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Inhibition of Duck Hepatitis B Virus DNA Replication by Antiviral Chemotherapy with Ganciclovir-Nalidixic Acid

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The aim of this study was to examine the effects of ganciclovir and nalidixic acid either alone or in combination on duck hepatitis B virus DNA replication in vivo with particular reference to production of viral supercoiled DNA and RNA. The most effective antiviral response was observed in the livers of ducks treated by the combination therapy for 28 days, which resulted in a substantial decrease in the amounts of viral supercoiled DNA, relaxed circular and single-stranded DNA, and also viral RNA. This combination treatment was not hepatotoxic over the study period.

The major therapeutic option for carriers of hepatitis B virus (HBV) is alpha interferon, which can control active virus replication (9). However, even in the most successful studies, the response rate in carefully selected patient groups has rarely exceeded 40% (1, 6, 19). One of the possible reasons cited for interferon failure is the persistence of viral supercoiled (SC) DNA in the liver (22).

Duck HBV (DHBV) infection has proved useful in providing insights into the mechanism of hepadnaviral DNA replication (17) and pathogenesis (7). Furthermore, several antiviral agents have been evaluated as potential inhibitors of replication in chronically infected ducks, including nucleoside analogs in vitro (11, 15) and in vivo (5, 8, 11, 20).

In an attempt to suppress DHBV SC DNA, we have been investigating the use of drugs that inhibit SC DNA generation and processing, including the DNA gyrase and topoisomerase inhibitors (3, 10). In this study, we have examined the effects of the DNA gyrase inhibitor nalidixic acid on hepadnaviral replication using a congenitally-infected-duck model. We have shown that the levels of viral SC DNA and RNA can be reduced by combination therapy with ganciclovir and nalidixic acid.

Pekin-Aylesbury crossbred ducks congenitally infected with an Australian strain of DHBV (3) were used in the study. Five-week-old ducks with stable virus titers were used for the treatments (20). The titer of DHBV DNA was determined by dot blot hybridization of alkaline-denatured duck sera, as described previously (20). Three drug regimens were used: two monotherapy treatments and one combination treatment. Eight ducks, weighing on average 2 kg, were each given a 28-day course of nalidixic acid (Winthrop Laboratories, Sydney, Australia) at 250 mg/kg of body weight twice a day. Another eight ducks were treated with a 21-day course of twice-daily intraperitoneal injections of ganciclovir (Syntex, Palo Alto, Calif.) at a total dose of 10 mg/kg/day. We have shown previously that there is no difference in the antiviral effects obtained with either a 3- or a 4-week course of ganciclovir in the duck model (references 13 and 20 and data not shown). Seven more ducks were given nalidixic acid at a dose of 250 mg/kg twice a day plus twice daily intraperitoneal injections of ganciclovir at 10 mg/kg/day for 28 days. Immediately after the treatment period, half of the ducks were sacrificed and liver tissue samples were removed for subsequent analysis whilst the remaining ducks were kept for follow-up study for a further 4 treatment-free weeks, after which they were also sacrificed and liver tissue was removed. Placebo-treated ducks received equivalent volumes of injectable saline in place of ganciclovir and/or lactose in gelatin capsules in place of nalidixic acid. During the treatment and follow-up period, weekly blood specimens were taken for monitoring levels of DHBV DNA as well as for liver and hematological function tests as described previously (13, 20). Liver tissue collected pretreatment, at the end of therapy, and after the follow-up period was processed for histology by standard procedures as described by Luscombe et al. (13). All animals received humane care in compliance with the guidelines of Fairfield Hospital's Animal Ethics Committee.

Viremia was measured by serum dot blot as described by Wang et al. (20). The liver DHBV DNA was analyzed by two DNA extraction procedures, one for total DNA (quantitation of viral burden and assessment of viral replicative intermediates) and the other for viral SC DNA (20, 21).

Total RNA was extracted from 200 mg of liver tissue by using an RNA extraction kit (Pharmacia, Milwaukee, Wis.) according to the manufacturer's directions and was analyzed by standard procedures for slot blot and Northern (RNA) hybridization (14). For the slot blot, serial doubling dilutions starting with 20 μg of total RNA were performed. For probing, DHBV DNA was prepared, labelled, and hybridized exactly as described previously (20). Densitometric analysis was performed on X-ray film by using an imaging densitometer (model GS-670; Bio-Rad Laboratories, Hercules, Calif.).

All the ducks tolerated the treatment protocol satisfactorily. Treatment of the ducks with either ganciclovir or nalidixic acid was not associated with any abnormality in weight loss or serum clinical chemistry. Birds treated with the ganciclovir-nalidixic acid combination also had normal weights but had on average a 15 to 20% decrease in hematocrit levels compared with those of the placebo- and monotherapy-treated animals (data not shown). There was no hepatotoxicity associated with any of the treatments. Markers of liver function in the treated animals were similar to those of the control placebo-treated ducks, and this was confirmed by extensive histological examination of liver tissue from the study animals, especially at the

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TABLE 1. Effects of antiviral agents alone and in combination on the DHBV copy number in liver samples of treated ducks compared with data for the age-matched control birds

Treatment group (sampling time)	VGE/hepatocyte[a,b]	P[c]
Six-week-old positive control (pretreatment)	249 ± 83	
Ten-week-old positive control (end of treatment)	498 ± 166	
Fourteen-week-old positive control (follow-up)	249 ± 83	
Ganciclovir (end of treatment)	83 ± 29	0.013
Ganciclovir (follow-up)	332 ± 10	NS
Nalidixic acid (end of treatment)	249 ± 48	NS
Nalidixic acid (follow-up)	415 ± 83	NS
Combination therapy (end of treatment)	10 ± 1	0.009[d]
Combination therapy (follow-up)	332 ± 10	NS

[a] Viral genome equivalent (VGE) per hepatocyte estimated by the method of Jilbert et al. (7), assuming a DHBV genome equivalent to be 3×10^{-6} pg of DNA and that each cell contains 5×10^{-6} μg of total cellular DNA.

[b] The values shown are the means ± the standard errors.

[c] The results for each treatment group were compared with those for the end-of-treatment controls by using an unpaired Student t test. A P value of <0.05 was regarded as significant. NS, not significant.

[d] $P < 0.05$ versus ganciclovir monotherapy; $P < 0.003$ versus nalidixic acid monotherapy.

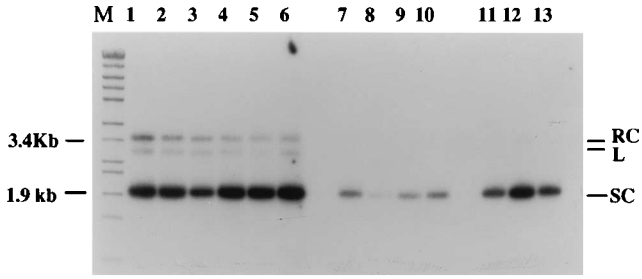

FIG. 1. Southern blot analysis of SC DNA extracted from liver tissue samples from ducks treated by nalidixic acid-ganciclovir combination therapy. Lanes 1 to 6, samples from age-matched controls at 6 weeks (lanes 1 and 2), 10 weeks (lanes 3 and 4), and 14 weeks (lanes 5 and 6) of age. Lanes 7 to 10, samples taken at the end of treatment; lanes 11 to 13, samples taken after the follow-up period. Lane M, molecular standards. RC, relaxed circular DHBV DNA; L, double-stranded linear DHBV DNA.

end of the treatment period (data not shown). No steatosis was observed in any of the treated birds.

Serum DHBV DNA levels in the placebo-treated congenitally infected ducks remained stable throughout the treatment period (20). Nalidixic acid had little effect on serum DHBV DNA levels, whereas treatment of ducks with either the ganciclovir regimen or the combination antiviral regimen produced a substantial decrease in viremia, with DHBV DNA levels becoming undetectable as shown by dot blot hybridization (data not shown). During the follow-up period for all treatments, the DHBV DNA in serum returned to detectable levels.

The effect of antiviral drug therapy on the hepatic levels of viral DNA at the end of the treatment and follow-up periods was determined by using semiquantitative dot blot analysis (7, 20), and the results of the analysis are shown in Table 1. The concentrations of viral DNA in the livers of the six age-matched placebo-treated ducks were similar to each other, fluctuating only twofold over the 8-week study period. For the four ducks treated with ganciclovir alone, a significant reduction ($P < 0.05$) in the level of hepatic viral DNA was observed. In the follow-up liver samples, the viral DNA returned to the control levels. Examination of the end-of-treatment liver samples from the four ducks treated with nalidixic acid did not reveal any change in the total level of viral DNA compared with the end-of-treatment level for control birds. Also, analysis of the follow-up liver samples from the other four ducks treated with nalidixic acid showed no change in viral DNA levels. With the combination therapy, all ducks responded similarly, with a significant drop in viral DNA levels compared with the end-of-treatment levels for controls (Table 1). Upon cessation of treatment, hepatic viral DNA levels returned to those of the age-matched controls. The differences in the viral DNA levels of the control animals detected during the study period reflect natural biological variation which has been observed previously (13, 20).

The total DNA from the liver samples used to obtain the data in Table 1 was also subjected to Southern blot analysis. The results showed that there was a substantial reduction in the level of viral replicative intermediates at the end of treatment with ganciclovir, confirming previous observations (20). In addition, no detectable bands of viral DNA were seen at the end of the combination therapy period. However, this technique is not as sensitive as dot blot hybridization in detecting copy numbers of viral DNA (20). Nalidixic acid treatment had no effect on the viral DNA replicative intermediates. After cessation of treatments, all replicative forms returned to pretreatment levels.

The second DNA extraction procedure enriched for viral SC DNA in liver samples (20, 21). Extracted samples were analyzed by Southern hybridization, revealing a band which migrated at 1.9 kb and corresponded to the viral SC DNA (13, 20, 21). Following therapy with ganciclovir or nalidixic acid, no substantial difference between the amount of SC DNA and the control amount was detected (data not shown). However, in the end-of-treatment samples from the combination-treated ducks there was a decrease in the level of SC DNA (Fig. 1, lanes 7 to 10) from both that of the age-matched controls and that of the ducks in the follow-up period. Densitometric analysis of the autoradiograph revealed a 70% reduction in the level of viral SC DNA at the end of treatment compared with levels for the controls (Fig. 1, lanes 1 to 6). The amount of viral SC DNA after the follow-up period was still 30% below the control level (Fig. 1, lanes 11 to 13). There was little variation in the levels of viral SC DNA of the control samples (Fig. 1, lanes 1 to 6).

Ganciclovir treatment resulted in a two- to threefold reduction (Fig. 2B) from the level of RNA in the age-matched controls (Fig. 2A), whilst nalidixic acid had no effect (Fig. 2C). In contrast, an eightfold drop in the hepatic DHBV RNA levels of two ducks treated by combination therapy was observed (Fig. 2D). When the combination treatment ceased, DHBV RNA levels of the birds kept for follow-up study returned to match that of controls (Fig. 2D). This observation was confirmed by densitometric analysis. Northern analysis of this RNA revealed no selective inhibition of any of the three major viral RNA transcripts of DHBV (18) (data not shown).

This report describes the inhibition of hepadnaviral SC DNA generation and processing in vivo by nalidixic acid-ganciclovir combination therapy. Orally administered nalidixic acid alone at a dose of 250 mg/kg of body weight twice a day did not significantly inhibit virus replication in ducks chronically infected with DHBV. Analysis of serum and liver tissue samples collected from nalidixic-acid-treated ducks revealed that the drug had a minimal effect on viral replicative intermediates, including SC DNA. Ganciclovir, whilst inhibiting replicative intermediate DHBV DNA, also had little effect on

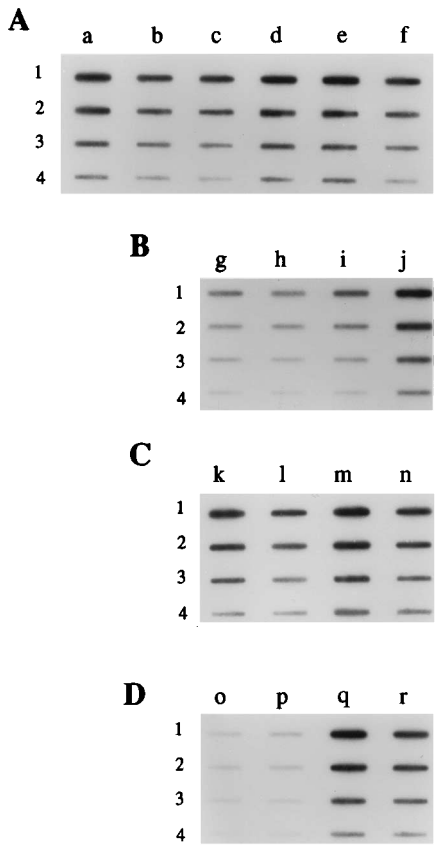

FIG. 2. Quantitative slot blot analysis of DHBV RNA in liver samples from ducks treated with ganciclovir (B), nalidixic acid (C), and the nalidixic acid-ganciclovir combination (D) and from control animals (A). Purified RNA from each sample was applied to row 1 (1-in-2 dilution; 20 μg of total RNA) and in doubling dilutions of up to 1:8 (rows 2 to 4). Lanes g, h, k, l, o, and p, samples taken at the end of treatment; lanes i, j, m, n, q, and r, follow-up samples; lanes a to f, DHBV DNA from age-matched controls at 6 weeks (lanes a and b), 10 weeks (lanes c and d), and 14 weeks (lanes e and f) of age.

DHBV SC DNA or RNA. In contrast, the combination treatment caused substantial decreases in the levels of total intrahepatic viral DNA, viral SC DNA, and viral RNA. The myelosuppression observed in this study is a recognized complication of ganciclovir treatment (4), but no hepatotoxic effect on any of the treated birds, as shown by liver function tests or histological assessment of the liver tissue, was observed.

Our previous studies have demonstrated that ganciclovir alone can substantially reduce hepadnaviral DNA replication both in humans (12) and in congenitally infected ducks (20). However, ganciclovir treatment failed to reduce either DHBV SC DNA levels (20) or viral RNA levels (Fig. 2). When treatment was withdrawn, viral DNA replication promptly returned to pretreatment or higher levels (rebound). Posttherapy relapse and/or rebound is a major problem common to all antiviral agents which have been used to treat chronic hepatitis B, including alpha interferon, adenine arabinoside, and phosphonoformate (16, 22). Viral SC DNA has been shown to have a finite half-life in vitro (2). Thus, to be successful, future therapies for chronic hepatitis B may of necessity include agents which can destabilize and eventually eliminate viral SC DNA as well as block overall viral DNA replication and promote immune elimination of virus-infected cells (19). At present, it is not possible to determine the mechanism(s) which resulted in the effect described in this report but further studies are in progress. Interactions of nalidixic acid with both HBV DNA and cellular DNA as well as their regulatory factors warrant further investigation.

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