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Evidence that Hepatocyte Turnover Is Required for Rapid Clearance of Duck Hepatitis B Virus during Antiviral Therapy of Chronically Infected Ducks

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Duck hepatitis B virus (DHBV) DNA synthesis in congenitally infected ducks is inhibited by 2′-deoxycarbocyclic guanosine (2′-CDG). Three months of therapy reduces the number of infected hepatocytes at least 10-fold (W. S. Mason, J. Cullen, J. Saputelli, T.-T. Wu, C. Liu, W. T. London, E. Lustbader, P. Schaffer, A. P. O'Connell, I. Fourel, C. E. Aldrich, and A. R. Jilbert, Hepatology 19:393–411, 1994). The present study was performed to determine the kinetics of disappearance of infected hepatocytes and to evaluate the role of hepatocyte turnover in this process. Essentially all hepatocytes were infected before drug therapy. Oral treatment with 2′-CDG resulted in a prompt reduction in the number of infected hepatocytes. After 2 weeks, only 30 to 50% appeared to still be infected, and less than 10% were detectably infected after 5 weeks of therapy. To assess the possible role of hepatocyte turnover in these changes, 5-bromo-2′-deoxyuridine (BUdR) was administered 8 h before liver biopsy to label host DNA in hepatocytes passing through S phase, and stained nuclei were detected in tissue sections by using an antibody reactive to BUdR. The extent of nuclear labeling after 5 weeks was the same as that before therapy (ca. 1%). However, biopsies taken after 2 weeks of therapy showed a ca. 10-fold elevation in the number of nuclei labeled with BUdR. This result suggested that a rapid clearance of infected hepatocytes by 2′-CDG was caused not just by the inhibition of viral replication but also by an acceleration of the rate of hepatocyte turnover. To test this possibility further, antiviral therapy was carried out with another strong inhibitor of DHBV DNA synthesis, 5-fluoro-2′,3′-dideoxy-3′-thiacytidine (524W), which did not accelerate hepatocyte turnover in ducks. 524W administration led to a strong inhibition of virus production but to a slower rate of decline in the number of infected hepatocytes, so that ca. 50% (and perhaps more) were still infected after 3 months of therapy. In addition, histopathologic evaluation of 2′-CDG-treated ducks revealed liver injury, especially at the start of therapy. No liver damage was observed during 524W therapy. These results imply that clearance of infected hepatocytes from the liver is correlated with hepatocyte turnover. Thus, in the absence of immune clearance or other sources for the accelerated elimination of infected hepatocytes, inhibitors of virus replication would have to be administered for a long period to substantially reduce the burden of infected hepatocytes in the liver.

Hepadnaviruses cause both transient and chronic infections of the liver. Transient infections generally have a mild course and resolve in less than 6 to 12 months, with no residual liver damage. Chronic infections, particularly in mammals, often lead to severe liver damage, cirrhosis, and hepatocellular carcinoma (18, 19, 27, 28). The spontaneous loss of virus production that sometimes occurs in chronically infected individuals as well as the loss that occurs in response to interferon therapy in other patients is associated with the cessation of active liver disease (22), though existing damage may remain. Unfortunately, interferon therapy is of low efficacy, especially in individuals infected perinatally, and there has therefore been an active search for new antiviral agents that would terminate or control chronic infections in patients who are not helped by interferon. Of those agents that have so far been tested, inhibitors of viral DNA synthesis appear, from cell culture experiments and in vivo trials in animals, to be the most promising (10, 13–16, 20, 25).

The utilization of inhibitors of virus replication to treat chronic hepadnavirus infections must, however, take account of the evidence that infection of individual hepatocytes is both chronic and productive, with an increase in the normally low amount of hepatocyte turnover probably occurring only as a consequence of the immune response to viral antigens. Therefore, effective therapy of patients lacking a strong immune response to the virus infection would seem to require not only inhibition of virus replication but also some additional mechanism for the destruction or curing of the infected hepatocytes. We recently reported that 2′-deoxycarbocyclic guanosine (2′-CDG), a strong inhibitor of human hepatitis B virus (HBV) and duck hepatitis B virus (DHBV) replication (3, 23, 24), caused a rapid loss of infected hepatocytes from the livers of ducks congenitally infected with DHBV, with a decline from 100 to <10% infected hepatocytes in less than 3 months (20). Hepatocytes in congenitally infected ducks appear to have a life time of ca. 2 to 3 months (20), but if dying hepatocytes are replaced by division of neighboring hepatocytes, which would

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also be infected, the almost complete loss of infected hepatocytes in response to 2′-CDG therapy is unexplained. We therefore investigated the loss of infected hepatocytes in more detail. We present results which argue that the antiviral efficacy of 2′-CDG was a consequence, in part, of an acute liver toxicity caused by this agent. Though the toxicity abated after a few weeks, it appeared sufficient to cause a major turnover of hepatocytes. In contrast, 5-fluoro-2′,3′-dideoxy-3′-thiacytidine (524W), a cytosine analog (2, 4, 10, 21, 30), was both nontoxic and an effective inhibitor of DHBV replication in vivo. We present evidence that antiviral therapy with 524W for up to 12 weeks produced a lesser decline in the number of infected hepatocytes than a 5-week treatment with 2′-CDG. Therefore, inhibition of virus replication, by itself, is not sufficient in the short term to eliminate virus from the liver.

MATERIALS AND METHODS

Ducks and treatment protocol. Experiments with ducks were reviewed and approved by the Institutional Animal Care and Use Committee of the Fox Chase Cancer Center. Eighteen ducks congenitally infected with DHBV and four uninfected ducks were entered into this study. At 3 to 5 months of age, ducks were randomly assigned into three groups. Thirteen ducks (nine DHBV-infected and four uninfected ducks) were treated orally every other day with 2′-CDG (100 μg/kg of body weight in a volume of 1 ml) for up to 9 weeks, at which time the interval between treatments was increased, as indicated. Another five ducks received placebo (distilled water). Four DHBV-infected ducks received twice-daily treatments with 524W (50 mg/kg of body weight in a volume of ca. 10 to 15 ml). Blood samples were obtained before therapy, every 4 days for 2 weeks at the start of therapy, and once a week thereafter. 2′-CDG was gift from ViraChem, Inc., and 524W was a gift from Burroughs Wellcome Company. Liver biopsies were performed 2 to 4 weeks (time zero) before therapy and at the indicated times during therapy as previously described (1). Eight hours before selected liver biopsies (done between 8 a.m. and 12 noon), ducks were injected intraperitonealy with 5-bromo-2′-deoxyuridine (BUdR; 50 mg/kg) to label host cell DNA. Biopsy samples (0.2 to 0.3 g) were divided into three parts. One piece was immediately frozen and stored at −80°C for DHBV DNA analyses. A second piece was fixed in formalin for histopathology. A third piece was fixed in ethanol-acetic acid for detection of DHBV antigens and BUdR-containing cell DNA by reaction with specific antibodies as previously described (11, 20).

Assays for DHBV DNAs. Serum and liver samples were tested for viral DNA. Serum DHBV DNA was detected by a DNA spot hybridization assay, using 5 μl of serum, as previously described (11); alternatively, virus was collected from 50 μl of serum by centrifugation through a 10 to 20% (wt/vol) sucrose step gradient containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.001 M EDTA for 3 h at 45,000 rpm in a Beckman SW-60 rotor at 4°C. The virus-containing pellets were resuspended in 2 mg of pronase per ml–0.1% sodium dodecyl sulfate–0.1 M NaCl–0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA, incubated for 1 h at 37°C, and then subjected to agarose gel electrophoresis and Southern blot analysis. Liver samples (0.05 to 0.1 g) were homogenized in 0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA and divided into two parts, one for isolation of total viral DNA and one for isolation of non-protein-bound, covalently closed circular (CCC) viral DNA (11). Nuclear counts were determined in the extracts following staining with ethidium bromide as previously described (11). Five micrograms of total DNA or CCC DNA extracted from 10^6 liver cells was subjected to electrophoresis on 1.5% agarose gels. Southern blot analysis was carried out essentially as described by Wahl et al. (29). Viral DNAs were detected by hybridization with a ^{32}P-labeled probe representing the complete viral genome. Quantitation of hybridized radioactive DNA was done with either an AMBIS scanning radioanalytic imaging system or a Fuji BAS1000 Bio Imaging analyzer.

Serologic assays. Sera were monitored for DHBV surface antigen (DHBsAg) by using a quantitative radioimmunoassay. Briefly, plastic beads (Precision Plastic F61334; lot 4914) were coated with antibody by incubation overnight at room temperature in a 1:1 mixture of mouse monoclonal antibodies (immunoglobulin G) 1.Hl and 7C.12, reactive to the pre-S and S domains of the DHBV envelope proteins, respectively (24a), at a final concentration of 2 μg of protein per ml in 50 mM Tris-HCl (pH 9.5)–0.1% (wt/vol) sodium azide. (Similar assay results were obtained if either monoclonal antibody alone was used to coat the beads.) The beads were then washed three times with phosphate-buffered saline (PBS) and blocked by incubation for 3 h at room temperature with PBS containing 2% (vol/vol) normal duck serum (PBSS). Samples to be tested were serially diluted in PBSS, 200 μl of each dilution was added to a bead, and the samples were incubated overnight at room temperature. The beads were then washed three times with PBS. To detect bound DHBsAg, ^{125}I-labeled 7C.12 immunoglobulin G (~10 μCi/μg) in PBSS was added to each bead. Following a 3-h incubation at room temperature, the beads were washed five times with PBS, and bound ^{125}I was then detected with an Abbott gamma counter.

Histopathology. Formalin-fixed liver tissue sections embedded in paraffin, sectioned at 6-μm thickness, and stained with hematoxylin and eosin were graded, without knowledge of prior treatment, on a subjective scale. Liver injury was assessed on the basis of the degree of hepatic inflammation, hepatocyte vacuolization, hepatocyte necrosis, variation in hepatocyte nuclear size, biliary hyperplasia, and Kupffer cell activation.

RESULTS

2′-CDG therapy caused a very rapid loss of infected hepatocytes from the liver of ducks congenitally infected with DHBV. A previous study has shown that after 3 months of oral administration of 100 μg of 2′-CDG per kg of body weight, given every other day, >90% of DHBV-infected hepatocytes have disappeared from the livers of treated ducks (20). To determine how rapidly DHBV-infected hepatocytes disappeared during 2′-CDG therapy, we first treated congenitally DHBV-infected ducks for only 5 weeks. Blood samples were taken before and during therapy and analyzed for the presence of DHBV, by blot hybridization to detect virion DNA (26, 29). Liver biopsy samples taken before treatment and again after 5 weeks of 2′-CDG therapy were also analyzed for DHBV DNA by Southern blot hybridization. Consistent with our previous report (20), administration of 2′-CDG caused the viremia, as determined by assays for virion DNA, to drop ca. 10-fold after 4 days of therapy and to nearly undetectable levels within a few weeks of the initiation of treatment (data not shown). To determine the effect of therapy on the amount of viral DNA in the liver, we assayed for total viral DNA (Fig. 1A) and for CCC DNA (Fig. 1B), the template for transcription of viral RNAs. In the ducks treated with placebo (ducks 2746, 2747, and 2749), viral DNA levels in the liver remained relatively constant. In contrast, in the four ducks treated with 2′-CDG, the amount of viral DNA replication intermediates declined to nearly undetectable levels (Fig. 1A) and the amount of CCC DNA dropped about 10-fold (Fig. 1B).

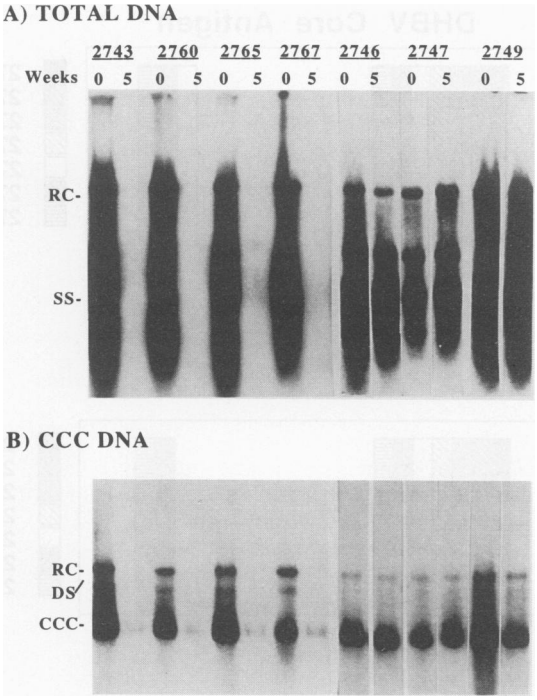

FIG. 1. Administration of 2′-CDG for 5 weeks caused a major reduction in the amount of DHBV DNA in the livers of congenitally infected ducks. Liver tissues taken before therapy (lanes 0) and after 5 weeks of therapy (lanes 5) were extracted for total DNA (A) and non-protein-bound nucleic acids (e.g., CCC DNA) (B), which were analyzed by Southern blot hybridization as described in Materials and Methods. Ducks 2743, 2760, 2765, and 2767 received 2′-CDG, and ducks 2746, 2747, and 2749 received the placebo. RC, 3-kbp relaxed circular DHBV DNA; DS, 3-kbp double-stranded, linear DHBV-DNA; CCC, 3-kbp CCC DHBV DNA; SS, 3-kb minus-strand DHBV DNA.

To determine if this early decline in the amount of viral DNA in the liver was associated with a similar decline in the number of hepatocytes producing detectable levels of viral antigens, liver tissue sections were stained with antibodies to DHBsAg and to DHBV core antigen (DHBcAg). The results are summarized in Fig. 2A and B. In agreement with the CCC DNA results, it was found that, on average, only 3 and 10% of hepatocytes were positive for DHBcAg and DHBV pre-S antigen detection, respectively.

The apparent clearance of 90% of the infected hepatocytes after only 5 weeks of therapy was unexpected. We therefore designed the next experiment to determine if the loss of infected hepatocytes might be very much faster than we had anticipated. Again, ducks were treated every other day with 2′-CDG. The first liver biopsy was performed 2 to 3 weeks before the beginning of treatment, and another biopsy was done after 2 weeks of therapy. After 6 weeks of therapy, the ducks were sacrificed and liver specimens were again collected and analyzed. As shown in Fig. 3, after 2 weeks of therapy, there was a threefold decrease in the amount of replicative forms of viral DNA in the liver and a twofold decrease in the amount of CCC DNA. After 6 weeks of therapy, viral DNA replication intermediates were almost at undetectable levels and viral CCC DNA levels had dropped ca. 10-fold below the levels prior to 2′-CDG administration. The results for CCC DNA were, to a large extent, paralleled by a decline in the amount of DHBsAg in the serum during 2′-CDG therapy, implying that DHBsAg levels in the serum provided an accurate reflection of changes in the amount of viral transcriptional template (CCC DNA) in the liver during short-term therapy (Fig. 4); however, as described later, this relationship was not observed during short-term therapy with another DNA synthesis inhibitor.

Assays for viral pre-S antigen in individual hepatocytes suggested a ca. 70% decline in the proportion of infected hepatocytes after 2 weeks of therapy, results again consistent with the decline in amount of CCC DNA in the liver (Fig. 2C and D). A greater decline was observed after 2 weeks of therapy in the fraction of hepatocytes reacting with DHBcAg; whether this was due to an actual loss of core antigen from hepatocytes that were still infected, or to some technical artifact, is unknown. In summary, the results suggested that even after 2 weeks of 2′-CDG therapy, there was at least a twofold decline in the number of infected hepatocytes.

Disappearance of DHBV-infected hepatocytes may result from a transient acceleration of hepatocyte turnover by 2′-CDG. Productive hepadnavirus infections are maintained by the presence of viral CCC DNA, the transcriptional template, in the nucleus of the infected cell. This DNA appears to be stable, at least in nondividing cultures of primary duck hepatocytes (3) (BUdR-labeled CCC DNA in reference 1a). Therefore, a rapid loss of CCC DNA and of viral gene expression from the liver would presumably require the death of at least some infected hepatocytes and might also be aided by the progression of surviving hepatocytes through the cell cycle to replace the dying cells. With the expectation that cell turnover might have a role in virus clearance from the liver, we performed BUdR pulse-labeling by injection of BUdR 8 h prior to selected liver biopsies. BUdR-labeled hepatocyte nuclei were detected by staining liver tissue sections with an anti-BUdR immunoglobulin. The results of this analysis are summarized in Fig. 5 and illustrated in Fig. 6. Before treatment, 0.5 to 1.5% of hepatocyte nuclei were BUdR positive. However, after 2 weeks of therapy with 2′-CDG, an average of 10% of hepatocytes were labeled by BUdR. This elevated labeling was primarily in lobular zone 1 and, to a lesser extent, zone 2. Interestingly, after 5 weeks of therapy, the percentage of hepatocytes labeled by BUdR injection was again at the pretreatment level. Assuming that the BUdR labeling gives a quantitative estimate of the fraction of dividing hepatocytes, our results suggested a rate of turnover of hepatocytes of ca. 15%/day after 2 weeks of therapy, i.e., a rate sufficient to regenerate the complete liver mass in as little as 7 days. Thus, the antiviral effect of 2′-CDG may depend not only on its ability to inhibit virus replication but also on its ability to produce a severe, albeit transient, hepatotoxicity.

To determine if the early hepatotoxicity described above was due to 2′-CDG alone or to a synergistic effect of viral infection and drug treatment, we also treated uninfected ducks with 2′-CDG. Liver biopsies were performed 2 to 3 weeks before treatment and again 2 weeks after initiation of therapy. BUdR was injected before both liver biopsies. As shown in Fig. 5C, before treatment, 0.05 to 0.1% of hepatocytes were positive for BUdR staining. This result suggested that uninfected duck hepatocytes have a slightly lower rate of turnover than the DHBV-infected hepatocytes. However, after 2 weeks of therapy, 8% of the hepatocyte nuclei, on average, were positive for BUdR staining. This result showed that 2′-CDG itself was primarily responsible for the transient acceleration of hepatocyte turnover.

To further characterize the effect of 2′-CDG, liver damage was also assessed by histologic evaluation performed on for-

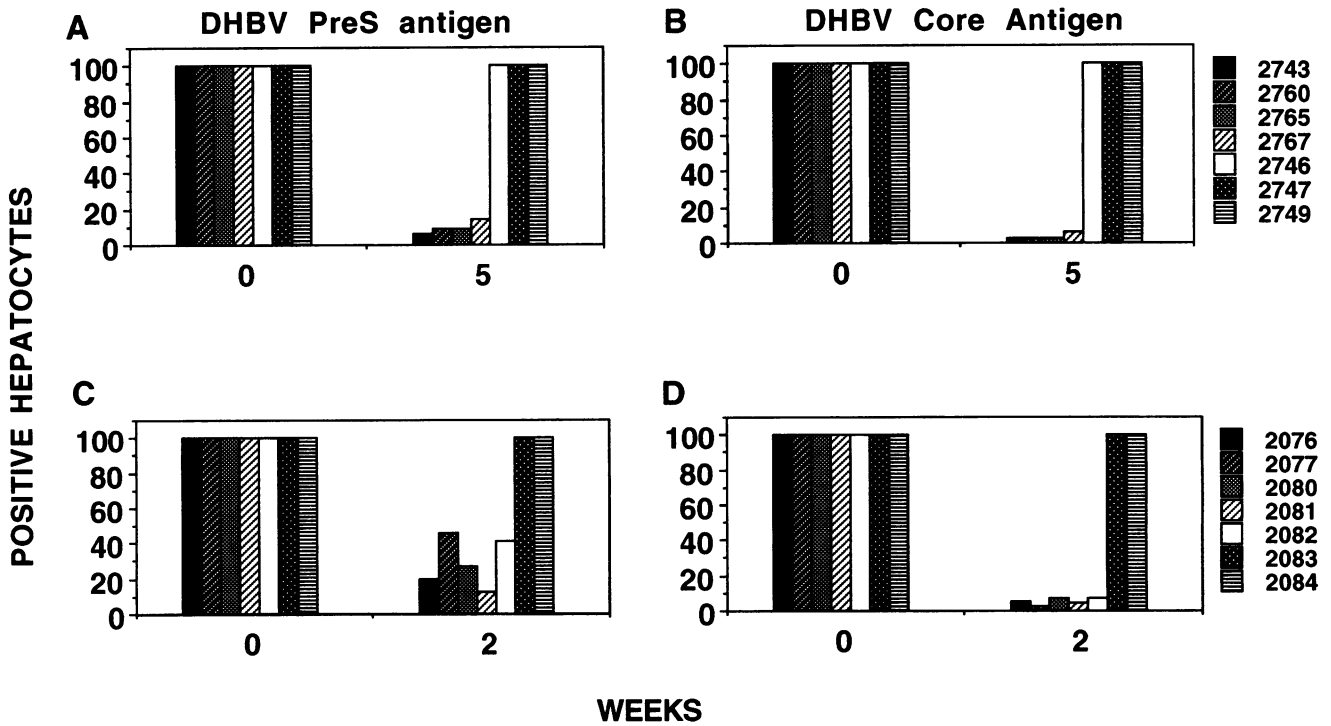

FIG. 2. 2′-CDG therapy induced a dramatic decrease in the number of hepatocytes positive for viral antigens. Immunostaining for DHBV pre-S antigen (A and C) and for DHBVcAg (B and D) was carried out on fixed liver tissues as described in Materials and Methods. Ducks 2743, 2760, 2765, 2767, 2076, 2077, and 2080 to 2082 were treated with 2′-CDG; ducks 2746, 2747, 2749, 2083, and 2084 received a placebo (H_2O).

malin-fixed liver sections. Before treatment, the hepatocytes of all ducks were normal. Several birds had subjective scores of 1 due to mild inflammatory background. By 2 weeks of 2′-CDG treatment, there was a characteristic, prominent vacuolization of hepatocytes and variation in nuclear size which was associated with lobular disarray. Although administration of 2′-CDG did not result in an appreciable elevation of rates of hepatocyte turnover at 5 weeks, there was evidence of moderate liver injury associated with long-term therapy. This was characterized by a continued but less prominent hepatocyte vacuolization and a mild disruption of lobular architecture. Inflammation was mild in all treated ducks despite the morphologic changes in hepatocytes. The results for all the biopsies are summarized in Table 1. Despite the histologic evidence, it should be noted that this regimen of 2′-CDG administration did not produce any clinically apparent effects in the ducks, including loss of body weight, even when continued for up to 6 months (20).

Evidence that inhibition of virus replication was, by itself, insufficient to rapidly clear DHBV from the livers of congenitally infected ducks. As a test of the hypothesis that the rapid antiviral effect of 2′-CDG was due to its ability to promote hepatocyte turnover, we evaluated the effect of another antiviral compound, 524W (10), on DHBV replication in ducks that were chronically infected as a result of congenital transmission. 524W appears to be less toxic in in vivo assays selective for inhibition of HBV replication (2). A pilot study using ducklings congenitally infected with DHBV established that 524W inhibited DHBV replication at a dose of 50 mg/kg of body weight, given orally twice a day, and that the drug was well tolerated (data not shown). We therefore treated four adult ducks for 12 weeks with 524W, using the same regimen found effective in ducklings. Liver biopsies were performed 2 to 3 weeks before treatment and again after 2, 6, and 12 weeks of therapy.

Viremia was reduced as effectively by 524W as by 2′-CDG (data not shown), dropping to levels of $<5 \times 10^6$/ml (20). However, even after 6 weeks of 524W therapy, at least 60 to 80% of the hepatocytes still appeared to be infected, and at least 40 to 60% were still infected after 12 weeks of therapy (Fig. 7). As shown in Fig. 8A, a significant decline of replicative forms of viral DNA in the liver was observed, consistent with the activity of 524W as an inhibitor of viral DNA synthesis. In contrast, the level of CCC DNA was changed more slowly, dropping an average of 20% after 2 weeks, 40% after 6 weeks, and 60% after 12 weeks of therapy (Fig. 8B and 9B).

In three of four ducks, the amount of circulating DHBsAg dropped more than 100-fold by 12 weeks (Fig. 9A). This lack of proportionality between the reduction of CCC DNA in the liver and DHBsAg in the serum was unexpected, especially with the decline in DHBsAg vastly exceeding the decline in CCC DNA. One interpretation is that there has been a decline in CCC DNA copy number in infected hepatocytes and that, at low copy number, the S promoter is less efficiently transcribed than the core or pre-S promoter because of promoter occlusion (9). The effect is probably not due simply to inhibition of viral DNA synthesis, since after 21 to 22 weeks of 2′-CDG administration, the decline in circulating DHBsAg exceeded that in CCC DNA in the liver by only 3- to 4-fold; in addition, even after 36 to 37 weeks of therapy, the discrepancy was only 10-fold (ducks 2743, 2760, and 2765; Fig. 4 and data not shown).

The number of hepatocytes with labeled nuclear DNA after BUdR injection was unchanged after 2 or 6 weeks of 524W

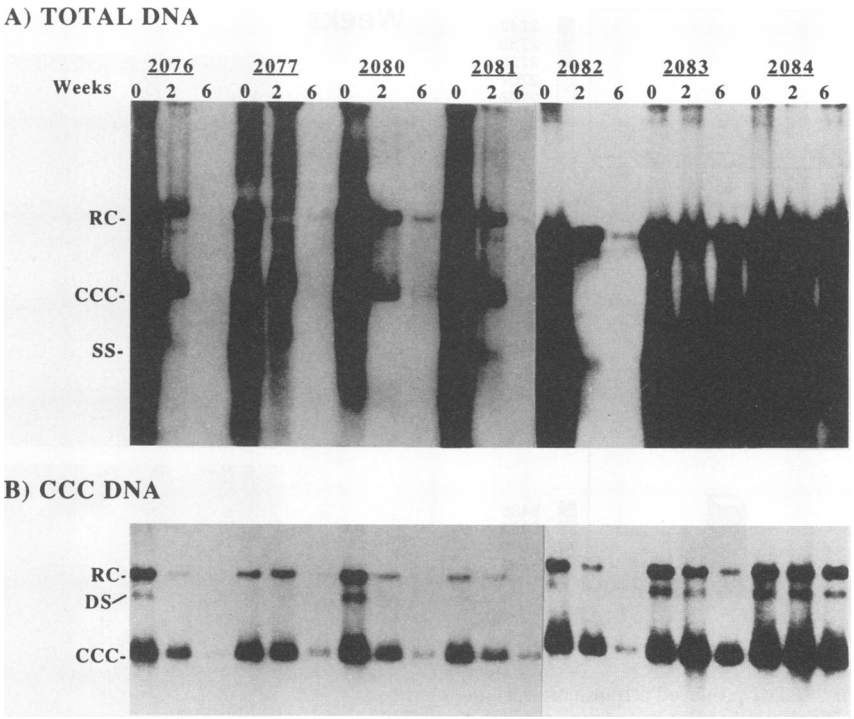

FIG. 3. As little as 2 weeks of 2′-CDG therapy produced a decrease in the amount of DHBV replicative intermediate DNA and CCC DNA in the livers of congenitally infected ducks. Liver biopsies obtained before therapy (lanes 0) and after 2 (lanes 2) and 6 (lanes 6) weeks of therapy were extracted for total DNA (A) and CCC DNA (B) and analyzed as in Fig. 1. Ducks 2076, 2077, and 2080 to 2082 received 2′-CDG, and ducks 2083 and 2084 received placebo. The positions of migration of relaxed circular (RC), double-stranded (DS), CCC, and single-stranded (SS) DNAs are indicated.

administration, suggesting that hepatocyte turnover was unaffected during 524W therapy (Fig. 7B). Histopathologic evaluation also failed to reveal any prominent changes throughout the 12 weeks of therapy (Table 1). These results suggest that inhibition of virus replication, by itself, was not sufficient to rapidly reduce the number of infected hepatocytes in the liver. However, the gradual decline in CCC DNA that was observed suggests that an eventual clearance of most infected hepatocytes would occur with longer-term therapy. The decline in CCC DNA levels may be a reflection of the natural turnover of hepatocytes. If cell death is random and the lifetime of infected hepatocytes is ca. 3 months, as suggested by BUdR labeling, two-thirds of the hepatocytes would have died and been replaced during therapy, apparently by division of surviving, mature hepatocytes.

Inhibition of DHBV replication occurring during 2′-CDG therapy could be maintained by spaced administration of the drug. We have previously shown that 2′-CDG therapy does not completely eradicate DHBV from any treated ducks (20), apparently because therapy with an inhibitor of virus replication is not sufficient, by itself, to eliminate all infected hepatocytes and congenitally infected ducks probably do not develop a DHBV-specific hepatitis. In mammals with chronic infections, unlike the situation in congenitally infected ducks, there is often an associated hepatitis that may serve to completely eliminate infected hepatocytes once their number is sufficiently reduced by an antiviral regimen. However, in some patients, this may not occur efficiently, and such individuals may require prolonged maintenance on an antiviral drug. 2′-CDG was previously found to be long acting both in tissue culture and in vivo (3). This observation raised the possibility that a more

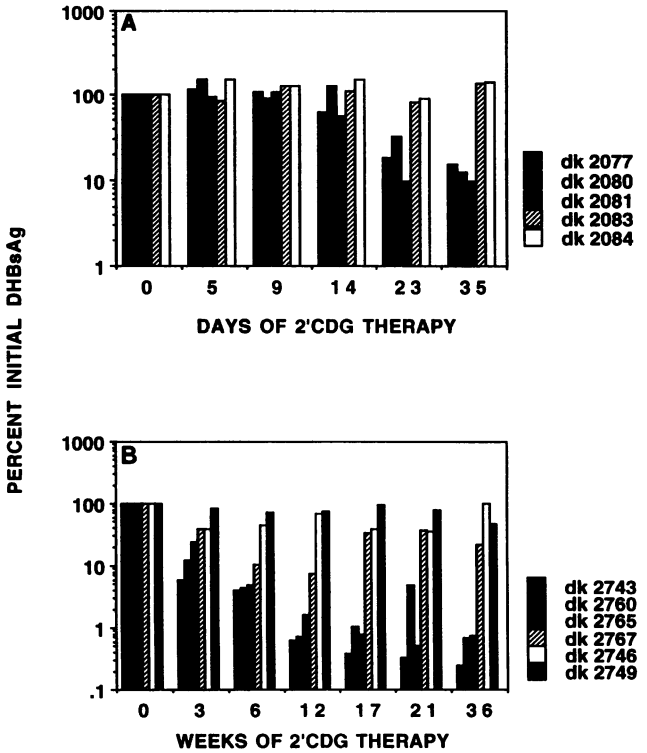

FIG. 4. Reduction in circulating DHBsAg in 2′-CDG-treated ducks. Assays for DHBsAg were carried out as described in Materials and Methods. (A) Results from the ducks (dk) represented in Fig. 4; (B) longer-term study of the ducks represented in Fig. 1.

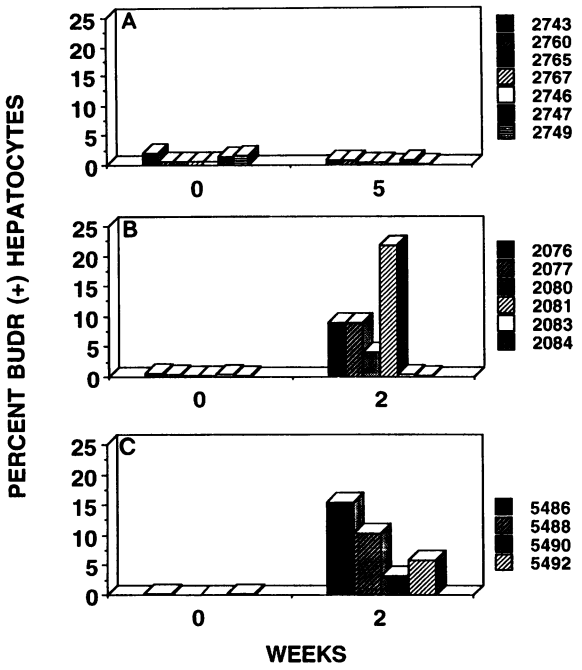

FIG. 5. Administration of 2′-CDG produced a transient acceleration of hepatocyte turnover. To detect hepatocytes passing through S phase, BUdR was injected 8 h before liver biopsy. Immunofluorescent staining was carried out, using an anti-BUdR antibody, on fixed liver specimens. Results for DHBV-infected ducks (A and B) and for DHBV-negative ducks (C) are shown as a function of the number of weeks of 2′-CDG or placebo administration. Ducks 2746, 2747, and 2749 (A) and ducks 2083 and 2084 (B) received placebo; the remainder received 2′-CDG.

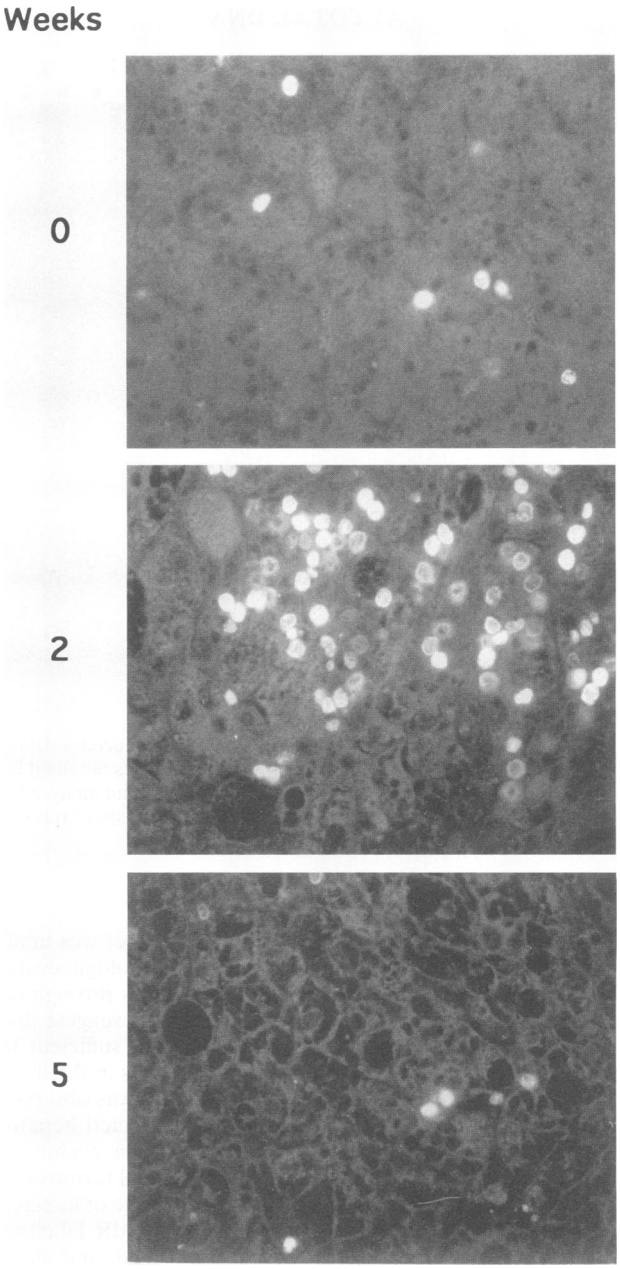

FIG. 6. BUdR labeling of hepatocyte nuclear DNA was dramatically increased after 2 weeks of 2′-CDG administration. The panels illustrate representative data from ducks represented in Fig. 1 and 3.

widely spaced administration of 2′-CDG at the dose used in the assays described above might be sufficient to maintain an infection at low levels without producing the hepatic alterations previously found during long-term administration. Accordingly, the 2′-CDG schedule in the four ducks represented in Fig. 1 was reduced after 9 weeks from every other day to twice weekly. During the treatment, blood samples were taken once a week and were analyzed for DHBV by dot blot hybridization. After 17 weeks of therapy on this reduced schedule, viremia was still suppressed (data not shown). The analysis of liver biopsy samples taken at this time revealed that viral DNA replication intermediates and CCC DNA were still almost undetectable, except in duck 2767, as shown in the Fig. 10 (sample 1). As expected from this result, 5% of the hepatocytes were positive for DHBV pre-S antigens in ducks 2743, 2760, and 2765 but elevated in duck 2767, in which 40% of the hepatocytes contained detectable levels of viral antigen. This change in CCC DNA in the liver of duck 2767 was paralleled by an elevation in DHBsAg in the serum (Fig. 4). As rebound of DHBV replication had occurred in only one duck on this twice-weekly regimen, this treatment protocol was extended for 5 more weeks and then reduced to one dose of 2′-CDG per week. After 6 weeks of treatment once per week, the ducks were sacrificed and virus expression in the liver was determined. As shown in Fig. 10 (sample 2), CCC DNA and replicative forms of viral DNA in the liver remained relatively unchanged, even in duck 2767, from values observed for the twice-weekly treatment. The fraction of hepatocytes expressing pre-S protein was also virtually unchanged from the preceding biopsy. Histologic evaluation detected no significant liver injury in ducks receiving once-weekly doses of 2′-CDG. Therefore, widely spaced administration of 2′-CDG or similar long-acting nucleoside analogs may be useful in controlling infections in patients with inadequate immune responses or as the primary antiviral in patients with an active hepatitis.

DISCUSSION

Antiviral therapy with the nucleoside analog 2′-CDG was studied to determine why the vast majority of infected hepatocytes disappeared after 3 months of therapy, as described in an earlier study (20). Further study of the in vivo antiviral activity of this compound was also carried out because of its

TABLE 1. Histopathological assessment of liver injury in ducks treated with antiviral agents[a]

Duck no.[b]	Drug	Grade at indicated time of biopsy (wk of therapy)				
		0	2	5–6	26	37
		None,[c] none[d]	Every other day,[c] twice daily[d]	Every other day,[c] twice daily[d]	Twice/wk[c]	Once/wk[c]
2743 (+)	2′-CDG	1		2	2	1/0
2760 (+)	2′-CDG	1/0		2	1	1
2765 (+)	2′-CDG	1/0		1/0	2	1
2767 (+)	2′-CDG	1/0		1	2	1
2746 (+)	None	1		1/0	1/0	1/0
2747 (+)	None	1/0		1/0[e]		
2749 (+)	None	1/0		1/0	1/0	1[f]
2076 (+)	2′-CDG	1/0	2	2		
2077 (+)	2′-CDG	1/0	2	2		
2080 (+)	2′-CDG	1	2	1		
2081 (+)	2′-CDG	1	2	1		
2082 (+)	2′-CDG	1/0	2	2		
2083 (+)	None	1/0	1/0	1/0		
2084 (+)	None	0	1/0	0		
5486 (−)	2′-CDG	1/0	2	1		
5488 (−)	2′-CDG	1/0	2	1		
5490 (−)	2′-CDG	1/0	1	1		
5492 (−)	2′-CDG	1/0	2	2		
2703 (+)	524W	1/0	1/0	1/0	1/0[d,g]	
2704 (+)	524W	1/0	1/0	1	1[d,g]	
2706 (+)	524W	1	1/0	1/0	1[d,g]	
2707 (+)	524W	1/0	1/0	1/0	1/0[d,g]	

[a] Liver injury was assessed on the basis of the degree of hepatic inflammation, hepatocyte vacuolization, hepatocyte necrosis, variation in hepatocyte nuclear size, biliary hyperplasia, and Kupffer cell activation as follows: 0, no evidence of liver injury; 1/0, scant inflammatory infiltrates, minimal evidence of changes itemized above; 1, mild inflammation and evidence of itemized changes; 2, mild to moderate inflammation with disruption of normal lobular architecture, evidence of hepatocyte necrosis and prominent hepatocyte vacuolization, and distinct variation in hepatocyte nuclear size; 3, moderate to intense inflammation, extensive hepatocyte necrosis, variation in nuclear size, and biliary hyperplasia.

[b] (+), DHBV infected; (−), uninfected.

[c] 2′-CDG regimen at biopsy.

[d] 524W regimen at biopsy.

[e] Duck 2747 was sacrificed at 11 weeks because of the development of significant amyloid deposits in the liver that made continuation in the study impractical. Amyloidosis is a common condition in domestic ducks.

[f] Duck 2749 had developed significant amyloidosis by 37 weeks.

[g] Twelve weeks, twice daily.

novel behavior in primary cultures of duck hepatocytes, in which even a single administration of the drug inhibited viral DNA synthesis for at least a week, even when the cultures were fed daily with drug-free medium (3); moreover, a preliminary study had indicated that the antiviral activity was probably also long lasting in vivo (3). We have shown in the present study that after only 2 weeks of 2′-CDG therapy, ~70% of the hepatocytes no longer expressed detectable levels of virus antigen. Moreover, there was an approximately equivalent (i.e., ~2-fold) drop in the amount of viral CCC DNA in the liver, consistent with the interpretation that half of the hepatocytes were no longer infected and the remainder contained pretreatment copy numbers of CCC DNA. That is, both observations suggested that a substantial fraction of hepatocytes were no longer infected after 2 weeks of drug treatment.

The virologic changes produced by 2′-CDG were found to correlate with a transient elevation of hepatocyte turnover. Equivalent virologic changes in the liver were not produced by 524W over the same time span. 524W was as potent an inhibitor of virus production as 2′-CDG but was nontoxic and failed to accelerate hepatocyte turnover. 524W produced only, on average, a 40% drop in CCC DNA levels after 6 weeks of administration and a 60% drop in CCC DNA levels after 12 weeks. This loss of CCC DNA can probably be attributed to the natural rate of hepatocyte death in the congenitally infected duck.

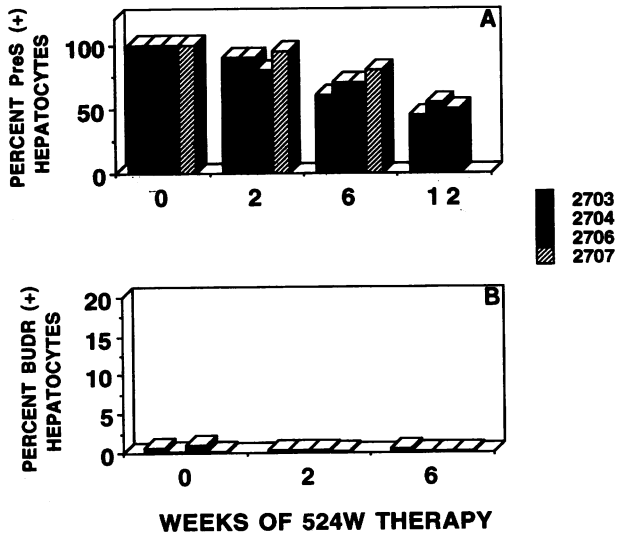

FIG. 7. Effects of 524W therapy on the number of pre-S antigen-positive hepatocytes and on BUdR labeling of nuclear DNA. All four ducks were treated with 50 mg of 524W per kg of body weight twice a day. Detection of pre-S antigen positive and BUdR-positive hepatocytes in sections of fixed liver tissue was carried out as described in Materials and Methods. Detection of viral antigens in the 12-week biopsy sample of duck 2707 was not possible because of a high background signal in the tissue sections from this biopsy sample.

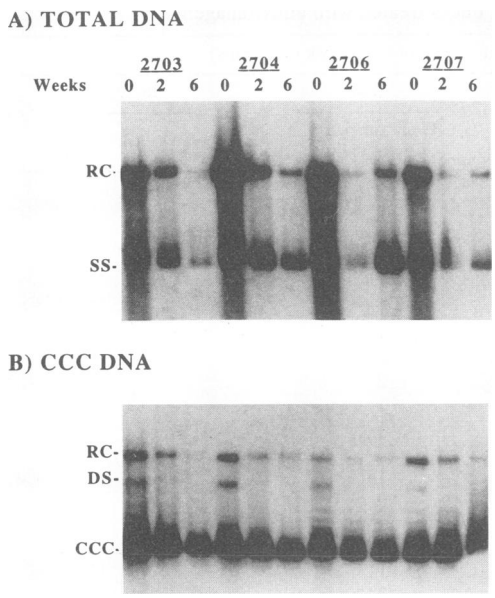

FIG. 8. 524W therapy caused a reduction in the amount of intermediates in DHBV DNA replication and in viral CCC DNA in the livers of congenitally infected ducks. Liver biopsies were performed before therapy (lanes 0) and after 2 (lanes 2) and 6 (lanes 6) weeks of therapy. Total DNA (A) and CCC DNA (B) were subjected to Southern blot analysis as described in Materials and Methods. The positions of migration of relaxed circular (RC), CCC, and single-stranded (SS) DNAs are indicated.

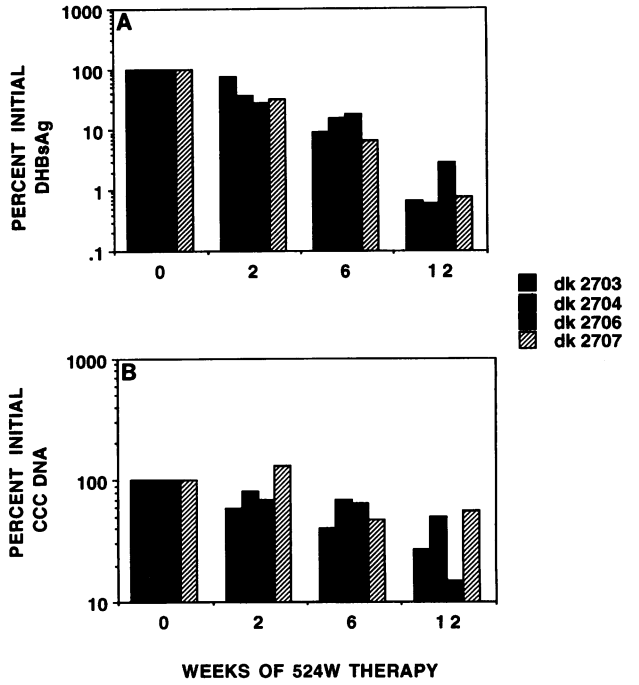

FIG. 9. DHBsAg in the sera of 524W-treated ducks (dk) declined more rapidly than CCC DNA levels in the liver. CCC DNA results were taken from Fig. 8 and data not shown. DHBsAg in the serum was measured as described in Materials and Methods.

An obvious question raised by these experiments is the role that the death of infected hepatocytes might play in freeing the liver of DHBV during antiviral therapy. With 2′-CDG, we observed that the increase in BUdR-labeled hepatocytes at 2 weeks of therapy was primarily in lobular zone 1 and, to a lesser extent, in zone 2. This would be consistent with the hypothesis that 2′-CDG was not only toxic to mature hepatocytes but also inhibited division of those that survived, so that dying hepatocytes would be replaced via progenitor cell proliferation (20). Unfortunately, our histologic evidence is insufficient to establish this hypothesis. In contrast to the results with 2′-CDG, there was no liver toxicity during therapy with 524W, and hepatocyte replacement presumably occurred by the normal process of proliferation of mature and, at the start, infected hepatocytes. If the two drugs really are so different in their effects upon hepatocyte proliferation, it would be predicted that infected hepatocytes surviving initial therapy with 2′-CDG would retain pretreatment copy numbers of CCC DNA, since they could not divide, whereas the average CCC DNA copy number would decline in infected hepatocytes during treatment with 524W, since these hepatocytes could divide. The levels of CCC DNA in the liver and DHBsAg in the serum are consistent with this possibility, in that DHBsAg levels declined much more rapidly than CCC DNA levels in ducks treated with 524W, as might be expected if transcription of the S gene and/or accumulation of the S gene product needed for particle assembly was impaired at a low CCC DNA copy number. It remains to be determined, however, whether all the hepatocytes present after 12 weeks of 524W therapy are infected or if the CCC DNA copy number in infected cells has really remained at pretreatment levels in these ducks.

With respect to the fate of cytoplasmic forms of viral DNA during cell division, Guidotti et al. (7) have recently obtained evidence that immature viral nucleocapsids are rapidly destroyed when hepatocytes divide. This was determined by using a transgenic mouse line expressing the HBV nucleocapsid subunit. In normal mice, the subunits accumulated in the nuclei of hepatocytes, where they assembled into nucleocapsids. When hepatocyte proliferation was induced by partial hepatectomy, the capsids first appeared, postmitotically, in the cytoplasm, as also observed by Yeh et al. (31) in cell cultures, but then disappeared. Since they did not concomitantly appear in the serum, a simple interpretation is that they were destroyed within the cytoplasm of the postmitotic hepatocyte. Nucleocapsids that are intermediates in virus replication assemble in the cytoplasm; therefore, the instability observed in the transgenic mice may represent a phenomenon uniquely associated with cell division, rather than an intrinsic instability of immature virus capsids within the cytoplasm.

In summary, the simplest model to explain our results might be that death of infected hepatocytes, either at the normal rate or at the accelerated rate caused by 2′-CDG therapy, produced a direct loss of precursors of CCC DNA and that CCC DNA was also lost more gradually by dilution, and perhaps by inefficient postmitotic resegregation to the nucleus, as hepatocytes proceeded through the cell cycle during therapy with 524W. The implication of these findings is that both the death of some infected hepatocytes and the proliferation of other infected hepatocytes are important and perhaps essential components of antiviral therapy with an inhibitor of virus replication. Therefore, any event which significantly accelerated hepatocyte turnover, such as an active hepatitis, might contribute substantially to antiviral therapies with nontoxic drugs that act as inhibitors of hepadnavirus replication. Finally, clearance of transient hepadnavirus infections appears to involve the curing of infected hepatocytes or their progeny

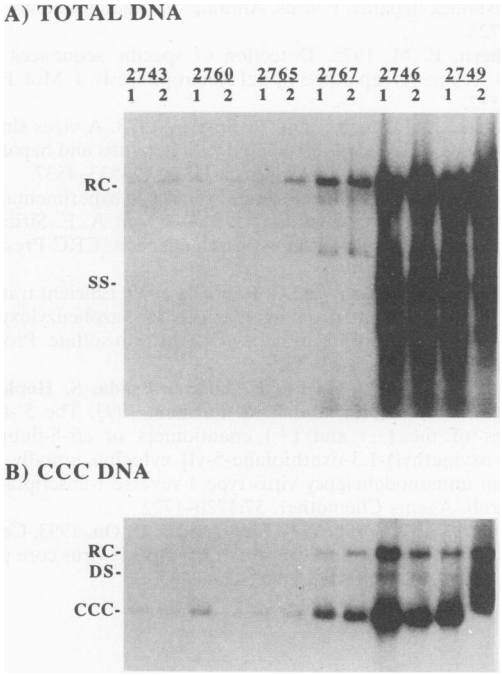

FIG. 10. Inhibition of DHBV replication could be maintained by administration of 2′-CDG as seldom as once a week. Ducks 2743, 2760, 2765, and 2767 were treated with 100 μg of 2′-CDG per kg every other day for 9 weeks, at which time the drug regimen was reduced to twice a week. After 17 weeks on this reduced regimen, liver biopsy samples were taken (lanes 1). The treatment was extended for 5 more weeks, and the drug regimen was again reduced, to only once a week. After 6 weeks on this regimen, the ducks were sacrificed (lanes 2). Ducks 2746 and 2749 were placebo controls. Total DNA (A) and CCC DNA (B) were extracted for Southern blot analysis as described Materials and Methods. The migration positions of DHBV relaxed circular (RC), double-stranded (DS), CCC, and single-stranded (SS) DNAs are indicated.

(12). If cytokines produced by the immune response, like antiviral drugs, inhibit virus replication within hepatocytes (5, 6, 8), proliferation of infected hepatocytes may also be important in virus elimination during recovery from transient infections.

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