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Short Communication

Reversion of duck hepatitis B virus DNA replication in vivo following cessation of treatment with the nucleoside analogue ganciclovir

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Abstract

In order to define, in more detail, the virological events which occur after completion of antiviral chemotherapy, ducks congenitally infected with the duck hepatitis B virus (DHBV) were treated for 4 weeks with the nucleoside analogue ganciclovir and followed up over a 7-day period. Specimens of serum and liver were collected daily during follow-up for virological analysis. Treatment resulted in a substantial reduction in both viraemia and liver DHBV DNA replicative intermediates. However, after cessation of treatment, viraemia returned to detectable levels within 4 days. In the liver, the viral supercoiled DNA (SC DNA) was the form least affected by therapy and returned to near control levels by day 2 post-treatment. The other hepatic replicative intermediates reached pretreatment levels within 4 days of cessation of therapy. This study has defined the kinetics of relapse of viral replication after completion of antiviral therapy in the duck hepatitis B model. Of all viral replicative forms, the SC DNA appears to be the one which is most resistant to nucleoside analogue therapy and is presumably responsible for the relapse phenomenon observed post-treatment.

Keywords: Duck hepatitis B virus; Ganciclovir; Relapse; Viral supercoiled DNA

Intensive research efforts are presently ongoing to develop antiviral drugs for the therapy of chronic hepatitis B virus (HBV) infection. In the last 10 years, interferon-α therapy has become the cornerstone of management for chronic hepatitis B. Unfortu-

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nately, favourable responses are only observed in patients with active liver disease who are HBeAg-positive and have low levels of serum viral DNA (Hoofnagle and Di Bisceglie, 1990). The response rate for interferon-α therapy, even in these highly selected patient groups, is limited and recurrence of disease is common (Lok, 1994).

A major reason for treatment failure in chronic hepatitis B appears to be the persistence of the key replicative intermediate of HBV, viral supercoiled (SC) DNA (Yokosuka et al., 1985). The HBV SC DNA is the major transcriptional template and is the first species generated following initial infection of the hepatocyte. Viral SC DNA is found in the nucleus of infected hepatocytes and the host cell RNA polymerase II transcribes a number of viral RNA transcripts from it. The largest of these, the pregenomic RNA, migrates to the cytoplasm where it is reverse transcribed and replicated within immature viral cores, producing a relaxed circular double-stranded DNA molecule with a single-stranded gap that is found in the mature virions (Summers and Mason, 1982). In persistent infections, not all complete nucleocapsids are enveloped by hepatitis B surface antigen (HBsAg) and exported as infectious virus. Instead, some re-enter the replication cycle via an intracellular conversion pathway and are transported back to the nucleus (Tuttleman et al., 1986). This allows amplification of SC DNA which establishes a pool of viral transcriptional templates in the cell without the need for semi-conservative replication or multiple rounds of re-infection (Tuttleman et al., 1986). The viral SC DNA copy number is maintained at approximately 50 molecules per cell in chronically infected liver (Miller and Robinson, 1984; Tuttleman et al., 1986) and is regulated in a negative-feedback pathway by the envelope proteins (Summers et al., 1990, 1991).

A key factor to be considered in antiviral therapy of chronic HBV is the rate at which the viral SC DNA decays when viral replication is inhibited. Studies in our laboratory, using the duck hepatitis B virus (DHBV) model for chronic hepatitis B (Summers and Mason, 1982), have shown that the half-life of DHBV SC DNA in vitro is approximately 4 days (Civitico and Locarnini, 1994). Accordingly, if viral replication can be fully inhibited long term, the viral SC DNA and infection should, theoretically at least, be eradicated. However, it has been shown in two independent studies using the duck model that hepadnaviral SC DNA is largely unaffected by treatment with purine nucleoside analogues which normally inhibit viral DNA (Wang et al., 1991; Mason et al., 1994). In these studies and in similar studies in man (Yokosuka et al., 1985; Locarnini et al., 1989), as soon as treatment was withdrawn, relapse in viral replication was observed. Ganciclovir, a purine nucleoside analogue, has been shown to block DHBV DNA replication in vitro (Yokota et al., 1990) and has also been effective in controlling severe human hepatitis B virus infection (Angus et al., 1993). Using the duck hepatitis B-ganciclovir model (Wang et al., 1991; Luscombe et al., 1994) we have investigated the relapse phenomenon in more detail by collecting daily specimens of liver and sera for 7 days immediately after treatment was completed.

One-day-old Pekin Aylesbury cross-bred ducks congenitally infected with an Australian strain of DHBV were obtained from a commercial supplier. Sera were tested for DHBV DNA by dot-blot hybridization (Guo and Bowden, 1991) to ensure only birds with a positive viraemia were selected for use. These birds were bled subsequently at 6 weeks of age to confirm their virological status, prior to commencement of treatment.

Fourteen 6-week-old ducks positive for DHBV DNA were treated by the intraperitoneal route (i.p.) with ganciclovir (Syntex, Palo Alto, CA) at a dosage of 10 mg/kg/day for a period of 4 weeks and serial weekly serum bleeds were taken from each duck. An additional 7 ducks were used as untreated controls; these ducks received no drug, but were otherwise housed, bled and handled identically to the ducks receiving ganciclovir therapy. On cessation of treatment all ducks were bled daily and three birds, two treated and one control duck, were culled each day for the next 7 days. Livers from the culled ducks were harvested and stored in TNE (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA) at −70°C for subsequent analysis. On autopsy, duck J (see Fig. 1) was found to have peritonitis and the liver tissue was unsuitable for analysis.

DHBV DNA from serum and liver tissue was analyzed by molecular hybridization using radioactively labelled cloned viral DNA probes as described previously (Wang et al., 1991). Serum was analyzed by dot-blot hybridization and total viral DNA and viral SC DNA were prepared from liver and analyzed by Southern hybridization (Wang et al., 1991). Slot-blot hybridization of total viral DNA was also used to establish the number of viral genome equivalents (VGE) per cell (Jilbert et al., 1988).

Ganciclovir treatment resulted in a substantial decrease of DHBV viraemia during the 4 weeks of therapy (Fig. 1) in all 14 treated ducks. The limit of detection for the dot-blot hybridization assay was 1 pg, equivalent to 3×10^5 viral genomes (Jilbert et al., 1988). During the ganciclovir treatment period there was one positive signal observed (Fig. 1, duck K, week 3 of treatment), but this was most likely due to a sample mix-up rather than a treatment breakthrough. After cessation of treatment, relapse of viraemia could be detected as early as day 3 in two birds and had returned in most ducks by day 4. Viraemia returned to near pretreatment levels by day 5 post-treatment (Fig. 1).

The level of viral DNA in the corresponding liver samples was determined by semi-quantitative slot-blot analysis (Table 1). Previous studies by our laboratory have determined that at the end of 4 weeks ganciclovir therapy, the level of viral DNA per hepatocyte has decreased to between 25 and 50 VGE (Wang et al., 1991; Luscombe et al., 1994). A similar level of inhibition was observed in this study on the first day after treatment had been terminated. There was a slight rise in the level of VGE per hepatocyte by the second day after therapy, which increased to around 75% of the control level on day 3. Thereafter, the levels of total viral DNA were approximately comparable to the control levels.

The various DHBV replicative forms that constitute the total viral DNA from these liver samples were then examined using Southern blot hybridization (Fig. 2). The results from this analysis confirmed the substantial reduction of DHBV DNA observed within the liver in the first few days post-treatment. There was a reduction in all the DHBV replicative intermediates, including the double-stranded relaxed circular (RC), double-stranded linear (L) and single-stranded (SS) species. Consistent with the results of the semi-quantitative slot-blot analysis, the DHBV replicative forms returned to detectable levels on day 3 post-therapy and all species were detected at similar levels to those of the control by day 4. In the total viral DNA profiles, there did not appear to be a disparate relapse of any particular viral replicative form over another, and all forms were observed to return uniformly. Other than the RC, L and SS species detected by Southern analysis, a fourth viral replicative intermediate (RI) migrating between the L and SS

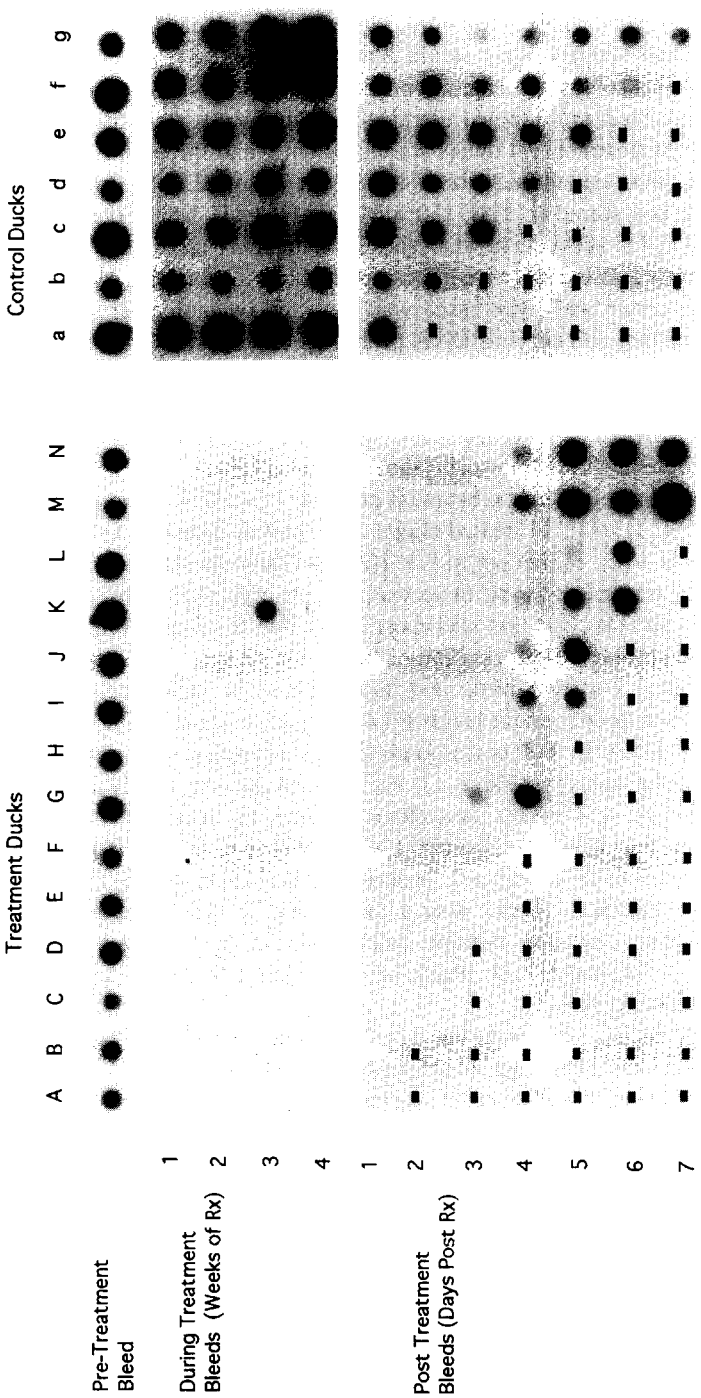

Fig. 1. Serum dot-blot for DHBV DNA before, during and after ganciclovir therapy. A–N represents ducks treated for 4 weeks and a–g untreated control ducks. During treatment, ducks had serial weekly bleeds taken and after treatment daily bleeds were collected up to 7 days. In the post-treatment period, ducks were culled daily (bar) to obtain liver tissue, so subsequent bleeds were not available. Std-cloned DHBV DNA standards at 1000, 100, 10 and 1 pg.

Table 1
Effect of cessation of ganciclovir therapy on the copy number of DHBV DNA within the liver

Follow-up period	Duck [a]	VGE/hepatocyte [b]
Day 1	A	30
	B	56
	a	772
Day 2	C	266
	D	190
	b	799
Day 3	E	626
	F	585
	c	800
Day 4	G	600
	H	568
	d	727
Day 5	I	975
	e	1066
Day 6	K	882
	L	899
	f	890
Day 7	M	754
	N	800
	g	867

[a] Ducks A–N and a–g refer to the treated and control birds, respectively, as shown in Fig. 1.
[b] Viral genome equivalent (VGE) per hepatocyte as estimated by Jilbert et al. (1988) assuming a DHBV genome equivalent to be 3×10^{-6} pg DNA and that each cell contains 5×10^{-6} μg total cellular DNA.

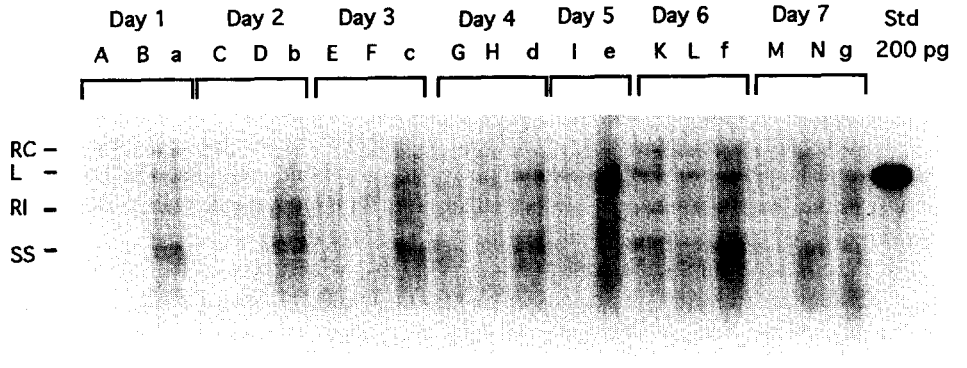

Fig. 2. Southern blot analysis of total liver DNA from ducks following cessation of treatment. The treated (A–N) and control ducks (a–g) are the same as described in Fig. 1. RC, L, RI and SS correspond to relaxed circular, double-stranded linear, replicative intermediate and single-stranded DHBV DNA, respectively. Std-200 pg of cloned DHBV DNA.

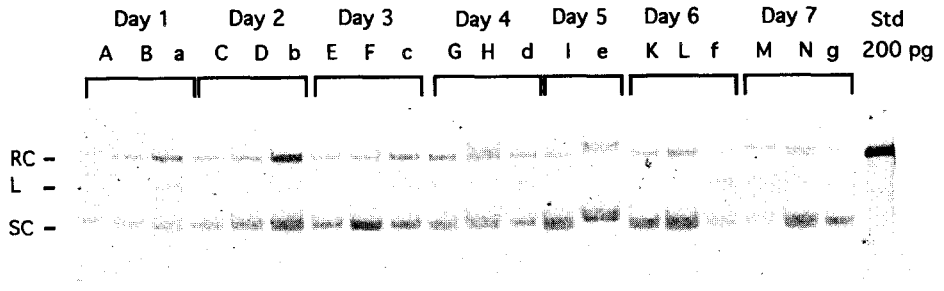

Fig. 3. Southern blot analysis of DHBV SC DNA extracted from liver tissue of ducks following cessation of ganciclovir treatment. Treated and control ducks are as described in previous figure legends. RC, L and SC correspond to relaxed circular, double-stranded linear and supercoiled DHBV DNA. Std-200 pg of cloned DHBV DNA.

forms was observed. From previous studies (Tuttleman et al., 1986), the SS viral DNA is composed of negative strand DNA and the RI species is at least partially double-stranded. This was confirmed here by labelling with strand-specific probes (results not shown).

To detect the DHBV SC DNA in the liver samples (Fig. 3), it was necessary to use an alternative DNA extraction procedure which enriches for non-covalently protein bound DNA (Wu et al., 1990). In contrast to the data obtained on day 1 post-treatment for circulating virus (Fig. 1) and total viral hepatic DNA (Fig. 2), the DHBV SC DNA remained at a detectable, although diminished, level (Fig. 3, ducks A and B). This result is consistent with previous studies that show the apparent resistance of DHBV SC DNA to ganciclovir therapy (Wang et al., 1991). The level of DHBV SC DNA increased on day 2 post-treatment close to the level found in the untreated control and was at a comparable level by day 3 (Fig. 3). The specificity of the band migrating as DHBV SC DNA was established by a mobility shift to L DNA after digestion with *Eco*RI (data not shown).

The mechanism of action of ganciclovir on DHBV replication is believed to be primarily by inhibition of second-strand DNA synthesis (Shaw et al., 1994). This would result in suppression of virion production and inhibition of the intracellular conversion pathway that maintains the level of viral SC DNA in the nucleus. In the present study, short-term treatment with ganciclovir reduced viraemia to below the level of detection and the total hepatic viral DNA was diminished to only trace amounts. The DHBV SC DNA was the least affected viral intermediate and remained at detectable levels even after 4 weeks of treatment.

The failure to eliminate the hepadnaviral SC DNA, which acts as the transcriptional template, appears to be the main reason for the observed relapse of replication. When the kinetics of the relapse phenomenon were examined, relapse was found to occur rapidly, within a few days of treatment cessation. Within the liver, the viral SC DNA was the first viral species to return to control or pretreatment levels, with the other hepatic replicative forms becoming detectable on day 3 and reaching control levels by day 4. The relapse of these other replicative forms was relatively uniform, as detected by

Southern hybridization, with no particular viral intermediate dominating during the relapse period. Comparable relapse kinetics were observed in the peripheral blood compartment with the return of detectable viraemia being observed in two ducks by day 3 and eventually relapsing in the remaining birds by day 4.

The amount of viral SC DNA is regulated by a feedback inhibition pathway regulated by the pre-S/S protein (Summers et al., 1990, 1991). Our results are consistent with this model. We have previously shown that at the end of ganciclovir therapy the hepatic levels of the viral envelope proteins are reduced (Luscombe et al., 1994), hence upon cessation of therapy any viral genomic DNA will be preferentially transported to the nucleus to produce SC DNA. As the level of pre-S/S returns to normal, cytoplasmic nucleocapsids containing the genomic DNA will be exported causing a reversion of viraemia.

The data presented in this report has shown that ganciclovir is capable of suppressing DHBV DNA replication, but has failed to eliminate the SC DNA species. This study has clearly documented how quickly relapse occurs and defined the kinetics of the relapse phenomenon. To be successful, future therapies for chronic hepatitis B will need to not only control overall viral DNA replication (Shaw et al., 1994) and promote immune clearance of virus-infected hepatocytes (Hoofnagle and Di Bisceglie, 1990), but also destabilize and eventually eliminate hepadnaviral SC DNA as well.

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