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Oral Famciclovir Against Duck Hepatitis B Virus Replication in Hepatic and Nonhepatic Tissues of Ducklings Infected in Ovo

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Detection of hepadnaviral DNA in extrahepatic tissues of human and animal models of hepatitis B virus (HBV) has raised the question of whether virus replication in organs other than the liver could be targeted for the treatment of chronic hepatitis B. Since duck hepatitis B virus (DHBV) replication is dynamic in the liver, kidney, pancreas, and spleen of newly hatched ducklings infected in ovo, we used the duck model and the new antiherpesvirus agent, famciclovir (FCV), to determine whether antiviral effect of nucleoside analogues on DHBV replication is pluripotential. Day-old ducklings hatched from eggs laid by a DHBV-carrier duck were bled and administered FCV (25 mg/kg/bd) orally for periods of 1, 2, 3, 6, 9, and 12 days. Seventeen (17) hours after the last dose of each regimen the duckling(s) was bled and postmortem samples of liver, kidney, pancreas, and spleen were snap-frozen and stored at −70°C. Analysis of plasma samples of ducklings treated for 2 days and longer by dot-blot hybridisation showed that levels of DHBV DNA were reduced significantly compared to levels in samples collected before treatment begun. Southern blot hybridisation of tissue DNA corroborated these results and showed that DHBV DNA replicative intermediates in all the tissues examined were reduced to levels that reflected the amount of virus released into the blood of each treated duckling. It is concluded from these results that if antiviral agents could be transformed to active metabolites in any infected tissues including the liver, replication of hepadnaviruses would be inhibited. We also note that the ability of young ducklings to metabolise FCV to the parent compound, penciclovir, suggests that hatchlings could be used for screening antiviral compounds under development.

KEY WORDS: liver and extrahepatic organs, Pekin ducklings, chronic hepatitis B, famciclovir

INTRODUCTION

Persistence of duck hepatitis B virus (DHBV) in the wild is maintained through vertical transmission from duck to offspring via infection in ovo. This mode of transmission leads to a widespread infection of the developing embryo. Ducklings predictably hatch from infected eggs with active virus replication in the liver and nonhepatic tissues [Halpern et al., 1983; Tagawa et al., 1985; Jilbert et al., 1987; Hosoda et al., 1990]. This pantropism results in high levels of viraemia in newly hatched ducklings, most of which grow into chronically infected ducks [Tsiquaye et al., 1985]. DHBV-carrier adult ducks have been considered an ideal model for studies of efficacy of antiviral drugs [Tsiquaye et al., 1986]. However, nonreplicative viral DNA in extrahepatic tissues of adult ducks (our unpublished observation) would neither contribute to viraemia nor be susceptible to inhibitory effects of chemotherapeutic agents. Hence chronically infected adult ducks may be unsuitable for studies on action of antiviral drugs at multiple infected sites. This study was therefore conducted to determine whether inhibition of DHBV replication by antiviral drugs occurs in the liver as well as extrahepatic tissues of newly hatched infected ducklings. We used the guanosine analogue famciclovir (FCV), an antiherpetic drug [Boyd et al., 1988], which inhibits DHBV replication in DHBV-carrier adult ducks [Tsiquaye et al., manuscript in preparation].

MATERIALS AND METHODS

Drug Administration to Ducklings

FCV was kindly provided by SmithKline Beecham. An aqueous solution of the drug (6.25 mg/ml) was aliquoted, stored at −20°C, and thawed just before use.

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Treated ducklings were orally administered two daily doses (25 mg/kg/dose) at 7 hours apart for 1, 2, 3, 6, 9, and 12 days. At about 17 hours after termination of therapy, the ducklings were exsanguinated and plasma samples stored at −20°C. Liver, kidney, pancreas, and spleen samples were snap-frozen in liquid nitrogen and stored at −70°C until required for analysis. A total of eleven ducklings, hatched in our laboratories from eggs laid by the dam of a mating pair of in-bred DHBV-positive ducks, were kept overnight and bled for pre-treatment plasma samples. Eight (8) ducklings were used. Two pairs of ducklings were treated for 9 and 12 days, respectively, and one duckling each for 1, 2, 3, and 6 days. Plasma and tissue samples were obtained from 1-, 4-, and 14-day-old ducklings which served as untreated controls.

Detection of DHBV DNA in Plasma and Tissues

Plasma samples (50 μl) were diluted fourfold in phosphate buffered saline (PBS), pH 7.3, spotted onto Hybond N+ membrane (Amersham International, England) in dot-blot manifold (Bio-Rad Laboratories, Richmond, California), and denatured with 150 μl of solution containing 0.67 M NaOH and 0.67 M NaCl. Each spotted sample was neutralised with 300 μl of 0.5 M Tris HCl, pH 7.4, containing 3 M NaCl. DNA on the membrane was fixed for 2 minutes with 0.4 M NaOH. The membrane was rinsed briefly in 5 × SSC and air-dried (Amersham's protocol). Total DNA was extracted according to the method described previously [Tagawa et al., 1985] from samples of kidney, liver, pancreas, and spleen obtained from treated and control ducklings. DNA samples were electrophoresed in 1.2% agarose gels and Southern blotted onto Hybond N+ membrane [O'Connell et al., 1983]. The membrane was fixed as described above, rinsed in 1 M ammonium acetate, and air-dried. Prehybridisation and hybridisation steps at 42°C and the series of final washings were performed according to Amersham's protocol. The hybridisation probe was purified cloned DHBV DNA (the plasmid was a gift from Prof. Hans Will) labelled to a high specific activity (3–6 × 10^8 cpm/μg) with [α-^{32}P]-dCTP by random priming [Feinberg and Vogelstein, 1983]. After hybridisation the membranes were air-dried and exposed for periods of up to 14 days at −70°C to Fuji RX 100 X-ray film in a cassette with two Hi-speed X intensifying screens for autoradiography.

RESULTS

Dot hybridisation analysis of serial plasma samples from a group of untreated ducklings showed that levels of viraemia fluctuate (data not shown). Therefore the apparent reduction of viraemia (Fig. 1A, lane 2) in the duckling that received 1-day treatment with FCV (25 mg/kg/bd) was considered to be within the range observed in untreated animals. Furthermore, since hybridisable DHBV DNA (Fig. 1B) in the liver of the duckling was high, it was interpreted that a day's treatment was insufficient to produce an effect on plasma DHBV. However, significant reduction of the level of

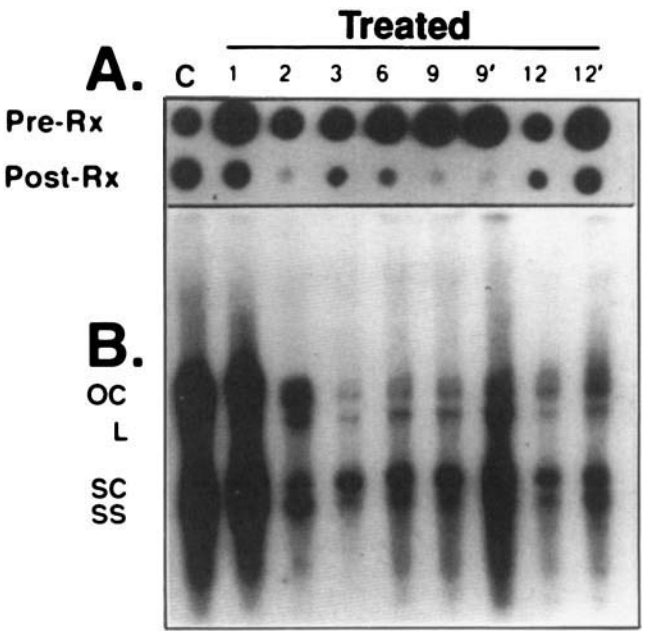

Fig. 1. DHBV DNA hybridisation analysis of 50 μl of plasma (**A**) and liver (**B**) samples of FCV-treated ducklings and untreated, 14-day-old control duckling, C. Southern transfer of total liver DNA (30 μg per lane) electrophoresed in 1.2% agarose gel and dot-blotted plasma samples were hybridised with α-^{32}P-labelled DHBV DNA probe. The duration of treatment (days) of each duckling is indicated above each lane and the ducklings were 1 day old when treatment started. Each lane in this figure and the corresponding lanes in Figures 2–4 contained tissue DNA from the same duckling. The primed numbers 9′ and 12′ are the second of duplicate ducklings treated for 9 and 12 days, respectively. The migration positions of DHBV forms are indicated: L (linear), OC (open circular), SC (supercoiled), and SS (single-stranded).

viraemia was observed in the duckling treated for 2 days. Compared to pretreatment plasma samples, DHBV DNA levels in plasma of ducklings treated for 3 days and longer were markedly reduced from an estimated range of (>128–32 pg/50 μl) to (8–0.5 pg/50 μl of plasma). Southern blot analysis of liver samples of ducklings treated for 2, 3, 6, 9, and 12 days showed a decrease of all replicative forms of DHBV DNA except supercoiled DNA (Fig. 1B).

The results of analysis of total DNA extracted from pancreas, kidney, and spleen from the ducklings are presented in Figures 2, 3, and 4, respectively. Each figure shows a comparison of hybridisable viral specific DNA in tissues of treated and control ducklings. Figure 2 shows a decline in single-stranded DNA present in 30 μg of total DNA extracted from pancreas of treated ducklings. The reduction occurred promptly and was detected after a day's treatment. The single-stranded replicative intermediate was totally inhibited in the pancreas of one of each pair of ducklings treated for 9 and 12 days. But a relative decline in single-stranded DNA forms occurred in the kidney after 6 days of treatment (Fig. 3). The level of reduction of viral DNA was qualitatively similar for ducklings treated for 6 and 9 days. But the two ducklings treated for 12 days showed the highest inhibition of synthesis of replicative inter-

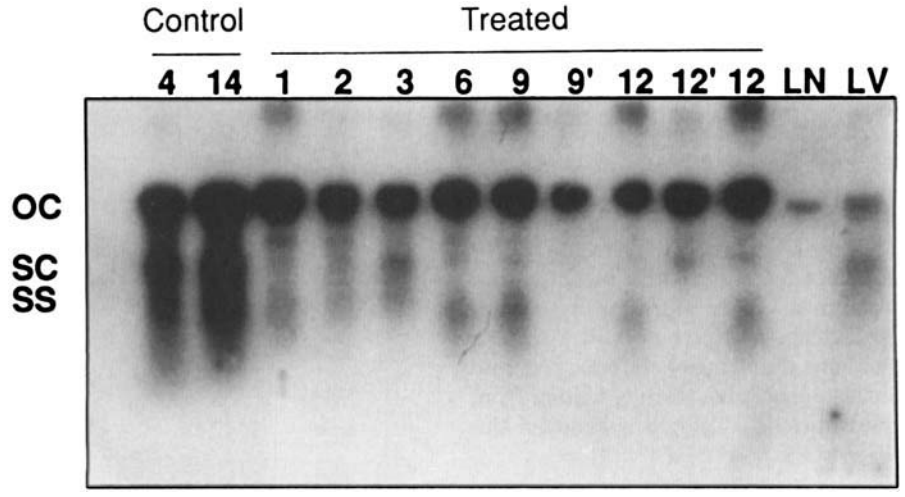

Fig. 2. Southern blot analysis of DHBV DNA in the pancreas of FCV-treated and untreated ducklings. Total pancreas DNA (30 μg per lane) were electrophoresed in 1.2% agarose gel. Untreated control ducklings were 4 and 14 days old when tissue samples were obtained. Lanes LN and LV contained 1 pg cloned linear DHBV DNA and 50 ng total DNA from DHBV-infected liver, respectively; sheared salmon sperm DNA (20 μg) was added to these samples to effect efficient Southern transfer.

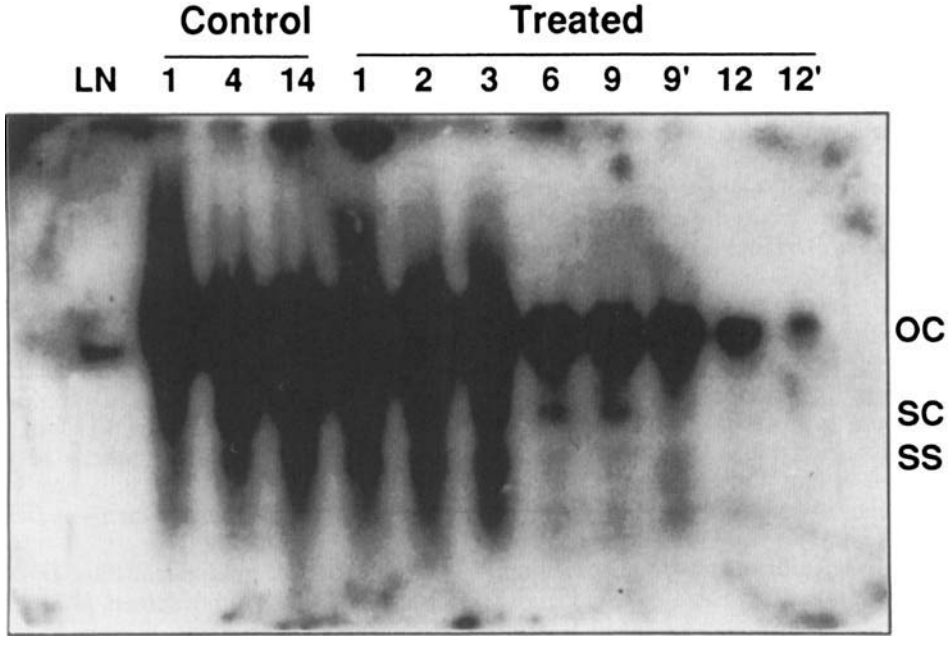

Fig. 3. Southern blot hybridisation of kidney samples (40 μg of total DNA per lane). Lane LN contained 1 pg linear cloned DHBV DNA. Control samples were obtained from untreated 1-, 4-, and 14-day-old ducklings.

mediates. The absence or apparent reduction of supercoiled DNA in the kidney of some of the animals is considered to be artifact of a nick to the open circular form. The pattern of inhibition of virus replication in the samples of spleen (Fig. 4) was similar to that observed in the pancreas of treated ducklings. Reduction in the level of single-stranded DNA synthesis was evident after a day's treatment but was much more significant in ducklings treated for 2 days and longer except that no antiviral effect was apparent in the spleen of the duckling treated for 3 days.

DISCUSSION

FCV, a diacetyl 6-deoxy derivative of penciclovir (PCV), is an oral prodrug which is quickly absorbed and sequentially deacetylated in the intestinal wall and

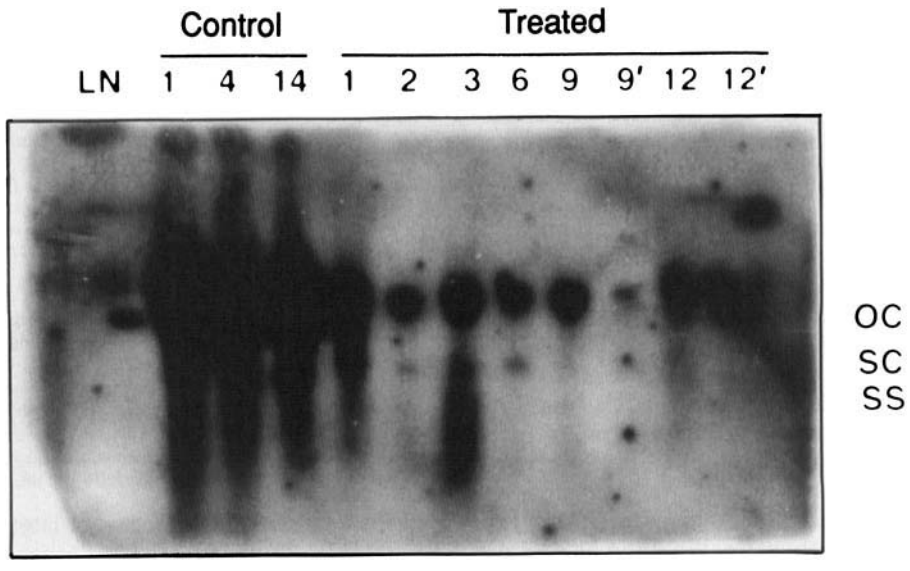

Fig. 4. Southern blot analysis of DHBV DNA in spleen (35 μg total DNA per lane) of FCV-treated ducklings and 1-, 4-, and 14-day-old untreated ducklings. Lane LN contained 1 pg of linear cloned DHBV DNA.

liver to yield 6-deoxypenciclovir. In human liver homogenates the latter is oxidised by xanthine oxidase to PCV, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine [Vere Hodge et al., 1989]. Xanthine oxidase is present in human kidney, liver, lung, and spleen [Diem and Lentner, 1975]. Hence oxidation of the 6-deoxy metabolite in these organs would produce local concentrations of the parent compound PCV which when transformed to triphosphate would exhibit antiviral effect. Data presented in this study show that oral FCV inhibits DHBV replication in liver, kidney, pancreas, and spleen of ducklings infected in ovo. A marked reduction of the level of viraemia was observed after 2-day administration of 25 mg/kg/bd of FCV. The reduction is significant in the content of the problems that have been identified in the treatment of chronic hepadnavirus infections [Mason and Taylor, 1989]. One specific concern relates to those cases where active virus replication in extrahepatic tissues might complicate interpretation of antiviral effect. We suggest that if hepadnavirus replication was inhibited exclusively in the liver without a simultaneous antiviral activity against active replication in nonhepatic tissues, levels of viraemia during therapy would remain essentially unchanged from pretreatment levels. Consequently, no antiviral effect would be observed in plasma/serum. On the other hand, a dosage regimen that established a balanced distribution of a therapeutic agent to multiple sites of virus replication would produce local antiviral activities after activation of the drug. The demonstration of anti-DHBV activity in this study shows that the young ducklings efficiently metabolised FCV to the parent compound, PCV, which was activated to exert an antiviral effect not only in the liver but also in the nonhepatic tissues examined. But it is not known whether host or viral protein(s) are involved in the transformation of nucleoside analogues to produce an antihepadnaviral effect.

Souther blot analysis of total liver DNA from treated ducklings showed that synthesis of single-stranded DHBV DNA was not completely inhibited in the liver. This may be due to the higher level of DHBV replication in hepatocytes than in other organs [Tagawa et al., 1985]. No dose-response study of FCV was conducted in infected ducklings prior to this study but we administered a daily dose of 25 mg/kg/bd which is therapeutic in DHBV-carrier adult ducks [Tsiquaye et al., manuscript in preparation]. It is possible that a larger dose may be required for ducklings in which a more dynamic replication occurs than in adult ducks [Tsiquaye et al., 1985]. Nevertheless, a relative reduction of single-stranded DNA synthesis occurred in all organs analysed. There is obviously an inherent physiological problem associated with collecting multiple blood samples from day-old ducklings for pharmacokinetic studies. However, our observation emphasises the need for determination of dosage regimen that would be effective in ducklings and adult ducks. The observation that supercoiled DNA in all tissues is refractory to antiviral action of metabolised FCV suggests that the data presented here are consistent with the chain-termination mechanism of nucleoside analogues. Therefore the absence of supercoiled DNA in kidney and spleen samples from ducklings treated for 12 days may be an artifact produced during DNA extraction of tissue samples presumably through a nick which caused the covalently closed circular DNA to adopt a relaxed circular conformation.

The evaluation of efficacy of therapy of persistent hepadnavirus infections in humans and animal models to date has been based on the analyses of viral DNA in

liver biopsy and/or plasma samples only. This study was therefore conducted to examine the evaluation a stage further. We analysed nonhepatic organs as well as liver and plasma of treated ducklings for anti-DHBV activity. The results presented here may have major significance in the treatment of chronic hepadnavirus infections. The data amply indicate that FCV was targeted nonselectively against active DHBV replication in both liver and the examined nonhepatic tissues to produce reduced levels of viraemia. This implies that efficacy of any treatment can be assessed readily provided virus replication is active in the infected tissue(s) and the administered drug can be transformed locally to exert an antiviral effect. Although DHBV does not induce the whole spectrum of sequelae of HBV infections, the duck model can provide important information to our understanding of the interactions between hepadnaviruses and antiviral agents, especially of host cells' responses to chemotherapy. We have also shown that DHBV-infected hatchlings could be used for evaluating the effects of potential antiviral agents on systemic hepadnavirus infection. Because of the low body weight at hatching, they can be particularly useful for studies of compounds that may be available in limited quantities during development.

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