recovered from HBx-def HBV-injected mice. (a) Nucleotide sequences of the HBx region of HBV determined by direct sequencing of PCR products using serum samples obtained from three mice (#1, #2 and #3 in Fig. 6a). The sequences were compared with that of inoculated HBV. Note that one of the three mice (#2) had a unique sequence different from the original sequence before introduction of the stop codon (C1395T). (b) Nucleotide sequences of the HBx gene determined by cloning and sequencing of PCR-amplified DNA from mice #1 and #2. Note that only one of 63 clones showed the introduced stop codon mutation. As we used a large amount of HBV plasmids, special care was taken to avoid contamination of DNA. Water was used as a negative control for all experiments and we observed no inappropriate amplification in these experiments.

It is thus still uncertain why the HBx protein is indispensable for virus replication *in vivo*. However, the fact that HBV cannot replicate in the absence of HBx protein may allow development of therapeutic medicine by disturbing the unknown action of HBx. To this end, it is interesting to identify a substance that binds to HBx.

The indispensability of the X protein for virus replication is a common feature shared by HBV and WHV (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Both of them cause chronic infection, inflammation, fibrosis and cancer. In contrast, DHBV, which can replicate without DHBx expression, does not cause such a pathological situation (Meier *et al.*, 2003). Further analysis of the X protein may pave the way to clarify the mechanism of cancer development caused by HBV infection.

METHODS

Human hepatocyte chimeric mice experiments. Care of $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously (Tateno *et al.*, 2004). The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously (Tateno *et al.*, 2004).

hAlb in mouse serum was measured with a Human Albumin ELISA Quantification kit (Bethyl Laboratories Inc.) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquotted and stored in liquid nitrogen until use.

Analysis of HBV markers. HBsAg and HBeAg were measured using a commercially available ELISA kit (Abbott). For quantitative analysis of HBV DNA, 10 μl mouse serum sample or 100 μl of culture supernatant was used. DNA was extracted from these samples using the SMITEST R&D (Genome Science Laboratories) and dissolved in 20 μl H_2O, and HBV DNA was quantified by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems). Amplification was performed as described previously (Tsuge *et al.*, 2005). The lower detection limit of this assay is 300 copies. For detection of small amounts of HBV DNA, we also performed nested PCR. The amplification conditions were as described previously (Tsuge *et al.*, 2005).

Plasmid construction. The construction of wild-type (WT) HBV 1.4 genome length, pTRE-HB-wt, was described previously (Tsuge *et al.*, 2005). We used pTRE2 vector without pTet-off vector and doxycycline because a sufficient amount of HBV transcripts was produced from internal HBV promoters, and transcription from the pTRE2 promoter is negligible under these conditions. The nucleotide sequence of the HBV genome that we cloned into plasmid pTRE-HB-wt was deposited in GenBank under accession number AB206817. A modified plasmid, pTRE-HB-X-def, was generated by introducing a C-to-T point mutation at nt 1395 (aa 7) to create a stop codon (CAA to TAA) in the HBx gene (Fig. 1a). The substitution was introduced by using a QuikChange Site-Directed Mutagenesis kit (Stratagene). For the construction of the HBx gene expression plasmid, the HBx gene was amplified from pTRE-HB-wt and cloned into pcDNA3, pcDNA3-3 × HA, p3 × FLAG-CMV10 vectors and designated pcDNA-HBx, pcDNA3-HA-HBx, p3FLAG-HBx, respectively. Partially truncated HBx plasmids, with a deletion of the N-terminal 50 aa [HBx(51–154)] and the C-terminal 50 aa [HBx(1–50)], were also cloned into pcDNA3 or p3FLAG-CMV10 vectors.

Transfection of HepG2 cell lines with HBV expression plasmids. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum at 37 °C and under 5 % CO_2. For functional analysis of the HBx protein *in vitro*, the HBV or HBx-def HBV expression plasmid was transfected with/without HBx expression plasmid using *Trans*IT-LT1 (Mirus) reagent according to the instructions provided by the supplier. Three to five days after transfection, core-associated HBV DNA was extracted from cells for HBV DNA quantification (Noguchi *et al.*, 2005). For analysing the infectivity of recombinant HBV particles, HBV expression plasmids were transiently transfected into HepG2 cells. The cells were seeded to semi-confluence in 90 mm dishes. WT HBV particles were generated from cells transfected with 20 μg pTRE2-HB-wt by calcium phosphate precipitation. HBx-def HBV particles were also generated from cells co-transfected with 10 μg pTRE2-HB-X-def and 10 μg pcDNA-HBx. Three days after transfection, the culture medium was collected and stored in liquid nitrogen until use.

Analysis of cell-culture-produced HBV by sucrose density gradient sedimentation. Five millilitres of HBV-positive human serum (8 $\log_{10}$ copies ml^{-1}) or 50 ml cell culture supernatant (8 $\log_{10}$ copies ml^{-1}) was layered on a 20 % (w/w) sucrose cushion, and centrifuged at 24 000 r.p.m. (maximum 103 864 *g*) for 12 h at 4 °C with a Beckman SW28 rotor (Beckman Coulter). The precipitate was resuspended in 500 μl PBS. These HBV samples were layered on a linear 20–50 % (w/w) sucrose gradient. Centrifugation was carried out at 24 000 r.p.m. (maximum 102 445 *g*) for 21 h at 4 °C with a Beckman SW40 rotor. The gradients were fractionated into 500 μl samples, and the density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBV DNA by real-time PCR.

Analysis of replication intermediate of HBV. The cells were harvested 5 days after transfection and lysed with 250 μl lysis buffer [10 mM Tris/HCl (pH 7.4), 140 mM NaCl and 0.5 % (v/v) NP-40] followed by centrifugation for 2 min at 15 000 *g*. The core-associated HBV genome was immunoprecipitated by mouse anti-HBV core monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after SDS/proteinase K digestion, followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with SYBR Green using the 7300 Real-Time PCR System and the amounts of the replication intermediates were compared. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 5 s and extension at 72 °C for 6 s. The lower detection limit of this assay was 300 copies.

Immunocytochemistry of HepG2 cells transfected with pTRE2-HB-X-def and p3FLAG-HBx plasmids. HepG2 cells were seeded to semi-confluence in two-well chamber plates. Each 1 μg pTRE2-HB-X-def and p3FLAG-HBx plasmids was co-transfected using *Trans*IT-LT1 reagent (Mirus) according to the instructions provided by the supplier. The cells were harvested 24 h after transfection and then washed with PBS and fixed with 4 % (v/v) paraformaldehyde. After fixation, the cells were stained with mouse monoclonal antibody directed to FLAG (Sigma) or rabbit polyclonal antibody against hepatitis B core antigen (HBcAg; DAKO Diagnostika) as the primary antibody. The bound antibodies were detected with an Alexa Fluor 488-conjugated antibody against rabbit IgG or Alexa Fluor 568-conjugated antibody against mouse IgG, respectively (Molecular Probes). Nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Hydrodynamic injection of HBx expression plasmids. Hydrodynamic injection was performed as reported previously (Yang *et al.*, 2002) with slight modifications. As the human hepatocyte chimeric mice were quite small (12–15 g) and weak for the rapid injection and the stress, we reduced the amount of DNA solution and injection speed: 1 ml PBS containing 30 μg HBx expression plasmids was injected rapidly through the mouse tail vein within 30 s. For analysis of infectivity of HBx-def HBV particles, the plasmids were injected twice a week.

Western blot analysis. Mouse liver tissues or transfected HepG2 cells were cooled on ice and treated with RIPA-like buffer [50 mM Tris/HCl (pH 8.0), 0.1 % SDS, 1 % NP-40, 150 mM sodium chloride and 0.5 % sodium deoxycholate] containing protease inhibitor cocktail (Sigma). Cell lysates were separated on SDS–polyacrylamide gels [5–20 % (w/v)] (Bio-Rad) and then transferred onto nitrocellulose membranes (GE Healthcare) by electroblotting. The membranes were incubated with anti-haemagglutinin fusion epitope (anti-HA) monoclonal antibody (Roche) or with anti β-actin monoclonal antibody (Sigma) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare). Proteins were visualized via the ChemiDoc XRS system (Bio-Rad). Expression of HBc protein was quantified from the densities of the immunoblot signals by Quantity One software (Bio-Rad).

Immunohistochemical analysis of mouse liver. The liver specimens of HBV-infected mice were fixed with 10 % buffered paraformaldehyde and embedded in paraffin blocks for histological

examination. The liver sections were stained with haematoxylin–eosin or subjected to immunohistochemical staining using an antibody against HBcAg (DAKO Diagnostika), anti-HA antibody or HSA (Bethyl Laboratories Inc.). Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 and methanol. Immunoreactive materials were visualized by using a streptavidin–biotin staining kit (Histofine SAB-PO kit; Nichirei) and diaminobenzidine.

Sequence analysis of the HBV genome. Genome-length HBV DNA was amplified by PCR as described by Günther *et al.* (1995). HBV genome-length PCR products were subjected to 1 % agarose gel electrophoresis and the 3.2 kbp band was extracted using a QiaEx II Gel Extraction kit (Qiagen). Direct sequencing, cloning and sequencing (Ohishi *et al.*, 2004) were performed in an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) with a Big Dye Terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Statistical analysis. All data are expressed as mean ± SD. Differences between groups were examined for statistical significance by using Student's *t*-test. A P value <0.05 denoted the presence of a statistically significant difference.

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REFERENCES

Ando, T., Sugiyama, K., Goto, K., Miyake, Y., Li, R., Kawabe, Y. & Wada, Y. (1999). Age at time of hepatitis Be antibody seroconversion in childhood chronic hepatitis B infection and mutant viral strain detection rates. *J Pediatr Gastroenterol Nutr* **29**, 583–587.

Arbuthnot, P., Capovilla, A. & Kew, M. (2000). Putative role of hepatitis B virus X protein in hepatocarcinogenesis: effects on apoptosis, DNA repair, mitogen-activated protein kinase and JAK/STAT pathways. *J Gastroenterol Hepatol* **15**, 357–368.

Bouchard, M. J., Wang, L. H. & Schneider, R. J. (2001). Calcium signaling by HBx protein in hepatitis B virus DNA replication. *Science* **294**, 2376–2378.

Chen, H. S., Kaneko, S., Girones, R., Anderson, R. W., Hornbuckle, W. E., Tennant, B. C., Cote, P. J., Gerin, J. L., Purcell, R. H. & Miller, R. H. (1993). The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. *J Virol* **67**, 1218–1226.

Colgrove, R., Simon, G. & Ganem, D. (1989). Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. *J Virol* **63**, 4019–4026.

Dandri, M., Schirmacher, P. & Rogler, C. E. (1996). Woodchuck hepatitis virus X protein is present in chronically infected woodchuck liver and woodchuck hepatocellular carcinomas which are permissive for viral replication. *J Virol* **70**, 5246–5254.

Dandri, M., Petersen, J., Stockert, R. J., Harris, T. M. & Rogler, C. E. (1998). Metabolic labeling of woodchuck hepatitis B virus X protein in naturally infected hepatocytes reveals a bimodal half-life and association with the nuclear framework. *J Virol* **72**, 9359–9364.

Doria, M., Klein, N., Lucito, R. & Schneider, R. J. (1995). The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. *EMBO J* **14**, 4747–4757.

Ganem, D. & Schneider, R. J. (2001). *Hepadnaviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 2923–2969. Edited by D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman & S. E. Straus. Philadelphia, PA: Lippincott Williams & Wilkins.

Günther, S., Li, B. C., Miska, S., Kruger, D. H., Meisel, H. & Will, H. (1995). A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* **69**, 5437–5444.

Henkler, F., Hoare, J., Waseem, N., Goldin, R. D., McGarvey, M. J., Koshy, R. & King, I. A. (2001). Intracellular localization of the hepatitis B virus HBx protein. *J Gen Virol* **82**, 871–882.

Jacob, J. R., Ascenzi, M. A., Roneker, C. A., Toshkov, I. A., Cote, P. J., Gerin, J. L. & Tennant, B. C. (1997). Hepatic expression of the woodchuck hepatitis virus X-antigen during acute and chronic infection and detection of a woodchuck hepatitis virus X-antigen antibody response. *Hepatology* **26**, 1607–1615.

Keasler, V. V., Hodgson, A. J., Madden, C. R. & Slagle, B. L. (2007). Enhancement of hepatitis B virus replication by the regulatory X protein *in vitro* and *in vivo*. *J Virol* **81**, 2656–2662.

Klein, N. P., Bouchard, M. J., Wang, L. H., Kobarg, C. & Schneider, R. J. (1999). Src kinases involved in hepatitis B virus replication. *EMBO J* **18**, 5019–5027.

Leupin, O., Bontron, S., Schaeffer, C. & Strubin, M. (2005). Hepatitis B virus X protein stimulates viral genome replication via a DDB1-dependent pathway distinct from that leading to cell death. *J Virol* **79**, 4238–4245.

Meier, P., Scougall, C. A., Will, H., Burrell, C. J. & Jilbert, A. R. (2003). A duck hepatitis B virus strain with a knockout mutation in the putative X ORF shows similar infectivity and *in vivo* growth characteristics to wild-type virus. *Virology* **317**, 291–298.

Melegari, M., Wolf, S. K. & Schneider, R. J. (2005). Hepatitis B virus DNA replication is coordinated by core protein serine phosphorylation and HBx expression. *J Virol* **79**, 9810–9820.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A. & other authors (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927–933.

Murakami, S. (2001). Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* **36**, 651–660.

Noguchi, C., Ishino, H., Tsuge, M., Fujimoto, Y., Imamura, M., Takahashi, S. & Chayama, K. (2005). G to A hypermutation of hepatitis B virus. *Hepatology* **41**, 626–633.

Ohishi, W., Shirakawa, H., Kawakami, Y., Kimura, S., Kamiyasu, M., Tazuma, S., Nakanishi, T. & Chayama, K. (2004). Identification of rare polymerase variants of hepatitis B virus using a two-stage PCR with peptide nucleic acid clamping. *J Med Virol* **72**, 558–565.

Raney, A. K. & McLachlan, A. (1991). The biology of hepatitis B virus. In *Molecular Biology of the Hepatitis B Virus*, pp. 1–37. Edited by A. McLachlan. Boca Raton, FL: CRC Press.

Seeger, C. & Mason, W. S. (2000). Hepatitis B virus biology. *Microbiol Mol Biol Rev* **64**, 51–68.

Sitterlin, D., Bergametti, F., Tiollais, P., Tennant, B. C. & Transy, C. (2000a). Correct binding of viral X protein to UVDDB-p127 cellular protein is critical for efficient infection by hepatitis B viruses. *Oncogene* **19**, 4427–4431.

Sitterlin, D., Bergametti, F. & Transy, C. (2000b). UVDDB p127-binding modulates activities and intracellular distribution of hepatitis B virus X protein. *Oncogene* **19**, 4417–4426.

Su, Q., Schroder, C. H., Hofmann, W. J., Otto, G., Pichlmayr, R. & Bannasch, P. (1998). Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. *Hepatology* **27**, 1109–1120.

Tang, H., Banks, K. E., Anderson, A. L. & McLachlan, A. (2001). Hepatitis B virus transcription and replication. *Drug News Perspect* **14**, 325–334.

Tang, H., Delgermaa, L., Huang, F., Oishi, N., Liu, L., He, F., Zhao, L. & Murakami, S. (2005). The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. *J Virol* **79**, 5548–5556.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K. & other authors (2004). Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**, 901–912.

Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y. & other authors (2005). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* **42**, 1046–1054.

Wang, W. L., London, W. T. & Feitelson, M. A. (1991). Hepatitis B X antigen in hepatitis B virus carrier patients with liver cancer. *Cancer Res* **51**, 4971–4977.

Xu, Z., Yen, T. S., Wu, L., Madden, C. R., Tan, W., Slagle, B. L. & Ou, J. H. (2002). Enhancement of hepatitis B virus replication by its X protein in transgenic mice. *J Virol* **76**, 2579–2584.

Yang, P. L., Althage, A., Chung, J. & Chisari, F. V. (2002). Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci U S A* **99**, 13825–13830.

Zhang, Z., Torii, N., Hu, Z., Jacob, J. & Liang, T. J. (2001). X-deficient woodchuck hepatitis virus mutants behave like attenuated viruses and induce protective immunity *in vivo*. *J Clin Invest* **108**, 1523–1531.

Zoulim, F., Saputelli, J. & Seeger, C. (1994). Woodchuck hepatitis virus X protein is required for viral infection *in vivo*. *J Virol* **68**, 2026–2030.

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Importance of Serum Concentration of Adefovir for Lamivudine-Adefovir Combination Therapy in Patients with Lamivudine-Resistant Chronic Hepatitis B▽

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Lamivudine (LMV)-adefovir pivoxil (ADV) combination therapy suppresses the replication of LMV-resistant hepatitis B virus (HBV), although its efficacy in suppressing HBV varies among patients. This study analyzed the clinical, virological, and pharmaceutical factors that influence the effect of the combination therapy. Patients negative for hepatitis B virus e antigen (HBeAg) and with low HBV DNA titers immediately prior to the combination therapy effectively cleared serum HBV DNA ($P = 0.0348$ and $P = 0.0310$, respectively). The maximum concentration of ADV in serum (ADV C_{max}) was higher in patients who showed HBV DNA clearance ($P = 0.0392$), and the cumulative clearance rates of HBV DNA were significantly higher in patients with ADV C_{max} equal to or greater than 24 ng/ml ($P = 0.0284$). HBeAg negativity and lower HBV DNA at the start of the combination therapy and higher ADV C_{max} were found to be independent factors for serum HBV DNA clearance. Serum creatinine increased significantly during the combination therapy, and the ADV C_{max} was higher in patients with low creatinine clearance rates. In conclusion, higher serum concentrations of ADV are associated with a good response to therapy based on clearance of HBV DNA in serum. However, care should be taken to prevent worsening of renal function due to high ADV serum concentrations.

Hepatitis B virus (HBV) infection is a serious global health problem. The risk of chronic HBV infection in immunocompetent adults is generally less than 5% but increases significantly in young children and immunocompromised adults (15, 36). Chronically infected individuals often develop chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and more than 500,000 people die every year from advanced liver diseases (6). Complete elimination of the virus is difficult, and patients are generally treated with interferon and nucleoside/nucleotide analogues, which suppress viral replication and prevent the progression of liver disease by combating inflammation (11, 23, 33). However, the emergence of drug-resistant viral mutants and hepatitis flare-up (breakthrough hepatitis) is a serious concern for such suppressive therapies (7, 9, 20, 32).

Lamivudine (LMV) is the first approved nucleoside analogue that terminates viral DNA synthesis by inhibiting chain elongation (31). Serum HBV DNA levels decrease soon after commencement of LMV therapy. However, long-term therapy frequently results in the emergence of drug-resistant HBV mutants (8, 24). In one study, the rate of LMV resistance increased from 24% in patients treated for 1 year to 70% after 4 years of treatment (21). LMV resistance is usually associated with amino acid substitutions in the YMDD motif of the viral reverse transcriptase (RT) (rtM204V/I/S) (4, 5, 19, 26). Additional substitutions, rtL180M and rtV173L, then further enhance the mutated transcriptase activity (1, 12, 26). The emergence of resistant mutants also often results in viral breakthrough and subsequent breakthrough hepatitis (21). The nucleotide analogue adefovir dipivoxil (ADV) potently suppresses the replication of both wild-type and LMV-resistant HBV both *in vitro* and *in vivo* (17, 25, 27, 37). LMV-ADV combination therapy is therefore recommended as a standard therapy for breakthrough hepatitis in Japan. Although both the combination therapy and ADV monotherapy are reported to be efficacious in patients with LMV-resistant HBV (28), the combination therapy carries a lower risk of emerging LMV-ADV double-resistant mutants (13, 14, 18). Recently, muta-

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TABLE 1. Baseline characteristics of 53 patients who received LMV-ADV combination therapy

Characteristic[a]	Value		P value
	VR (n = 39)	Non-VR (n = 14)	
Sex (male/female)	31/8	8/6	NS[c]
CH/LC	24/15	8/6	NS
HBV genotype C	39	14	
At start of LMV monotherapy			
Age (yr)	54[b] (31–70)	52[b] (27–66)	NS
HBV DNA (log copies/ml)	6.7[b] (2.6–8.5)	6.7 (3.9–8.4)	NS
HBeAg (+/−)	14/25	10/4	0.0236
Duration of LMV monotherapy (wk)	96[b] (0–166)	69[b] (0–213)	NS
At start of LMV plus ADV combination therapy			
Age (yr)	56[b] (32–73)	54[b] (27–69)	NS
BMI (kg/cm^2)	22.3[b] (15.6–27.3)	22.2[b] (18.6–26.2)	NS
Breakthrough hepatitis (+/−)	25/14	8/6	NS
HBV DNA (log copies/ml)	5.6[b] (2.6–8.7)	7.2[b] (4.4–8.0)	0.0310
HBeAg (+/−)	15/24	10/4	0.0348
ALT (IU/liter)	44[b] (12–654)	39[b] (18–310)	NS
Cr (mg/dl)	0.74[b] (0.49–1.28)	0.73[b] (0.45–1.05)	NS
CL_{CR} (ml/min/1.73 m^2)	114.3[b] (56.7–163.1)	101.4[b] (74.9–180.7)	NS
Duration of combination therapy (wk)	186[b] (68–311)	168[b] (58–276)	NS

[a] CH, chronic hepatitis; LC, liver cirrhosis; ALT, alanine transaminase; Cr, creatinine; +, positive; −, negative.
[b] Median value.
[c] NS, not significant.

tions conferring resistance to both LMV and ADV (through a combination of rtA181T/V and rtI233V or rtA181T/V and rtN236T) have been reported (2, 30), although the incidence of these mutations remains lower than the incidence associated with monotherapy.

We recently observed that some patients on LMV-ADV combination therapy who developed LMV resistance showed a poor response to long-term combination therapy. Decrease of serum HBV DNA levels in these patients leveled off, and HBV DNA levels sometimes remained higher than 4 log copies/ml.

The present study investigated those factors that affect the virological response to LMV-ADV combination therapy. We considered the nucleotide and amino acid sequences of HBV reverse transcriptase virological factors and the LMV/ADV concentrations pharmacological factors and then correlated the results with the clinical data of the patients.

MATERIALS AND METHODS

Patients. Between July 2003 and May 2009, 59 consecutive patients with chronic hepatitis or cirrhosis due to LMV-resistant HBV infection were treated with LMV-ADV combination therapy at Hiroshima University Hospital. Of these, 53 patients who received the combination therapy for more than 48 weeks were analyzed in this study. Patients began to receive the combination therapy based on the following criteria: (i) increase in serum HBV DNA levels of ≥1 log copy/ml in comparison with the nadir level during LMV monotherapy with or without breakthrough hepatitis, (ii) detection of mutations in the HBV RT domain related to LMV resistance by direct sequence analysis before the combination therapy, and (iii) serum creatinine levels of <1.5 mg/dl. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethics committee. Written informed consent was obtained from each patient. Patients coinfected with hepatitis C virus or human immunodeficiency virus were excluded from the study. In addition, patients were not administered drugs that affected serum concentrations of LMV and ADV.

The 53 patients were divided into two groups according to virological response: virological responders (VR) and non-VR. Since cessation of the combination therapy in LMV-resistant chronic hepatitis B patients is likely to lead to severe acute exacerbation, response to the therapy was assessed under extended combination therapy. VR were defined by sustained negative serum HBV DNA (<2.6 log copies/ml by the Amplicor HBV Monitor test [Roche Diagnostics, Basel, Switzerland]) for at least 12 weeks, while non-VR showed sustained positive HBV DNA tests until the final observation. In cases of cessation of the combination therapy, the point of discontinuation was defined as the final observation point. Table 1 details the clinical and virological features of the two groups.

The patients were administered daily oral doses of 10 mg ADV and 100 mg LMV. Sera were collected from the patients every month during the combination therapy and stored at −80°C until they were used. Serum HBV DNA, liver function, complete blood count, and serum creatinine were measured every month.

Sequence analysis of the HBV polymerase RT domain. HBV DNA was extracted from 100 μl of stored serum samples using the Smitest R&D (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μl of sterile water. The extracted DNA was then amplified by nested PCR using 1 μl of DNA as a template for the first PCR. PCR was performed in 25 μl of reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM each deoxynucleoside triphosphate (dNTP), 20 pmol of each primer, and 1.25 units of LA *Taq* (Takara Bio Inc., Shiga, Japan) with the buffer supplied by the manufacturer. The first PCR products were diluted 10-fold, and 1 μl was used as a template for the second PCR. The primers used in this study were S2F (nucleotides [nt] 3189 to 3215; 5′-CAGGGATCCT CAGGCCATGCAGTGGAAC-3′) and X2R1 (nt 1606 to 1625; 5′-GTTCACG GTGGTCTCCATGC-3′) for the first PCR, and B2 (nt 65 to 84; 5′-GGCTCM AGTTCMGGAACAGT-3′) (where M is A or C) and X2R1 for the second PCR. The PCR protocol was as follows: initial denaturation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min. After amplification, the final PCR products were gel purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced using the dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence analysis was performed on an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

Measurement of serum concentrations of LMV and ADV. Serum concentrations of LMV and ADV were measured at the last time of observation in 39 of 53 patients who received the combination therapy. Blood sampling for trough values of LMV and ADV was performed at least 24 h after the drugs were taken. Subsequent blood sampling was performed 1 and 2 h after both of the drugs were taken for concentration measurement by liquid chromatography-tandem mass spectrometry (LC–MS-MS) analysis, using an LC-20A system (Shimadzu, Japan) and a Chromolith Performance RP-18e high-performance liquid chromatography (HPLC) column (Waters) for chromatography and an API4000 system

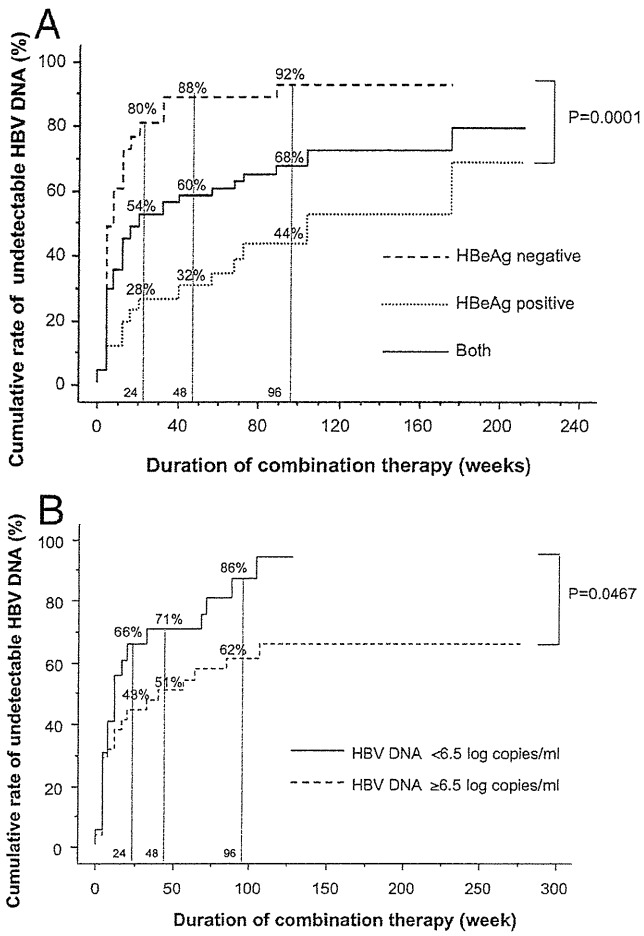

FIG. 1. Cumulative HBV DNA clearance rates in patients treated with lamivudine plus adefovir. Patients were assessed for HBeAg status (A) and HBV DNA levels (B).

(MDS Sciex, Canada) for mass detection and analysis. The instrument was operated in electrospray–positive-ionization mode, and the signal was detected by multiple-reaction monitoring. We defined the highest concentration for the three time points as the maximum concentration of LMV (LMV C_{max}) or ADV (ADV C_{max}) in serum. The AUC_{0-2} (the area under the drug concentration-time curve at 0 to 2 h) of LMV and ADV was calculated by the trapezoidal rule.

Statistical analysis of clinical data. The background characteristics and serum concentrations were compared using the chi-square test and the Mann-Whitney U test. The cumulative probability of undetectable HBV DNA was analyzed by the Kaplan-Meier method, and differences between the curves were tested by the log rank test. P values of less than 0.05 were considered statistically significant.

RESULTS

Effects of LMV-ADV combination therapy. Table 1 details the clinical characteristics of 39 VR and 14 non-VR subjects. Serum HBV DNA in 39 VR decreased to continuously undetectable levels, while serum HBV DNA in 14 non-VR decreased to plateau levels but never became undetectable by the final observation. A larger proportion of VR than non-VR were HBeAg negative prior to the start of LMV monotherapy. Similarly, a larger proportion of VR than non-VR patients were HBeAg negative and had lower serum HBV DNA immediately prior to the combination therapy. The cumulative clearance rates of HBV DNA were significantly higher in HBeAg-negative patients and in those with lower HBV DNA levels (<6.5 log copies/ml) just before the combination therapy than in patients positive for HBeAg or with HBV DNA levels equal to or greater than 6.5 log copies/ml (Fig. 1A and B). Out of 25 patients who were HBeAg positive immediately prior to combination therapy, none had seroconverted to anti-HBe after completing the combination therapy, and none of the total 53 showed viral breakthrough or breakthrough hepatitis during the combination therapy.

Genotyping of LMV- and ADV-resistant mutants. The nucleotide and amino acid sequences were determined for the RT domain in 47 of the 53 patients by the direct-sequencing method at the time just before HBV DNA clearance or at the nadir of HBV DNA levels after initiation of the combination therapy. Negative amplification of HBV DNA because of low HBV DNA values precluded such analysis in the remaining 6 patients. As shown in Table 2, the amino acid substitutions rtS85A and A181T, previously reported to confer ADV resistance (16, 40), were detected in 2 patients and 1 patient, respectively. The 2 patients with an rtS85A mutation also had YMDD motif mutations (Table 2), and their HBV DNA levels decreased gradually to undetectable levels at 62 and 177 weeks after the beginning of combination therapy, respectively. In contrast, HBV levels in the patient with a unique rtA181T mutation did not decrease to undetectable levels following 58 weeks of combination therapy until the patient was successfully treated with entecavir (ETV) monotherapy (reference 39 and data not shown).

Virological response to the combination therapy according to serum concentrations of LMV and ADV. To further explore the poor response of non-VR to therapy, drug concentration analysis was then undertaken in 29 VR and 10 non-VR, and the C_{max} and AUC_{0-2} values of LMV and ADV were compared. ADV C_{max} was significantly higher in VR than in non-VR (Fig. 2A), although the difference for ADV AUC_{0-2} was not statistically significant (Fig. 2B). The median values of ADV C_{max} and ADV AUC_{0-2} were 24 ng/ml and 37 ng · h/ml, respectively. The cumulative HBV DNA clearance rates were significantly higher in patients with high ADV C_{max} values (≥24 ng/ml) (Fig. 2C), and most of these patients belonged to

TABLE 2. Amino acid sequence substitutions in the HBV RT domain

Substitution[a]	No. of patients with substitution[a]	
	VR (n = 33)	Non-VR (n = 14)
rtM204 M		
Alone	7	3
+rtA181T	0	1
rtM204V/I		
Alone	19	10
+rtV214A/E	2	0
+rtQ215H	2	0
+rtV84I	1	0
+rtS85A	1	0
+rtS85A + rtV214E	1	0

[a] Two known ADV-resistant amino acid substitutions (A181T and S85A) are underlined.

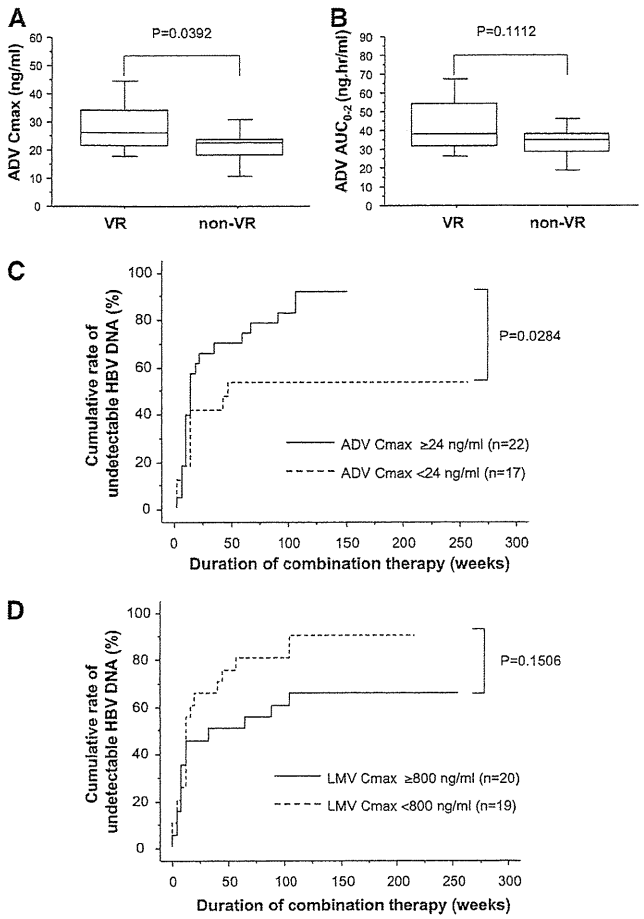

FIG. 2. Serum concentrations of ADV and effects of combination therapy. (A and B) Effects of the combination therapy based on ADV C_{max} (A) and AUC_{0-2} (B) determinations. In these box-and-whisker plots, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (C and D) Cumulative clearance rates of HBV DNA by ADV C_{max} (C) and LMV C_{max} (D).

the VR group (Table 3). However, the C_{max} and AUC_{0-2} of LMV were not significantly different between the VR and non-VR groups, and there was no difference in HBV clearance rates between patients with high or low C_{max} or AUC_{0-2} of

TABLE 3. Serum concentration of ADV and efficacy of LMV-ADV combination therapy

Parameter and value	No. (%) with value		*P* value
	VR	Non-VR	
AUC_{0-2} (ng · h/ml)			
≥37	15 (52)	3 (30)	0.2071
<37	14 (48)	7 (70)	
Total	29	10	
C_{max} (ng/ml)			
≥24	20 (69)	2 (20)	0.0097
<24	9 (31)	8 (80)	
Total	29	10	

TABLE 4. Multivariate analysis of factors associated with HBV DNA clearance in LMV-ADV combination therapy

Factor[a]	Category	*P* value	Odds ratio	95% CI
HBeAg	1 (positive)	0.0170	1	1.475–25.129
	2 (negative)		7.194	
HBV DNA (log copies/ml)	1 (≥6.5)	0.0485	1	1.178–22.367
	2 (<6.5)		4.185	
ADV C_{max} (ng/ml)	1 (<24)	0.0019	1	2.833–99.836
	2 (≥24)		16.818	

[a] At the start of LMV-ADV combination therapy. Factors: gender, age, background liver status, HBeAg, HBV DNA, ALT, Cr, RT mutation, ADV C_{max}, and ADV AUC_{0-2}.

LMV (Fig. 2D and data not shown). The AUC_{0-2} and C_{max} levels of both LMV and ADV did not correlate with the body mass index (BMI) (data not shown).

Analysis of independent predictive factors for VR. To analyze predictive factors for achieving VR, multivariate analysis was conducted. When factors appearing in Tables 1, 2, and 3 were analyzed simultaneously, higher ADV C_{max} and HBeAg negativity and lower HBV DNA at the start of the combination therapy were found to be independent factors for VR (Table 4). ADV C_{max}, in particular, was a strong determinant factor for VR (odds ratio, 16.818; 95% confidence interval [CI], 2.833 to 99.836).

Renal function and serum concentrations of the drugs. LMV and ADV are excreted from the kidney. Serum creatinine levels increased in 17 (32.1%) of 53 patients during the combination therapy, while the median serum creatinine levels increased significantly from 0.74 mg/dl at baseline to 0.86 mg/dl at the end of the observation period in 53 patients treated with LMV and ADV (Fig. 3A). The dose of ADV was reduced in 6 (11.3%) of the 53 patients to 5 mg/day or 10 mg every 2 days, and ADV administration was stopped in 3 (5.7%) patients due to elevated serum creatinine levels (≥1.5 mg/dl). The HBV DNA titers of 6 patients who reduced the dose of ADV never had a flare-up after the reduction. Five of the 6 patients belonged to the VR and one to the non-VR group. Serum creatinine levels returned to pretherapy values in all patients who reduced or stopped treatment with ADV. Next, we investigated whether the drug concentration was related to renal function. The C_{max} and AUC_{0-2} values of LMV and ADV were compared between patients whose creatinine clearance rates (CL_{CR}) were normal and those whose rates were low. As shown in Fig. 3B and C, both C_{max} and AUC_{0-2} of ADV were significantly higher in patients with CL_{CR} of <80 ml/min/1.73 m^2. In contrast, there was no relationship found between CL_{CR} and C_{max}/AUC_{0-2} of LMV (Fig. 3D and E).

DISCUSSION

The poor response of chronic HBV infection to nucleotide/nucleoside therapy is commonly attributed to amino acid substitutions in the RT domain of HBV polymerase. Several RT amino acid mutations that induce resistance to ADV have been reported, although the incidence is much lower than that reported for LMV. The HBV polymerase RT domain substi-

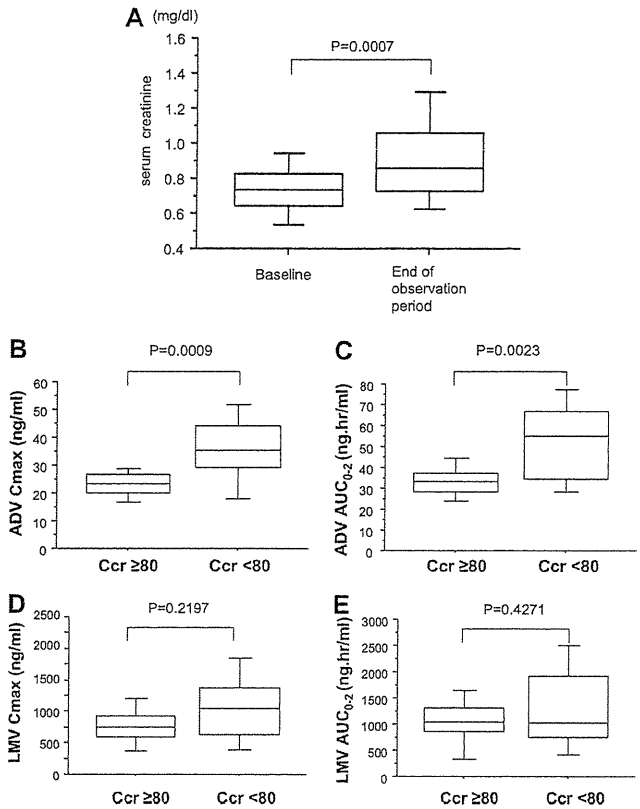

FIG. 3. (A) Comparison of serum creatinine concentrations just before the start of the combination therapy and at the end of the observation period. Renal function and concentrations of LMV and ADV are shown. (B to E) The ADV C_{max} (B), LMV C_{max} (D), and AUC_{0-2} of ADV (C) and LMV (E) were compared between patients with high (≥80 ml/min/1.73 m^2) and low (<80 ml/min/1.73 m^2) CL_{CR}. In these box-and-whisker plots, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

tutions rtV84, rtS85, rtA181, rtV214, rtQ215, rtI233, rtN236, and rtP237 are associated with ADV resistance (16, 40), and rtA181 and rtQ215 mutations are associated with cross-resistance to LMV and ADV (23, 35). To study the incidence and the effects of amino acid substitutions in the RT domain of HBV polymerase in patients receiving combination therapy, this study initially analyzed serum samples for amino acid sequences in the region. We identified the previously reported A181T and S85A substitutions, as well as substitutions at rt84, rt214, and rt215 that might confer resistance to ADV. However, all these mutations, except for A181T, were found in VR.

These results are consistent with a previous report that most of these mutations confer only limited resistance to ADV therapy (16). In contrast, one of 14 patients who failed to clear HBV DNA in the present study had an apparent ADV resistance mutation. This unique mutation, A181T, which disrupts a stop codon in the HBs gene, is reported to be involved in resistance against both LMV and ADV (39). Therefore, it became apparent in the present series of experiments that only one of 14 patients responded poorly to the combination therapy due to the emergence of a resistant viral clone.

However, none of the remaining 13 patients had amino acid substitutions known to induce resistance to ADV. This is consistent with a recent report by Lampertico et al. (22) citing 11% of patients who failed to clear serum HBV DNA despite 3 years of combination therapy. In addition, none of these patients had a known ADV-resistant strain of HBV. Yatsuji et al (40) also reported 6 of 132 patients with transiently fluctuating HBV DNA levels (from <2.6 to 3.1 log copies/ml) and wild-type genotypes for rtA181 and rtN236.

To further explore the poor response to combination therapy, the concentration of ADV was investigated with respect to the drug's efficacy. Although it is noted that ADV is converted to the diphosphate derivative in hepatocytes by adenylate kinase and inhibits viral DNA polymerase (3, 29), the detailed metabolic pathway remains unclear. According to experimental data from GlaxoSmithKline K.K., when chronic hepatitis B patients were administered oral doses of 10 mg ADV and 100 mg LMV, the ADV C_{max} and AUC_{0-24} were 20.1 ± 3.3 ng/ml and 231.5 ± 33.7 ng · h/ml, respectively (AUC_{0-2} data not shown). The reported 50% inhibitory concentration (IC_{50}) of ADV is 0.36 to 0.39 μM, equal to 180.5 to 195.6 ng/ml (38, 39) and much higher than the values obtained in this study (ADV C_{max}, 5.1 to 54.6 ng/ml). This difference might come from the fact that the concentration of orally administered ADV should be higher in portal blood but lower in the peripheral blood. At any rate, there have been no reports detailing effective serum concentrations of ADV. In this study, the ADV C_{max} was higher in VR, and cumulative clearance rates of HBV DNA were higher in patients with higher ADV C_{max} values. The reason for the lack of association between the efficacy of the combination therapy and the ADV AUC_{0-2} remains unclear and might be related to different absorption profiles or metabolic profiles for the drugs or lack of power due to the small number of patients analyzed. However, these results indicate that poor response to the combination therapy arises at least in part from a low serum concentration of ADV. Because 90.9% (20/22) of patients with ADV C_{max} values equal to or greater than 24 ng/ml could clear serum HBV DNA, it is expected that non-VR with ADV C_{max} values below 24 ng/ml can achieve VR by boosting the serum level of ADV. Therefore, it might be recommended to raise the serum level of ADV to over 24 ng/ml in such cases. Two choices are considered for boosting the serum concentration of ADV: increasing the dose of ADV or using drugs that affect the serum concentration of ADV, such as an inhibitor of organic anion transporters (10, 34).

Meanwhile, renal dysfunction sometimes occurs as a side effect of ADV, and serum creatinine levels actually increased in patients administered the combination therapy; 11.3% of patients had to reduce the dose of ADV, and 5.7% of patients had to discontinue ADV due to elevated serum creatinine levels. Furthermore, the serum concentration of ADV was higher in patients with low CL_{CR}. This finding suggests a possible worsening of renal dysfunction in patients treated with ADV due to the generation of a vicious cause-effect circle (a higher ADV concentration worsens renal function). Although we did not investigate the safety range of ADV concentrations

in this study, and the upper limit of the range is not known, it is considered important that adequate and precise doses of ADV should be prescribed to patients, especially those with impaired renal function, instead of simply increasing the serum concentration of ADV. This study suggests that monitoring the serum ADV concentration would be useful to fine tune the appropriate drug dosage.

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REFERENCES

1. **Allen, M. I., M. Deslauriers, C. W. Andrews, G. A. Tipples, K. A. Walters, D. L. Tyrrell, N. Brown, and L. D. Condreay.** 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology **27:**1670–1677.
2. **Angus, P., R. Vaughan, S. Xiong, H. Yang, W. Delaney, C. Gibbs, C. Brosgart, D. Colledge, R. Edwards, A. Ayres, A. Bartholomeusz, and S. Locarnini.** 2003. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology **125:**292–297.
3. **Balzarini, J., Z. Hao, P. Herdewijn, D. G. Johns, and E. De Clercq.** 1991. Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl) adenine, a potent anti-human immunodeficiency virus compound. Proc. Natl. Acad. Sci. U. S. A. **88:**1499–1503.
4. **Bozdayi, A. M., C. P. Eyigun, A. R. Turkyilmaz, I. Y. Avci, A. Pahsa, and C. Yurdaydin.** 2004. A novel pattern (sW195a) in surface gene of HBV DNA due to YSDD (L180M plus M204S) mutation selected during lamivudine therapy and successful treatment with adefovir dipivoxil. J. Clin. Virol. **31:** 76–77.
5. **Bozdayi, A. M., O. Uzunalimoğlu, A. R. Türkyilmaz, N. Aslan, O. Sezgin, T. Sahin, G. Bozdayi, K. Cinar, S. B. Pai, R. Pai, H. Bozkaya, S. Karayaçin, C. Yurdaydin, and R. F. Schinazi.** 2003. YSDD: a novel mutation in HBV DNA polymerase confers clinical resistance to lamivudine. J. Viral Hepat. **10:**256–265.
6. **Bruix, J., and J. M. Llovet.** 2003. Hepatitis B virus and hepatocellular carcinoma. J. Hepatol **39**(Suppl. 1):S59–S63.
7. **Buti, M., R. Jardi, M. Cotrina, F. Rodriguez-Frias, R. Esteban, and J. Guardia.** 1998. Transient emergence of hepatitis B variants in a patient with chronic hepatitis B resistant to lamivudine. J. Hepatol. **28:**510–513.
8. **Chang, T. T., C. L. Lai, R. N. Chien, R. Guan, S. G. Lim, C. M. Lee, K. Y. Ng, G. J. Nicholls, J. C. Dent, and N. W. Leung.** 2004. Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. J. Gastroenterol. Hepatol. **19:**1276–1282.
9. **Chayama, K., Y. Suzuki, M. Kobayashi, M. Kobayashi, A. Tsubota, M. Hashimoto, Y. Miyano, H. Koike, M. Kobayashi, I. Koida, Y. Arase, S. Saitoh, N. Murashima, K. Ikeda, and H. Kumada.** 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology **27:**1711–1716.
10. **Cihlar, T., D. C. Lin, J. B. Pritchard, M. D. Fuller, D. B. Mendel, and D. H. Sweet.** 1999. The antiviral nucleotide analogs cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. Mol. Pharmacol. **56:**570–580.
11. **Conjeevaram, H. S., and A. S. Lok.** 2003. Management of chronic hepatitis B. J. Hepatol **38**(Suppl. 1):S90–S103.
12. **Delaney, W. E., IV, H. Yang, C. E. Westland, K. Das, E. Arnold, C. S. Gibbs, M. D. Miller, and S. Xiong.** 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J. Virol. **77:**11833–11841.
13. **Fung, S. K., P. Andreone, S. H. Han, K. Rajender Reddy, A. Regev, E. B. Keeffe, M. Hussain, C. Cursaro, P. Richtmyer, J. A. Marrero, and A. S. Lok.** 2005. Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. J. Hepatol. **43:**937–943.
14. **Fung, S. K., H. B. Chae, R. J. Fontana, H. Conjeevaram, J. Marrero, K. Oberhelman, M. Hussain, and A. S. Lok.** 2006. Virologic response and resistance to adefovir in patients with chronic hepatitis B. J. Hepatol. **44:** 283–290.
15. **Ganem, D., and A. M. Prince.** 2004. Hepatitis B virus infection—natural history and consequences. N. Engl. J. Med. **350:**1118–1129.
16. **Ghany, M., and T. J. Liang.** 2007. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. Gastroenterology **132:**1574–1585.
17. **Hadziyannis, S. J., N. C. Tassopoulos, E. J. Heathcote, T. T. Chang, G. Kitis, M. Rizzetto, P. Marcellin, S. G. Lim, Z. Goodman, M. S. Wulfsohn, S. Xiong, J. Fry, C. L. Brosgart, and the Adefovir Dipivoxil 438 Study Group.** 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. N. Engl. J. Med. **348:**800–807.
18. **Ijaz, S., C. Arnold, S. Dervisevic, J. Mechurova, N. Tatman, R. S. Tedder, and N. V. Naoumov.** 2008. Dynamics of lamivudine-resistant hepatitis B virus during adefovir monotherapy versus lamivudine plus adefovir combination therapy. J. Med. Virol. **80:**1160–1170.
19. **Jardi, R., M. Buti, F. Rodriguez-Frias, M. Cotrina, X. Costa, C. Pascual, R. Esteban, and J. Guardia.** 1999. Rapid detection of lamivudine-resistant hepatitis B virus polymerase gene variants. J. Virol. Methods **83:** 181–187.
20. **Kobayashi, M., F. Suzuki, N. Akuta, Y. Suzuki, Y. Arase, K. Ikeda, T. Hosaka, H. Sezaki, M. Kobayashi, S. Iwasaki, J. Sato, S. Watahiki, Y. Miyakawa, and H. Kumada.** 2006. Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotype A, B, and C. J. Med. Virol. **78:**1276–1283.
21. **Lai, C. L., J. Dienstag, E. Schiff, N. W. Leung, M. Atkins, C. Hunt, N. Brown, M. Woessner, R. Boehme, and L. Condreay.** 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin. Infect. Dis. **36:**687–696.
22. **Lampertico, P., M. Viganò, E. Manenti, M. Iavarone, E. Sablon, and M. Colombo.** 2007. Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. Gastroenterology **133:**1445–1451.
23. **Lee, Y. S., D. J. Suh, Y. S. Lim, S. W. Jung, K. M. Kim, H. C. Lee, Y. H. Chung, Y. S. Lee, W. Yoo, and S. O. Kim.** 2006. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology **43:**1385–1391.
24. **Leung, N. W., C. L. Lai, T. T. Chang, R. Guan, C. M. Lee, K. Y. Ng, S. G. Lim, P. C. Wu, J. C. Dent, S. Edmundson, L. D. Condreay, R. N. Chien, and the Asia Hepatitis Lamivudine Study Group.** 2001. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology **33:**1527–1532.
25. **Marcellin, P., T. T. Chang, S. G. Lim, M. J. Tong, W. Sievert, M. L. Shiffman, L. Jeffers, Z. Goodman, M. S. Wulfsohn, S. Xiong, J. Fry, C. L. Brosgart, and the Adefovir Dipivoxil 437 Study Group.** 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N. Engl. J. Med. **348:**808–816.
26. **Melegari, M., P. P. Scaglioni, and J. R. Wands.** 1998. Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. Hepatology **27:**628–633.
27. **Perrillo, R., E. Schiff, E. Yoshida, A. Statler, K. Hirsch, T. Wright, K. Gutfreund, P. Lamy, and A. Murray.** 2000. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology **32:**129–134.
28. **Peters, M. G., H. Hann Hw, P. Martin, E. J. Heathcote, P. Buggisch, R. Rubin, M. Bourliere, K. Kowdley, C. Trepo, D. Gray Df, M. Sullivan, K. Kleber, R. Ebrahimi, S. Xiong, and C. L. Brosgart.** 2004. Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. Gastroenterology **126:**91–101.
29. **Robbins, B. L., J. Greenhaw, M. C. Connelly, and A. Fridland.** 1995. Metabolic pathways for activation of the antiviral agent 9-(2-phosphonylmethoxyethyl) adenine in human lymphoid cells. Antimicrob. Agents Chemother. **39:**2304–2308.
30. **Schildgen, O., H. Sirma, A. Funk, C. Olotu, U. C. Wend, H. Hartmann, M. Helm, J. K. Rockstroh, W. R. Willems, H. Will, and W. H. Gerlich.** 2006. Variant of hepatitis B virus with primary resistance to adefovir. N. Engl. J. Med. **354:**1807–1812.
31. **Severini, A., X. Y. Liu, J. S. Wilson, and D. L. Tyrrell.** 1995. Mechanism of inhibition of duck hepatitis B virus polymerase by (-)-beta-L-2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **39:**1430–1435.
32. **Suzuki, F., A. Tsubota, Y. Arase, Y. Suzuki, N. Akuta, T. Hosaka, T. Someya, M. Kobayashi, S. Saitoh, K. Ikeda, M. Kobayashi, M. Matsuda, J. Satoh, K. Takagi, and H. Kumada.** 2003. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology **46:**182–189.
33. **Suzuki, Y., H. Kumada, K. Ikeda, K. Chayama, Y. Arase, S. Saitoh, A. Tsubota, M. Kobayashi, M. Koike, N. Ogawa, and K. Tanikawa.** 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J. Hepatol. **30:**743–748.
34. **Uwai, Y., H. Ida, Y. Tsuji, T. Katsura, and K. Inui.** 2007. Renal transport of adefovir, cidofovir, and tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2). Pharm. Res. **24:**811–815.
35. **Villet, S., C. Pichoud, J. P. Villeneuve, C. Trépo, and F. Zoulim.** 2006. Selection of a multiple drug-resistant hepatitis B virus strain in a liver-transplanted patient. Gastroenterology **131:**1253–1261.

36. **Wright, T. L., and J. Y. Lau.** 1993. Clinical aspects of hepatitis B virus infection. Lancet **342:**1340–1344.
37. **Xiong, X., C. Flores, H. Yang, J. J. Toole, and C. S. Gibbs.** 1998. Mutations in hepatitis B DNA polymerase associated with resistance to lamivudine do not confer resistance to adefovir in vitro. Hepatology **28:**1669–1673.
38. **Yatsuji, H., N. Hiraga, N. Mori, T. Hatakeyama, M. Tsuge, M. Imamura, S. Takahashi, Y. Fujimoto, H. Ochi, H. Abe, T. Maekawa, F. Suzuki, H. Kumada, and K. Chayama.** 2007. Successful treatment of an entecavir-resistant hepatitis B virus variant. J. Med. Virol. **79:**1811–1817.
39. **Yatsuji, H., C. Noguchi, N. Hiraga, N. Mori, M. Tsuge, M. Imamura, S. Takahashi, E. Iwao, Y. Fujimoto, H. Ochi, H. Abe, T. Maekawa, C. Tateno, K. Yoshizato, F. Suzuki, H. Kumada, and K. Chayama.** 2006. Emergence of a novel lamivudine-resistant hepatitis B virus variant with a substitution outside the YMDD motif. Antimicrob. Agents Chemother. **50:**3867–3874.
40. **Yatsuji, H., F. Suzuki, H. Sezaki, N. Akuta, Y. Suzuki, Y. Kawamura, T. Hosaka, M. Kobayashi, S. Saitoh, Y. Arase, K. Ikeda, S. Watahiki, S. Iwasaki, M. Kobayashi, and H. Kumada.** 2008. Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. J. Hepatol. **48:**923–931.

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Original article

Differential effects of interferon and lamivudine on serum HBV RNA inhibition in patients with chronic hepatitis B

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Background: Lamivudine and interferon have been widely used for the treatment of patients with chronic HBV infection. Serum HBV RNA is detected during lamivudine therapy as a consequence of interrupted reverse transcription and because RNA replicative intermediates are unaffected by the drug. In this study, we aimed to determine the detectability of serum HBV RNA during sequential combination therapy of interferon and lamivudine.
Methods: HBV DNA and RNA in serum samples were quantified by reverse transcription of HBV nucleic acid extract and real-time PCR. Samples were analysed every 2 weeks to 3 months from three groups of patients: 10 male patients treated with nucleoside analogue monotherapy for 44–48 weeks (5 with lamivudine and 5 with entecavir), 6 males on sequential interferon and lamivudine combination therapy, and 3 males on lamivudine monotherapy for 20–24 weeks.
Results: HBV RNA was not detectable in any patients before treatment, but became detectable in 15 during antiviral treatment. Among the three groups, pre-treatment HBV DNA (8.1 ±2.4 versus 7.7 ±1.4 versus 5.1 ±0.3 $\log_{10}$ copies/ml; P=0.06), treatment and follow-up durations (45.5 ±2.0 versus 49.7 ±5.6 versus 48.7 ±6.4 weeks; P=0.32) were comparable. HBV RNA was detectable at the end of treatment or follow-up in all patients with monotherapy, but in none of those with sequential combination therapy (100% versus 0%; P<0.001).
Conclusions: Compared with lamivudine therapy with detectable serum HBV RNA in patients with chronic HBV infection, interferon treatment might reduce HBV DNA replication through the inhibition of HBV RNA replicative intermediates, resulting in the loss of serum HBV RNA.

Introduction

Although effective HBV vaccines have been available for more than two decades, HBV infection remains a global health problem. It is estimated that more than 350 million people are chronic carriers of HBV worldwide [1,2]. In the US, 1.2 million individuals have chronic HBV infection [3]. HBV infection causes a wide spectrum of clinical manifestations, ranging from acute or fulminant hepatitis to various forms of chronic liver disease, including inactive carrier state, chronic hepatitis, cirrhosis and even hepatocellular carcinoma [2,4].

HBV is a unique DNA virus that replicates via pre-genomic RNA. There are several key steps in HBV replication. Firstly, in the nucleus of infected hepatocytes, the asymmetric DNA in virions converts to

covalently closed circular DNA (cccDNA); the cccDNA is then transcribed to pre-genomic RNA. Next, the minus strand of viral DNA is synthesized by reverse transcriptase. Finally, there is synthesis of the plus strand to form mature genomic DNA [5]. Both interferon and nucleos(t)ide analogues have been approved for the treatment of chronic hepatitis B (CHB). All of these agents have viral suppression effects, whereas interferon has additional immunomodulatory properties [6]. Lamivudine is the first approved nucleoside analogue for the treatment of CHB; however, it does not affect the integrated HBV DNAs or their transcripts, the RNA replicative intermediates [7]. Thus, lamivudine, as well as other nucleos(t)ide analogues, needs indefinite duration of therapy for continued viral suppression. By contrast, interferon has a finite duration of therapy and a higher rate of hepatitis B surface antigen (HBsAg) seroclearance than nucleos(t)ide analogues [8]. Our study and others showed that serum HBV RNA could be detected during lamivudine therapy, as the consequence of unaffected RNA replicative intermediates as well as interrupted reverse transcription [9,10]. In addition, serum HBV RNA might serve as a predictor of early emergence of viral mutation during lamivudine therapy [10].

Previous clinical trials indicated that simultaneous combination therapy of interferon-α plus lamivudine leads to greater on-treatment viral suppression and higher sustained response rates than lamivudine monotherapy [8]. However, the detectability and patterns of serum HBV RNA in patients receiving sequential combination therapy of interferon and lamivudine compared with those on lamivudine monotherapy remain largely unknown. Thus, we explored the differential effects of interferon and lamivudine on serum HBV RNA inhibition in CHB patients with various treatment regimens.

Methods

Patients

We enrolled 19 CHB patients treated with nucleoside analogue alone or sequential combination therapy of conventional interferon and lamivudine. These patients were divided into three groups on the basis of treatment regimen. Group I consisted of 10 male patients (mean age 44.2 years; range 30–74) treated with nucleoside analogue monotherapy for 44–48 weeks (5 patients with lamivudine and 5 with entecavir). Group II consisted of six male patients (mean age 47.3 years; range 39–56). Five of these patients were treated with lamivudine for 34–52 weeks and then shifted to conventional interferon for 24–36 weeks; there was an overlap of the two drugs for 4–20 weeks. The remaining patient in this group was treated with conventional interferon for 36 weeks and then shifted to lamivudine for 32 weeks; there was an overlap of the two drugs for 12 weeks. Group III consisted of three male patients (mean age 51.3 years; range 41–64) who were treated with lamivudine for 20–24 weeks and then followed for 22–36 weeks. Serum samples from enrolled patients were obtained just before the initiation of therapy and every 2 weeks to 3 months until the end of treatment or follow-up. These samples were stored at -80°C until use. Informed consent was obtained from each patient.

Extraction of HBV nucleic acid and reverse transcription

Extraction of HBV nucleic acid and reverse transcription with subsequent quantification were performed as previously described [10]. Nucleic acid was extracted from 100 μl serum using the SMI TEST EX-R&D kit (Genome Science Laboratories, Tokyo, Japan) and dissolved in 18 μl of ribonuclease-free water. The extract was then divided into two aliquots of equal size, termed solutions I and II. Solution I was mixed with an equivalent amount of water for DNA quantification. Solution II underwent reverse transcription using a random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) with subsequent DNA plus cDNA quantification. The steps in reverse transcription were as follows: 25 pM random primer was added and the sample heated to 65°C for 5 min; the sample was then put on ice for 5 min; 5× reverse transcription buffer (4 μl), 10 mM dNTPs (2 μl), 0.1 M dithiothreitol (2 μl), 8 units of ribonuclease inhibitor and 100 units of M-MLV reverse transcriptase were added; the sample was incubated at 30°C for 10 min and 42°C for 60 min; and inactivation was carried out at 99°C for 5 min.

Quantification of HBV DNA and cDNA by real-time PCR

HBV DNA and cDNA quantification were performed as previously described [10]. A 1 μl aliquot of solution I and solution II were each amplified by real-time PCR with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Amplification was performed in a 25 μl reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM forward primer (5′-TTTGGGGCATGGACATTGAC-3′, nucleotides 1893–1912), 200 nM reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029–2049) and 1 μl of solution I or solution II. The steps in real-time PCR were as follows: incubation at 50°C for 2 min, denaturation at 95°C for 10 min, and PCR cycling comprising 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. The lower detection limit of this assay was 10^3 copies/ml. The HBV RNA quantity is obtained by subtracting the quantification result of solution I from solution II (that is, HBV nucleic acid determined by real-time PCR after reverse transcription reaction minus HBV DNA determined by real-time PCR).

Serological assays

Serum hepatitis B e antigen (HBeAg) and levels of antibodies against HBV e antigen (anti-HBe) were tested by chemiluminescent immunoassay (Architect HBeAg and Architect HBeAb, Abbott Japan, Tokyo, Japan).

Statistical analysis

Baseline characteristics including age, gender, serum alanine aminotransferase (ALT) level, HBeAg and HBV DNA level were compared among study groups. Continuous variables were expressed as mean ± SD and evaluated by Student's *t*-test for comparison between two groups and by Kruskal–Wallis test for comparison among three groups. Categorical variables were expressed as frequencies with proportions and compared using Pearson's χ^2 test; Fisher's exact test was applied when at least one cell of the table has an expected frequency <5. All of the tests were two-tailed and a *P*-value <0.05 was considered statistically significant.

Results

Demographic profiles of patients

Baseline characteristics of CHB patients treated with nucleoside analogues and/or conventional interferon are shown in Table 1. There was no significant difference in terms of age, gender ratio, ALT level, HBeAg status or HBV DNA level among the three groups.

Serum HBV RNA levels after nucleoside analogue therapy

The detectability of serum HBV RNA before, after and at the end of nucleoside analogue therapy or follow-up is shown in Table 2. Serum HBV RNA was undetectable in all patients before the initiation of nucleoside analogue therapy; however, it became detectable in 15 patients (79%) after therapy. Of 14 patients treated with lamivudine, serum HBV RNA was detectable in 10 (71%). By contrast, of five patients treated with entecavir, all (100%) had detectable serum HBV RNA. The peak serum HBV RNA level ranged from 4.2 to 7.0 $\log_{10}$ copies/ml in lamivudine-treated patients and from 7.2 to 9.6 $\log_{10}$ copies/ml in entecavir-treated patients.

Serum HBV RNA levels after interferon treatment in patients with prior lamivudine therapy

In patients with detectable serum HBV RNA after nucleoside analogue monotherapy, HBV RNA persisted until the end of therapy (group I; Table 2). Similarly, serum HBV RNA was persistently detectable after short-term lamivudine therapy (group III; Table 2). By contrast, those with sequential lamivudine and interferon therapy experienced undetectable

Table 1. Baseline characteristics of chronic hepatitis B patients treated with nucleoside analogues and/or conventional interferon

Characteristic	Group I[a]	Group II[b]	Group III[c]	*P*-value
Patients (3TC/ETV), *n*	10 (5/5)	6	3	–
Mean age, years ±SD	44.2 ±13.7	47.3 ±6.5	51.3 ±11.7	0.31[d]
Gender (male/female), *n*	10/0	6/0	3/0	1.0[e]
Mean ALT, U/l ±SD	164.4 ±105.5	180.3 ±121.2	154 ±142	0.88[d]
HBeAg, % (+/-)	70 (7/3)	17 (1/5)	66.7 (2/1)	0.13[e]
Mean HBV DNA, $\log_{10}$ copies/ml ±SD	8.1 ±2.4	7.7 ±1.4	5.1 ±0.3	0.06[d]

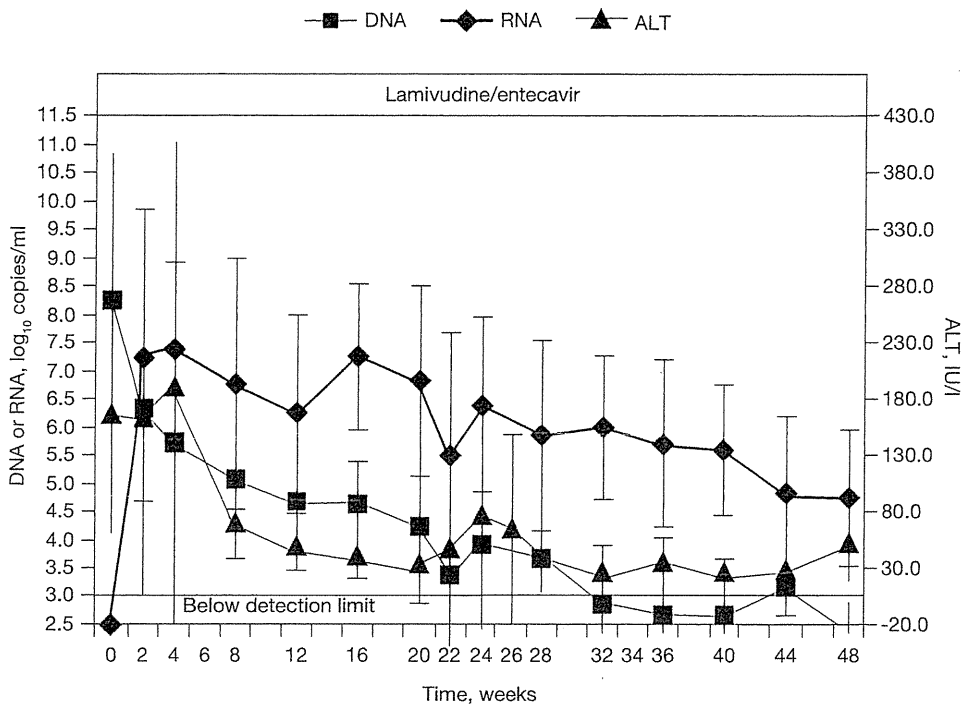
[a]Group I, lamivudine (3TC) or entecavir (ETV) monotherapy for 44–48 weeks. [b]Group II, sequential 3TC for 34–52 weeks and conventional interferon for 24–36 weeks; there was overlap of the two drugs for 4–20 weeks. [c]Group III, 3TC therapy for 20–24 weeks, then follow-up for 22–36 weeks. [d]Kruskal–Wallis test; [e]Fisher's exact test. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

Table 2. Serum HBV RNA in chronic hepatitis B patients treated with nucleoside analogues and/or conventional interferon

	Group I[a]	Group II[b]	Group III[c]	*P*-value
Patients (3TC/ETV), *n*	10 (5/5)	6	3	–
Detectability before treatment, %	0	0	0	1.0[e]
Detectability after treatment, *n* (%)	8/2 (80.0)	5/1 (83.0)	2/1 (66.7)	1.0[e]
Mean peak level, $\log_{10}$ copies/ml ±SD	7.4 ±1.9	6.1 ±0.9	4.6 ±0.1	0.08[d]
Mean total duration (treatment plus follow-up), weeks ±SD	45.5 ±2.0	49.7 ±5.6	48.7 ±6.4	0.32[d]
Detectability at end of treatment plus follow-up, *n* (%)	8/0 (100)	0/5 (0)	2/0 (100)	<0.001[e]

[a]Group I, lamivudine (3TC) or entecavir (ETV) monotherapy for 44–48 weeks. [b]Group II, sequential 3TC for 34–52 weeks and conventional interferon for 24–36 weeks; there was overlap of the two drugs for 4–20 weeks. [c]Group III, 3TC therapy for 20–24 weeks, then follow-up for 22–36 weeks. [d]Kruskal–Wallis test; [e]Fisher's exact test.

Figure 1. Sequential changes of serum HBV RNA, DNA and ALT levels in patients treated with lamivudine or entecavir monotherapy for 44–48 weeks with detectable serum HBV RNA (group I)



Serum HBV RNA was detectable until the end of treatment. ALT, alanine aminotransferase.

serum HBV RNA at the end of treatment (group II; Table 2).

Sequential changes of serum HBV RNA levels in patients with various treatments

The sequential changes of serum HBV RNA in patients with detectable HBV RNA in group I, II and III are shown in Figure 1, Figure 2 and Figure 3, respectively. As early as 2–4 weeks after starting nucleoside analogue therapy, serum HBV RNA could be detected in 13 patients (87%) and reached the peak level in 11 (73%).

Discussion

The specific presence of serum HBV RNA in patients with CHB infection was confirmed in our previous study using ribonuclease digestion [10]. Ribonuclease treatment reduced the amount of HBV DNA detected by real-time PCR after reverse transcription to about 1% of that originally detected [10]. In this study, detectable serum HBV RNA persisted during nucleoside analogue therapy including lamivudine and entecavir (group I), whereas it was inhibited under sequential lamivudine and interferon therapy (group II). The difference between these two groups is significant even in a small number of patients, demonstrating the substantial HBV RNA inhibitory effect of interferon. The decline in serum HBV RNA level was not simply the result of discontinuation of lamivudine, because serum HBV RNA was persistently detectable even after discontinuation of short-term lamivudine therapy in group III patients. The inhibition of serum HBV RNA was found in patients treated with lamivudine and then shifted to conventional interferon and in those on conventional interferon treatment and shifted to lamivudine. In the latter patients, the inhibition of serum HBV RNA might be due to the delayed therapeutic effect of interferon.

Several previous studies have proven that sequential combination therapy of lamivudine and interferon has a better efficacy than lamivudine monotherapy. For example, sequential lamivudine and interferon therapy increased HBeAg seroconversion rate [11] and had a higher response rate in terms of sustained HBeAg seroconversion, ALT normalization, HBV DNA loss and reduced rates of relapse after stopping therapy [12]. In addition, the initial use of lamivudine before interferon