BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 43–48 (1997)
ARTICLE NO. RC977766

Evaluation of Anti-Hepatitis B Virus (HBV) Drugs Using the HBV Transgenic Mouse: Application of the Semiquantitative Polymerase Chain Reaction (PCR) for Serum HBV DNA to Monitor the Drug Efficacy

Kazunori Kajino, Naohiro Kamiya,* Satoshi Yuasa,* Tomoko Takahara, Junko Sakurai, Ken-ichi Yamamura,† and Okio Hino[1]

*Department of Experimental Pathology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshimaku, Tokyo 170 Japan; *Pharmaceuticals Laboratory II, Yokohama Research Center, Mitsubishi Chemical Cooperation, 1000 Kamoshidacho, Midoriku, Yokohama, Kanagawa 227 Japan; and †Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto 862 Japan*

Received October 31, 1997

For evaluation of anti-hepatitis B virus (HBV) drugs, we have employed the HBV transgenic mouse in which virion-like particles can be assayed in the serum. Bis-pivaloyloxymethyl-9-(2-phosphonylmethoxyethyl)-adenine [bis (POM) PMEA] 100 mg/kg/day, 2′,3′-dideoxy-3′-thiacytidine [(+−)-BCH189] 200 mg/kg/day and a placebo were orally administered to mice twice a day for 14 days. Anti-viral effects were monitored by checking the levels of serum HBV DNA by the semiquantitative polymerase chain reaction, HBsAg and HBeAg by enzyme immunoassay, and replicative intermediates in the liver by Southern blotting. As expected, decrease from the $10^{0.5}$ to 10^{3} copies of HBV DNA per μl of sera detected before the treatment to the undetectable level was evident for all five animals treated with bis(POM) PMEA 100 mg/kg/day. However (+−)-BCH189 200 mg/kg/day, which is known to act as the inhibitor of reverse transcriptase for HBV or HIV *in vivo* and *in vitro,* did not suppress HBV DNA levels in the transgenic mouse. Thus, we were able to detect the effects of anti-HBV drugs semi-quantitatively, and confirm differences in drug efficacy.

The incidence of hepatitis B virus (HBV) infection is being reduced in these countries with HBV vaccination programs. However there are nearly 300 million HBV carriers in the world (1) with the high risk of chronic liver disease and hepatocellular carcinoma. There are at present no anti-HBV drugs which work as effectively as for example penicillin against streptococcus infections. One of the obstacles to developing a good anti-HBV drugs is the lack of an appropriate experimental system to evaluate their efficiency. HBV can normally only infect and replicate in human and chimpanzee hepatocytes. In 1986 and 1987, cell culture systems permitting replication and secretion of HBV DNA were established, and since then cell lines such as HB611 or HepG2 2.2.15 have been used for the evaluation of new anti-HBV drugs (2,3). However, in 1989 Araki et al. established a HBV transgenic mouse which features replication of HBV in the liver and release of virion-like particles into the blood (4). Nagahata et al. have used this mouse to evaluate anti-HBV drugs such as oxetanocin G or alpha interferon (5). In the present study we introduced semiquantitative PCR to monitor changes in the amounts of HBV DNA in sera linked to drug exposure. Bis(POM) PMEA [bis-pivaloyloxymethyl-9-(2-phosphonylmethoxyethyl)-adenine] is an oral prodrug of PMEA which has an inhibitory effect on HBV *in vitro* (6,7) and human immunodeficiency virus (HIV) replication *in vivo* (8). (+−)-BCH189 (2′,3′-dideoxy-3′-thiacytidine) is a racemic mixture of the cis-isomers, and its (−)-enantiomer is known to be lamivudine or 3TC which has an antiviral activity against HBV as well as HIV in clinical trials (9,10). Before the treatment the transgenic mice had $10^{0.5}$ to 10^{3} copies of HBV DNA per μl of sera. After administration of bis(POM) PMEA 100mg/kg/day, this decreased to the undetectable level in all five animals. Using the HBV transgenic mouse and semiquantitative PCR for HBV DNA, we could thus assay the efficacy of an anti-HBV drug accurately.

MATERIALS AND METHODS

Chemicals. The following chemicals were prepared at Mitsubishi Chemical Corporation, Yokohama Research Center, Japan. (+−)-

[1] Corresponding author. Fax: +81-3-5394-3815. E-mail: ohino@hgc.ims.u-tokyo.ac.jp.

0006-291X/97 $25.00

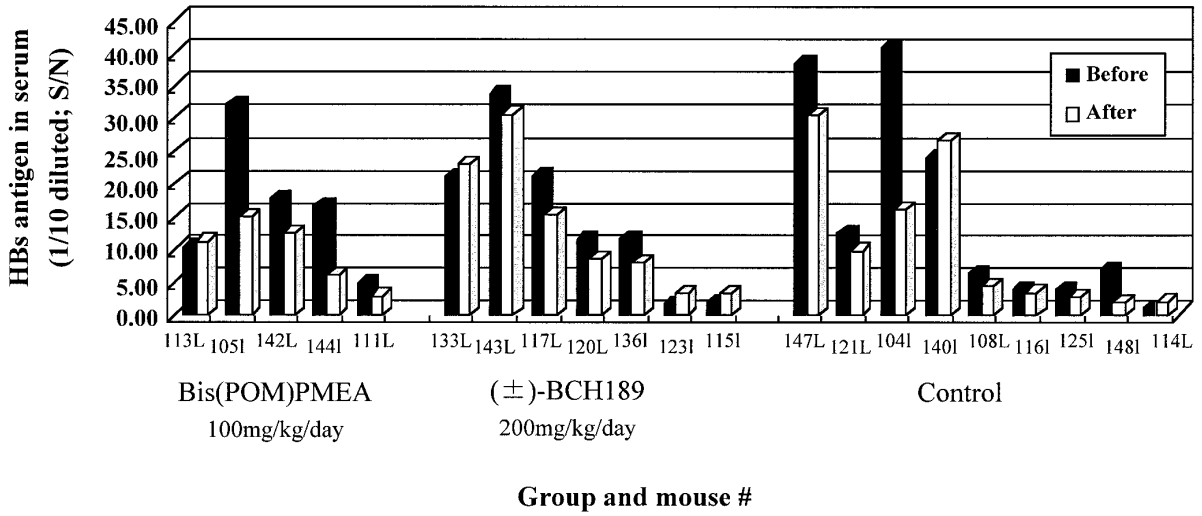

FIG. 1. HBs antigen titers in serum before and after the treatment. Serum samples were collected at days 0 and 15, and diluted 10 times with phosphate buffered saline before the assay. L or l in the mouse number indicate male or female respectively.

BCH189 was synthesized as described by Liotta et al. (11), and bis (POM) PMEA as described by Starett et al (12).

Animals. The HBV transgenic mouse line 1.2 HB-BS10 established by Araki et al. (4) has 1.2 copies of HBV DNA (subtype adr) integrated in the genome, and produces Dane-like particles which enter the blood. We employed the mice of this lineage, having integrated HBV DNA in the homozygotic state. The animals in groups A, B, C were treated with bis(POM) PMEA 100 mg/kg/day, (+−)-BCH189 200 mg/kg/day, and placebo, respectively, and were maintained with free access to water and food. Initially, 24 mice (9 to 14 weeks old) were assigned into three groups, but three mice in group A were later proved to contain undetectable level of serum HBV DNA and we removed these mice from our study. As a result, the groups A, B and C consisted of five, seven and nine mice, respectively. All the drugs were suspended in tragacance gum and orally administrated by stomach tube twice a day for 14 days (from days 1 to 14). On day 15, the animals were sacrificed and the livers were excised and stored at −70°C. Blood was collected from the tail three weeks before the initiation of treatment, on day 0, and on day 15. The isolated sera were kept at −70°C.

Assays for serum HBsAg and HBeAg. Serum was diluted 10 times with phosphate buffered saline and used for the enzyme immunoassay (EIA) of HBsAg and HBeAg, with the AXSYM and IMX systems (Dinabot), respectively. EIA results were expressed in terms of S/N ratio, and samples showing values higher than two were interpreted as positive, in line with the manufacturer's instructions. Sera obtained at days 0 and 15 were thawed and used simultaneously for the assay.

Semiquantitative polymerase chain reaction (PCR) of HBV DNA in serum. We followed the method described by Nagahata et al. (5) to perform the PCR of HBV DNA in serum, with modifications. Twenty five microliters of undiluted serum was digested in 100μg/ml proteinase K, 0.1% sodium dodecyl sulfate (SDS), 50mM TrisHCl

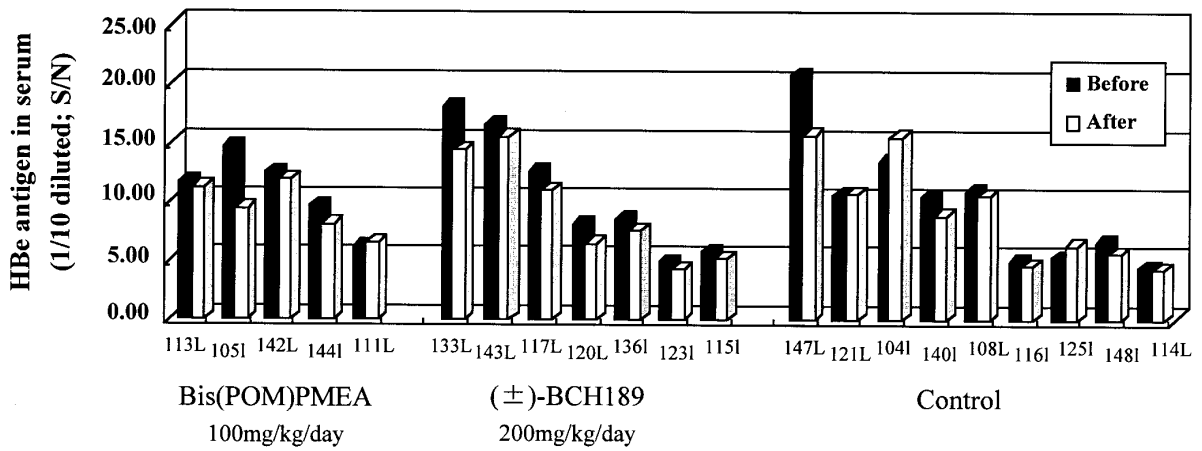

FIG. 2. HBe antigen titers in serum before and after the treatment. Serum samples were collected and diluted as described in the legend of Fig. 1.

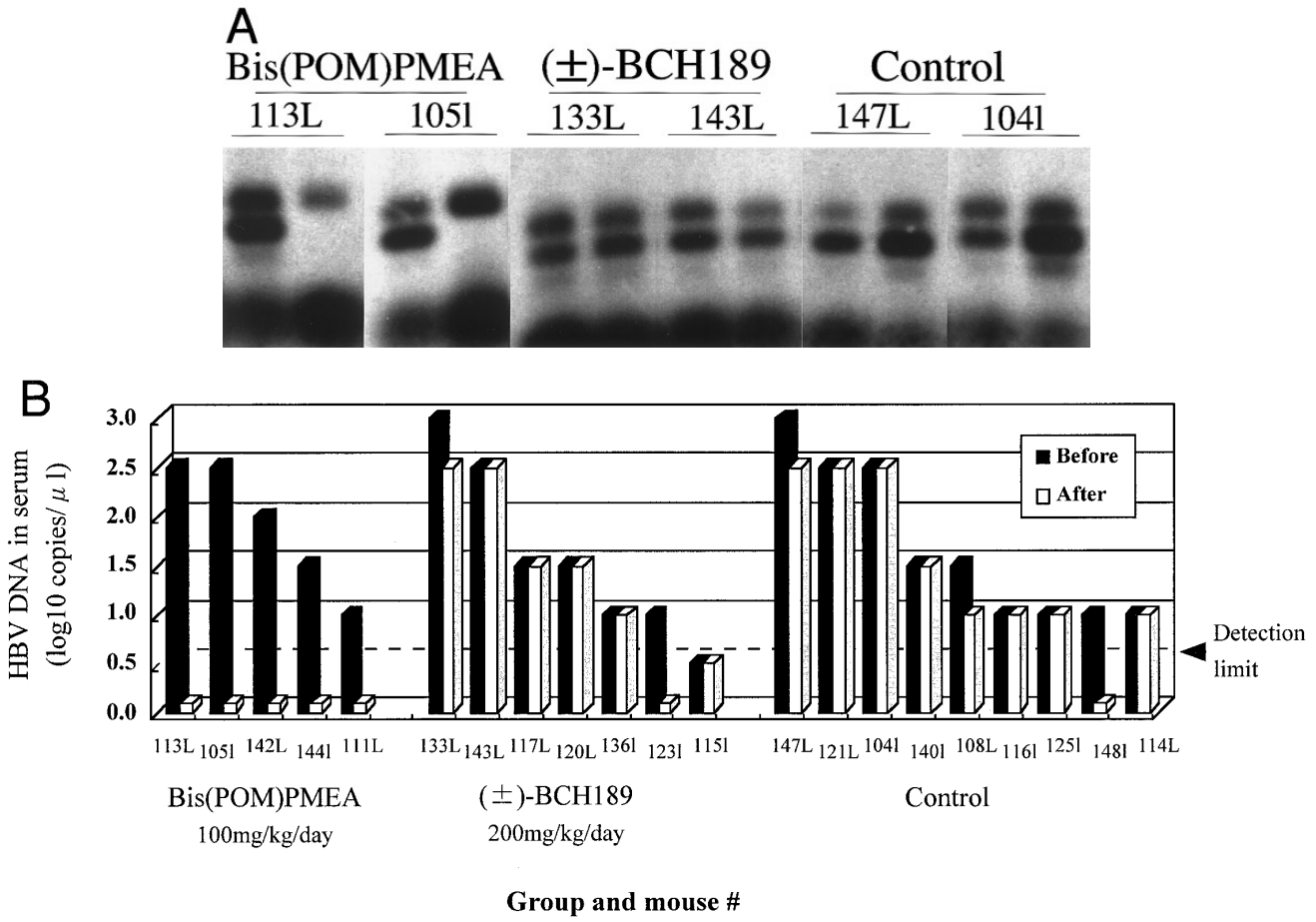

FIG. 3. HBV DNA in serum detected by a competitive PCR. (A) Representative data of two mice in each group. Upper or lower bands are derived from the competitor DNA or serum HBV DNA respectively. Concentration of the competitor DNA in this figure was $10^{1.5}$ copies/μl. Left and right lanes in each panel show the PCR products derived from the serum obtained at days 0 and 15 respectively. (B) Summarized data of serum HBV DNA titers in each group before and after the treatment. The detection limit was $10^{0.5}$ copies/μl.

(pH8.0), 100mM NaCl and 10mM EDTA, in a volume of 250 μl at 37°C overnight. A 40 μl aliquot of the digested sample was mixed with the known concentration (10^0 to 10^3 copies/μl) of competitor plasmid carrying the HBV DNA (subtype adr) XbaI-XhoI fragment with a deleted *Bam*HI site, and processed to phenol/chloroform extraction and ethanol precipitation with glycogen as a carrier. The precipitated DNA was washed once with 70% ethanol, dried in room air, and digested with *Bam*HI in a 10 μl of digestion buffer to prevent amplifying contaminating lymphocyte-derived DNA. Five microliter aliquots of *Bam*HI digested samples were used for PCR to amplify the HBV gap region. The primers applied were 5′-digoxigenin (DIG)-adr-1 (5′-DIG-GGG GTA CTT TAC CGC AAG) and adr-2 (5′-CCG CGT AAA GAG AGG TGC GC). Amplification conditions were 30 sec of denaturation at 94°C, 1 min 15 sec of annealing at 58°C, and 1 min 30 sec of extension at 72°C for 35 cycles; this was followed by 10 min at 72 °C and 5 min at 50 °C. PCR products were processed to phenol/chloroform extraction and ethanol precipitation. The precipitated DNA was washed once with 70% ethanol, dried in room air, and digested with *Bam*HI in 20 μl of buffer. The *Bam*HI digested post-PCR samples were electrophoresed on 1.25% agarose gels and transferred to nylon membranes (Amersham Hybond-N+) in 0.5 N NaOH. The membranes were dried, UV-crosslinked, and blocked with 1 % blocking reagent (Boehringer Mannheim). DIG-labeled products were detected with anti-DIG-AP Fab fragments (Boehringer Mannheim) and CDP-star (TROPIX) following the manufactures' recommendations. One or two bands could be seen per lane in exposed films; the upper was derived from the competitor plasmid and the lower from the HBV DNA in the serum. To estimate the HBV DNA titers, we did PCR with different concentrations of competitors at least three times for each sample to confirm whether its concentration is lower or higher than that of a competitor plasmid.

Southern blotting of HBV DNA in the liver. Fifty microgram samples of genomic DNA isolated from livers by the SDS/proteinase K method (13), were digested with *Eco*RI, electrophoresed on 1 % agarose gels and transferred to Nylon membranes (PALL, Biodyne B) in 0.4 N NaOH. Immobilized DNA was hybridized with ^{32}P-labeled HBV DNA (subtype adr) in 0.2 M NaHPO4 (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, 7 % SDS at 65 °C overnight, and washed in 1 × SSC (150 mM NaCl, 15 mM sodium citrate), 0.1 % SDS at 65 °C for 30 min. Kodak XAR-5 film was used for autoradiography, and the intensity of each signal was measured by densitometric scanning. The signal intensity of the integrated HBV DNA (shown by an arrowhead in Fig. 4A) was much stronger than that of the replicative intermediates (a bar in Fig. 4A). Therefore, to obtain a linearity between the amount of HBV DNA and the signal density, we used the three days' or seven days' exposure films to measure the signal intensity of the integrated HBV DNA or that of the replicative intermediates, respectively. The ratio of these two signal intensities was calculated in each animal, and used as an indicator of the intensity of HBV replication in hepatocytes. The average ratio in each group is shown in Fig. 4B.

RESULTS

All of the animals survived throughout the period of drug treatment, without showing any abnormal signs such as weakness, emaciation, or hair loss.

Changes in Serum HBV Markers

Before the treatment, serum HBV DNA in mice varied from $10^{0.5}$ copies/μl to 10^3 copies/μl, and at least two mice in each group exhibited more than 10^2 copies/μl (Fig. 3B). Fig. 1 summarized data for serum HBsAg before and after the treatment. In each group some animals showed a slight decrease, but there were no significant differences among the groups. As for HBeAg, there was essentially no treatment-associated change in any of the animals before and after the treatment (Fig. 2). In contrast, there was a significant decrease in the amount of serum HBV DNA in mice administered bis(POM) PMEA, HBV DNA no longer being detectable in all five mice (Fig. 3B). Five of seven (+−)-BCH189-treated mice and six of nine control mice did not show any change in serum HBV DNA.

Effects of Treatments on HBV DNA Replication in the Liver

As shown in Figs. 4A & 4B, the number of replicative intermediates of HBV DNA in the liver was lower in the bis(POM) PMEA-treated group than in the controls. In contrast, the (+−)-BCH189-treated group did not demonstrate any significant difference. Fig. 5 showed that the amount of HBV replicative intermediates in the liver correlated well with the serum HBV DNA titers.

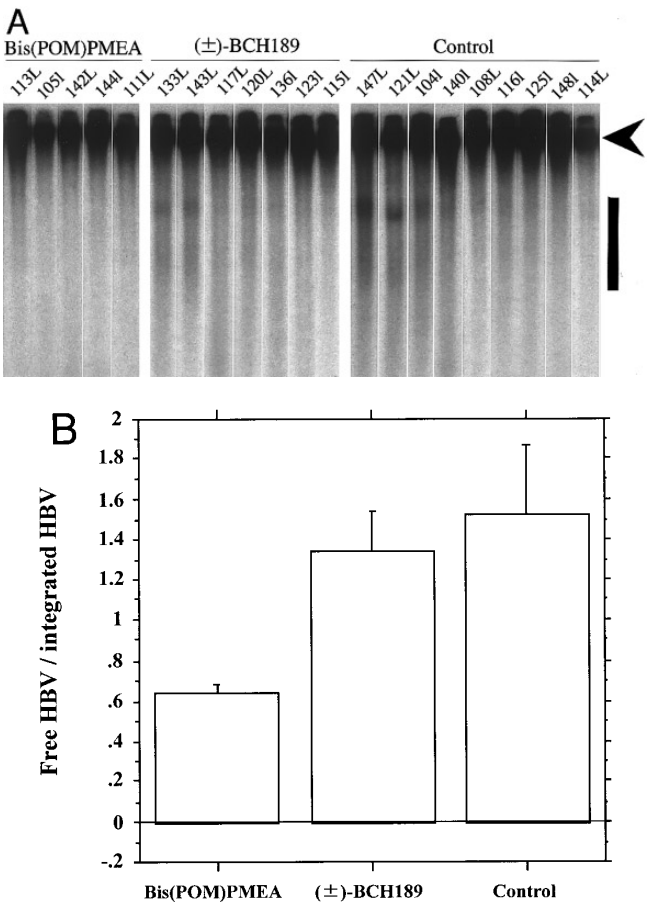

FIG. 4. HBV replicative intermediates in the livers of transgenic mice after the treatment. (A) HBV DNA in the liver detected by Southern blotting. An arrowhead or bar indicate the genomic-integrated HBV DNA or replicative intermediates of HBV respectively. (B) The average ratio of the free HBV DNA (replicative intermediates) to integrated HBV DNA in each group after the treatment. (See Materials & Methods as for the calculation of the ratio) The boxes or bars indicate the average ratio or standard errors respectively.

DISCUSSION

There have been several reports concerned with evaluation of the efficacy of anti-HBV drugs using tissue culture systems (2,3,6,7). However anti-HBV effects observed *in vitro* system do not always predict *in vivo* influence. There are many factors *in vivo* which affect the drug efficacy, such as its tissue distribution, metabolism and excretion, and toxicity. Woodchucks or ducks infected with woodchuck hepatitis virus (WHV) or duck hepatitis B virus (DHBV) have been introduced for assays of anti-WHV or anti-DHBV effects of test drugs, but animal experiments with natural HBV infection are very difficult. The HBV transgenic mouse, lineage 1.2 HB-BS10, allows HBV DNA replication, although there are several differences between the natural infection model and the mouse model. For instance, in the transgenic mouse, hepatitis usually does not occur, and a virion released from the liver into the serum cannot re-infect mouse hepatocytes. However as far as a tool to evaluate anti-HBV drugs is concerned, it is a very practicable model with a homogeneous genetic background.

The observed amounts of HBV DNA released into the serum in untreated transgenic mice at $10^{0.5}$ to 10^3 copies /μl, is about one-hundredth of that in human chronic HBV carriers which typically contain 10-500 pg/ml, i.e. $3\times10^3-1.5\times10^5$ copies/μl of HBV DNA (9,14). Maximum replication in the mouse thus appeared to be almost the same as the lowest replication level in man. The low quantity of serum HBV DNA is compatible with the relatively small amount of replicative intermediates in the liver. Although the level of HBV DNA in mouse serum was low compared with that in humans, it was high enough to be detected by our semiquantitative PCR. Using this approach, we could demonstrate that bis(POM) PMEA 100mg/kg/day, which is known to have anti-retroviral activity *in vivo* (15), almost completely abolished the serum HBV

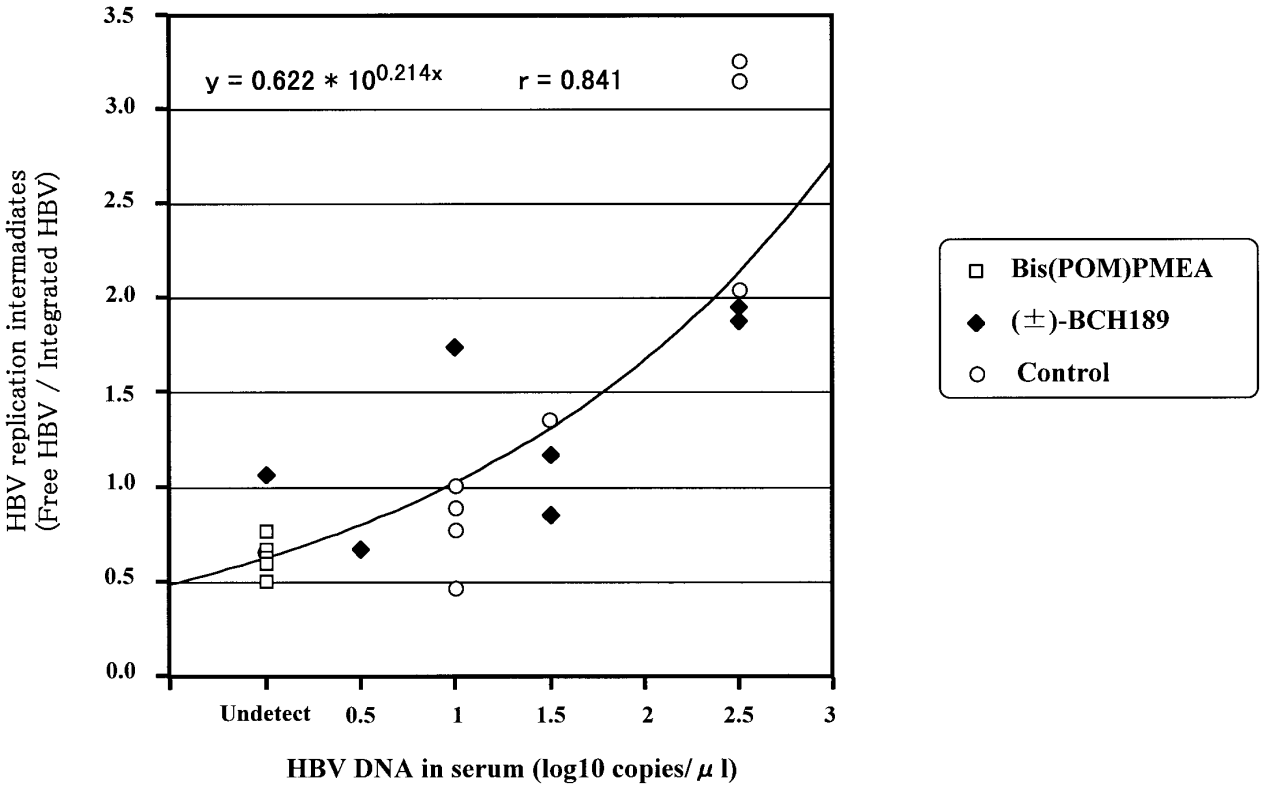

FIG. 5. Correlation of serum HBV DNA titers and HBV replicative intermediates in the liver.

DNA without any major complications. Furthermore, the correlation of HBV DNA titers between in serum and in the liver suggested that a disappearance of HBV DNA in serum reflected a decrease of HBV replicating intermediates in the liver.

Unexpectedly (+−)-BCH189 did not show any anti-HBV effects in the transgenic mice despite evidence of a capacity to inhibit replication of HBV *in vivo* and *in vitro* (3,9,14). Assuming that (+−)-BCH189 200mg/kg/day is equivalent to lamivudine 100 mg/kg/day, the dosage of lamivudine used in our study is more than 10 times of dosage used in human trials in which 25-300 mg/person/day suppressed the HBV replication within two weeks (9,14). During the lamivudine therapy of HBV, a mutant virus resistant to lamivudine has been reported to appear (16,17), with an amino acid replacement in its YMDD motif in the reverse transcriptase domain, i.e., methionine in the YMDD motif is replaced with valine or isoleucine. However we did not detect any similar mutation in HBV DNA of the transgenic mouse by the PCR and direct sequencing of the YMDD region (data not shown). Lamivudine becomes active after conversion to the 5′-triphosphate derivative by a cellular enzyme. It is possible that the activity or quantity of the enzyme is low and (+−)-BCH189 is therefore poorly phosphorylated in the mouse liver.

Although the HBV DNA in serum disappeared in bis(POM) PMEA-treated mice, neither the change of HBsAg nor that of HBeAg was significant. These results revealed that the serum HBsAg or HBeAg titers do not always reflect the amount of serum HBV DNA, particularly in the transgenic mouse in which mRNAs of HBV-associated antigens are transcribed not only from the episomal viral DNA but also from the integrated HBV DNA.

The present study proved that this transgenic mouse is a good model in which change of serum HBV DNA can be accurately assayed using semiquantitative PCR. However some anti-HBV drugs such as (+−)-BCH189 demonstrated clearly different effectiveness in humans in mice probably because of species-specific variation in drug metabolism.

ACKNOWLEDGMENTS

We thank Dr. Masaru Ubasawa for synthesis of bis(POM) PMEA and (+−)-BCH189, and Ms. Rumi Kobayashi, Yoshiko Yamashita, Michiko Yokoyama, Keiko Nagano and Midori Ueno for their technical assistance.

REFERENCES

1. Ayoola, E. A., Balayan, M. S., Deinhardt, F., Gust, I., Kureshi, A. W., Maynard, J. E., Nayak, N. C., Brodley, D. W., Ferguson, M., Melnick, J., Purcell, R. H., and Zuckerman, A. J. (1988) *Bull. World Health Org.* **66,** 443–455.

2. Ueda, K., Tsurimoto, T., Nagahata, T., Chisaka, O., and Matsubara, K. (1989) *Virology* **169,** 213–216.
3. Doong, S.-L., Tsai, C.-H., Schinazi, F., Liotta D. C., and Cheng, Y.-C. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9495–8499.
4. Araki, K., Miyazaki, J., Hino, O., Tomita, N., Chisaka, O., Matsubara, K., and Yamamura, K. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 207–211.
5. Nagahata, T., Araki, K., Yamamura, K., and Matsubara, K. (1992) *Antimicrobial Agents and Chemotherapy* **36,** 2042–2045.
6. Yokota, T., Konno, K., Shigeta, S., Holy, A., Balzarini, J., and De Clercq E. (1994) *Antiviral Chemistry & Chemotherapy* **5,** 57–63.
7. Heijtink, R. A., Kruining, J., De Wilde, G. A., Balzarini, J., De Clercq, E., and Schalm, S. W. (1994) *Antimicrobial Agents and Chemotherapy* **38,** 2180–2182.
8. Balzarini, J., Naesens, L., Herdewijn, P., Rosenberg, I., Holy, A., Pauwels, R., Baba, M., Johns, D. G., and De Clerq, E. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 332–336.
9. Dienstag, J. L., Perrillo, R. P., Schiff, E. R., Bartholomew, M., Vicary, C., and Rubin , M. (1995) *New England Journal of Medicine* **333,** 1657–1661.
10. Ingrand, D., Weber, J., Boucher, C. A., Loveday, C., Robert, C., Hill, A., and Cammack, N. (1995) *AIDS* **9,** 1323–1329.
11. Liotta, D. C., and Choi. W. B. (February 1990) U.S. Patent 900201.
12. Starett, J. E. Jr., Mansuri, M. M., Martin, J. C., Tortolani, D. R., and Bronson, J. J. (September 1990) U.S. Patent 583906.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. pp. 9.14–9.19, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Lai, C.-L., Ching, C.-K., Tung, A. K.-M., Li, E., Young, J., Hill, A., Wong, B. C.-Y., Dent, J., and Wu, P-C. (1997) *Hepatology* **25,** 241–244.
15. Naesens, L., Balzarini, J., Bischofberger, N., and De Clercq, E.(1996) *Antimicrobial Agents and Chemotherapy* **40,** 22–28.
16. Ling, R., Mutimer, D., Ahmed, M., Boxall, E. H., Elias, E., Dusheiko, G. M., and Harrison, T. J. (1996) *Hepatology* **24,** 711–713.
17. Tipples, G. A., Ma, M. M., Fischer, K. P., Bain, V. G., Kneteman, N. M., and Tyrrell, D. L. (1996) *Hepatology* **24,** 714–717.