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The use of ampligen alone and in combination with ganciclovir and coumermycin A1 for the treatment of ducks congenitally-infected with duck hepatitis B virus

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Summary

Ampligen, a known immunomodulator and interferon inducer, was used alone and in combination with other antiviral agents to treat ducks congenitally-infected with duck hepatitis B virus. These antiviral agents included the conventional nucleoside analogue ganciclovir and the prokaryotic DNA gyrase B inhibitor coumermycin A1. When used alone, ampligen decreased the amount of serum and liver viral DNA, but had no effect on circulating duck hepatitis B surface antigen (DHBsAg). In combination with ganciclovir, the antiviral effect appeared at least additive with a greater inhibition of viral DNA replication within the liver. The combination of ampligen with coumermycin A1 also resulted in inhibition of viral replication but to a lesser extent than ampligen alone. When all three agents were used together, viral DNA replication was again inhibited, but as with previous treatment regimes, serum DHBsAg levels remained unchanged. At the end of the treatment period for all regimes, analysis of viral DNA forms in the liver showed that the viral relaxed circular and supercoiled DNA forms had persisted. Within 1 week of cessation of therapy, viral replication had often

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returned to pre-treatment levels. Interferon-like activity was detected in the sera of the majority of the treated ducks during the ampligen therapy, but no clear relationship between the presence of interferon and antiviral effect could be established. These observations in the duck hepatitis B model may provide a rational basis for the use of combinations of antiviral and immunomodulatory regimes for the management of chronic hepatitis B infection in man.

Ampligen; Ganciclovir; Coumermycin A1; Duck hepatitis B virus; Combination antiviral chemotherapy

Introduction

Hepatitis B virus (HBV) infection is recognized as a major global public health problem with over 250 million chronic carriers (Szmuness, 1978). Chronic infection with HBV may result in the development of chronic active hepatitis, cirrhosis and primary hepatocellular carcinoma (Beasley et al., 1981). At present, there is little effective therapy for controlling active viral replication. Therapeutic trials with antiviral agents such as α-interferon (Alexander et al., 1981), adenine arabinoside (Hoofnagle et al., 1985), acyclovir (Weller et al., 1983), ganciclovir (Locarnini et al., 1989) and zidovudine (Farraye et al., 1989) as well as immunomodulatory regimes such as interferon α (Perillo et al., 1985) administered alone (Korenman et al., 1991) or in combination (Schalm et al., 1985; Perillo et al., 1988) have now been evaluated. To date, the clinical and virological outcome of these trials has been disappointing, with few benefits and in some cases, significant toxicity.

Duck hepatitis B virus is a model for human HBV infection (Zuckerman, 1987) and has provided some useful insights into the mechanism of HBV DNA replication (Summers and Mason, 1982). Several antiviral agents have been evaluated as potential inhibitors of viral replication in vivo (Sherker et al., 1986; Hirota et al., 1987; Haritani et al., 1989; Wang et al., 1991). These studies demonstrated that all intermediates of DHBV DNA replication, such as relaxed circular (RC), double-stranded linear (L), and single-stranded (SS) DNA were sensitive to conventional antiviral therapy but the supercoiled (SC) DNA form was resistant. Together with studies in humans (Yokosuka et al., 1985) these results indicate that persistence of SC DNA may be responsible for the viral relapse frequently observed after completion of therapy. We have recently shown that DNA gyrase inhibitors (e.g., nalidixic acid and coumermycin A1) and topoisomerase II inhibitors (amsacrine and ellipticine), inhibit DHBV SC DNA generation and processing in vitro (Civitico et al., 1990), and have also demonstrated that the DNA gyrase inhibitor nalidixic acid could be used in the treatment of congenital duck hepatitis B virus infection in vivo (Locarnini et al., 1991).

It is likely that successful treatment of chronic hepatitis B infection will

require combinations of antiviral and immunomodulatory agents (Thomas et al., 1986). Double-stranded RNAs (dsRNAs) are biological response modifiers having multiple activities including the induction of various lymphokines, such as α-interferon (Marcus and Sekellick, 1977). However, dsRNAs can cause toxic side-effects including renal failure, coagulopathies and circulatory collapse (Levy and Riley, 1984). Ampligen^{R} (HEM Pharmaceuticals Corporation, Rockville, MD) is a dsRNA, comprised of polyriboinosinic/polycytidylic (12:1) uridylic acid [poly $(I)_n$:poly $(C12\text{-}U)_n$], which induces interferon (Carter et al., 1972) without causing adverse reactions (Carter et al., 1976).

In an attempt to inhibit all viral DNA replicative species found in infected hepatocytes as well as stimulating endogenous immune responses to the virus we evaluated the effect of ampligen alone, ampligen in combination with the nucleoside analogue ganciclovir, the DNA gyrase inhibitor coumermycin A1, and all three agents together, using ducks congenitally-infected with DHBV.

Materials and Methods

Ducks

One-day-old Pekin-Aylesbury cross-bred ducks congenitally-infected with an Australian strain of DHBV (Frieman and Cossart, 1986) were obtained from a commercial supplier. At 1 week and again at 4 weeks of age, serum from the animals was tested and DHBV DNA concentrations measured by dot blot hybridization (Mason et al., 1983). Thirteen 5-week-old ducklings with stable and intermediate levels ($1\text{–}5 \times 10^8$ viral genome equivalents per ml of serum) of circulating viral DNA were chosen for the study. This level of viraemia permits convenient detection of DHBV DNA by dot blot hybridization.

Treatment regime

Of the thirteen ducks, ten were selected for the treatment study (ducks A to H and placebo 1 and 2) and the remaining three were included as age-matched untreated controls (ducks I, J and K, see Tables 1 and 2). The age-matched untreated control ducks were sacrificed and their livers removed at 5 weeks (duck I), 9 weeks (J) and 13 weeks (K) of age and processed as described below. Of the ten ducks in the treatment study, eight birds received ampligen whilst two birds served as placebo controls. The eight ampligen-treated carrier birds were assigned to four groups: ampligen alone (two birds, A and B), ampligen and ganciclovir (C and D), ampligen and coumermcyin A1 (E and F), ampligen with ganciclovir and coumermycin A1 (G and H). All eight ducks were treated with a 4 week course of intraperitoneal (IP) injections of Ampligen^{R} (HEM Pharmaceutical Company, Rockville, Maryland, USA; Lot 004–022588) at a dose of 7.5 mg/kg once a day as recommended by the manufacturer. The ampligen was administered as a 2.5 mg/ml solution prepared by dissolving the powder in sterile distilled water at 65°C. In the combination chemotherapy experiments, the six ducks (C to H) were initially treated with a 2 week course

of ampligen alone after which time this treatment continued for another 2 weeks in the presence of either ganciclovir (Syntex, Australia; lOmg/kg/day IP in two divided doses) (see Wang et al, 1991) or coumermycin Al (Sigma, USA; 10 mg/kg/day IP in two divided doses) or ganciclovir plus coumermycin A1, both at 10 mg/kg/day IP in two divided doses. Both compounds were prepared in sterile distilled water. All injections were administered individually in separate syringes. For each group, at the completion of treatment one of the ducks was sacrificed (ducks A, C, E and G) and the liver removed and processed as described below. The remaining ducks were observed for a further 4 weeks (ducks B, D, F and H) after which they were also sacrificed, the liver removed and processed as detailed below. Two placebo-treated positive control ducks received twice daily injections of sterile water for 4 weeks, then observed for a further 4 weeks, processed in a similar fashion to the treated animals. Weekly serum specimens were collected from all ducks throughout the study period. Blood samples were tested for markers of liver, renal and haematological function as well as for markers of DHBV replication (Wang et al., 1991). The ducks were weighed before, at the end, and 4 weeks after treatment. For autopsy, ducks were anaesthetised with sodium pentobarbitone and a large wedge of liver resected from both the right and left lobes. Liver specimens were then diced into small pieces and immediately frozen in TNE (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA) and stored at $-70°C$.

Detection of DHBV markers of replication

(a) Serum markers. To detect DHBV DNA in serum, 10 μl samples of sera were denatured with an equal volume of 1 M NaOH-2 M NaCl for 10 min at room temperature and applied to a nitrocellulose membrane (Hybond C extra, Amersham International, England) using a dot blot or slot manifold (Bio-Rad, Richmond, California). The immobilized DNA was then neutralized by the addition of 20 μl of 0.5 M Tris-HCl (pH 7.4)-2.5 M NaCl, the membrane removed from the apparatus and baked for 2 h at 80°C before prehybridization.

The amount of duck hepatitis B surface antigen (DHBsAg) in serum specimens collected before treatment, at the end of treatment and 4 weeks later was determined by solid-phase radioimmunoassay (SPRIA) as described by Qiao et al. (1990). The positive/negative (P:N) ratio for DHBsAg was calculated from known laboratory reference standard specimens.

(b) Liver markers. To detect DHBV DNA in liver two procedures were used: total DNA extraction and supercoiled DNA selection.

(i) Total DNA extraction. DNA was extracted from liver tissue and analyzed by dot-blot hybridization as previously described (Wang et al., 1991). Briefly, homogenised liver tissue was digested with 0.5 mg/ml pronase in the presence of 1% SDS at 37°C and then deproteinized by two phenol/chloroform (1:1) extractions. DNA was precipitated with ethanol, washed and the pellet

dissolved in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and then treated with RNase A (Boehringer–Mannheim, Germany) for 1 h at 37°C. The material was again extracted with phenol/chloroform, precipitated with ethanol and washed exhaustively before dissolving in nuclease-free water. Standardized amounts of cellular DNA (1 μg) was analysed by dot blot hybridization with each sample tested directly and in doubling dilutions for comparison of DHBV DNA levels. The cellular DNA was quantitated for each sample spectrophotometrically and also by ethidium bromide staining of samples after agarose gel electrophoresis. For Southern blot analysis, 10 μg of total cellular DNA was subjected to gel electrophoresis in 1% agarose, denatured and neutralized using standard procedures (Sambrook et al., 1989) and transferred by vacuum (Vacu-Aid, Hybaid, England) to a positively charged nylon membrane (Boehringer–Mannheim, Germany).

(ii) Supercoiled viral DNA extraction. To specifically enrich for the supercoiled (SC) form of the viral DNA, a modification of the method of Wu et al. (1990) was used as has been previously described (Wang et al., 1991). Homogenised liver tissue in 10 mM Tris-HCl (pH 7.6), 10 mM EDTA was treated with SDS and KCl (final concentration 0.5% and 0.5 M, respectively) followed by centrifugation to remove protein-bound DNA. The clarified supernatant material, containing the SC DNA, was extracted twice with an equal volume of phenol saturated with 50 mM Tris-HCl (pH 7.6) and once with an equal volume of Tris-saturated phenol/chloroform. DNA was precipitated with ethanol, washed twice and redissolved in TE. Southern hybridization was performed as described above.

Preparation of probe

A full length clone of the Australian strain of DHBV (Bowden and Dixon, unpublished results) ligated into the plasmid pT3T7 (Pharmacia, Sweden) was used for all molecular hybridization studies. The recombinant plasmid was propagated in *E. coli* and extracted using standard techniques (Sambrook et al., 1989). The cloned DHBV DNA was removed from the plasmid by digestion with *Eco*RI, separated by preparative gel electrophoresis and purified using a Prep-A-Gene DNA purification kit (Bio-Rad) by following the instructions of the manufacturer. DHBV DNA was labelled with [α-^{32}P]dCTP using an NEN Random Primer Plus Extension Kit (NEN Research Products, DuPont, Wilmington, DE) to a specific activity of 1×10^9 dpm/μg.

Hybridization conditions

Prehybridization of membranes was carried out at 42°C for at least 3 h in a hybridization oven (Hybaid, England). For nitrocellulose membranes, prehybridization solution was as previously described (Wang et al., 1991). For nylon membranes, the prehybridization solution consisted of 50% deionized formamide, 1% SDS, 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.15 M sodium citrate, pH 7.0) and 100 μg/ml denatured herring sperm DNA. The

heat-denatured radiolabelled probe was added to a concentration of at least 2×10^6 cpm/ml and hybridization proceeded overnight at 42°C. After hybridization, membranes were washed twice in 2 × SSC-0.1% SDS for 5 min at room temperature and twice in 0.1 × SSC-0.1% SDS for 30 min at 50°C. Membranes were dried and radiolabelled DNA was detected by exposure to Fuji medical X-ray film at −70°C between intensifying screens.

Assay of duck serum interferon:

(a) Cytopathic effect protection assay for duck interferon. Primary duck embryo fibroblasts (DEFs) were prepared from 10-day-old domestic duck embryos originating from a flock negative for DHBV (Dixon et al., 1990). Cells suspended in L.15 culture medium containing 10% foetal bovine serum (Bishop et al., 1990) were seeded into 96 well microculture plates at a rate of 5×10^5 per well and plates were incubated at 37°C in 5% $C0_2$. After 48 h or when the monolayers were confluent the growth medium was replaced with maintenance medium (1% foetal bovine serum) containing duplicate serial 2-fold dilutions (beginning at 1:5) of sera from the control and experimental ducks. Control duck interferon reference standard (1440 U per ml) was derived from poly I:poly C (Pharmacia Fine Chemicals, Sweden) -treated duck lymphocyte cultures in vitro using standard methods (Carter et al., 1976). Duck lymphocytes were prepared from heparinized blood samples with 4 ml of blood being overlayed onto 3 ml of Ficoll-Paque (Pharmacia, Sweden) gradient and then centrifuged at $400 \times g$ for 30 min at room temperature. The mononuclear cell layer (the middle cloudy layer) was collected, washed in serum-free Eagles Minimal Essential Medium (MEM), counted and seeded into Greiner plates in RPMI-1640 containing 10% fetal bovine serum (Bishop et al., 1990) and then stimulated to produce interferon α using poly 1:C (Carter et al., 1976).

Duck interferon activity was assayed in the DEF cultures. Samples including positive and negative controls in MEM were added to the DEF cultures and after 24 h the medium was removed and replaced with fresh maintenance medium containing Semliki Forest virus at a dilution of approx. 2.0×10^8 PFU/ml, a concentration which caused lysis of unprotected DEF monolayers within 48 h. The plates were reincubated for up to 48 h then scored visually for cytopathic effect (CPE). Medium was then removed, the cells washed and stained in a 0.05% amido black, 90% acetic acid, O.1 M sodium acetate solution. After fixing in 10% formaldehyde, 9% acetic acid, O.1 M sodium acetate the cells were dried, the dye extracted with 5O mM sodium hydroxide and the optical density of the dye measured by an ELISA plate spectrophotometer set at 630 nm. Interferon activity was expressed as units per litre (U/L) which was determined by calculating the 50% optical density end-point between the uninfected (lOO% viability) and infected (0% viability) controls. Interferon values were calculated by comparison with the reference duck interferon preparation described above. One unit of interferon was defined as

that amount which protected 50% of the indicator cells from viral cytopathology.

(b) Characterisation of interferon-like activity. Sera showing high levels of interferon-like activity from a range of ampligen-treated ducks were also analysed for thermal and pH stability as well as trypsin sensitivity (Stewart, 1981). Sera diluted 1:5 were subjected to the following treatments: heated to either 56°C or 80°C for 1.5 h; dialysed at 4°C against a 0.1 M HCl-glycine (pH 2) or 0.1 M NaOH (pH 12) buffer for 24 h before a further dialysis against phosphate-buffered saline (pH 7.5) for another 24 h; or incubated at 37°C for 2 h with trypsin (2.5 mg per ml). The treated samples were then added as duplicate 2-fold serial dilutions to DEF monolayer cultures and re-assayed for interferon-like activity as detailed above.

Results

The ten ducks [A-H, placebo 1 and 2] survived the treatment protocol without major ill effects. Treated birds showed no change in weight, and stable liver renal and haematological function as compared with placebo-treated or untreated age-matched positive controls (results not shown). Intraperitoneal injection of coumermycin A1 in all the treatment protocols was associated with accumulation of 5–10 ml ascites fluid during the 2 week treatment period which was resorbed on cessation of IP therapy (data not shown).

Effects of ampligen alone and in combination on ducks congenitally-infected with DHBV

(i) Serum DHBV DNA. The serum DHBV DNA levels observed in the two placebo-treated, congenitally-infected ducks over the study period (week 5 of life to week 13) were stable with only minor fluctuations (Fig. 1a), a characteristic feature of the duck hepatitis B model (Wang et al., 1991). In contrast, all eight ducks treated with ampligen alone for the first 2 weeks of treatment demonstrated a decrease in serum DHBV DNA levels (Fig. 1b: lanes 2 and 3, ducks A-H). In duck A this decrease continued with ampligen treatment alone for the subsequent 2 weeks (A, lanes 4 and 5), however, duck B demonstrated a fluctuating response (B, lanes 4 and 5). In the follow-up period (B, lanes 6–9) the serum viral DNA levels returned to that of pre-treatment. When combined with ganciclovir (C and D, lanes 4 and 5) the serum DHBV DNA levels also decreased; however, on withdrawal of treatment the viral DNA rebounded to above pre-treatment levels (D, lanes 6–9).

When ampligen was combined with coumermycin A1, inhibition of serum DHBV DNA levels was again maintained (E,F; lanes 4 and 5). Upon cessation of therapy, serum viral DNA remained at a level below that of the pre-treatment (F; lanes 6–9). When ducks were treated with all three agents, DHBV

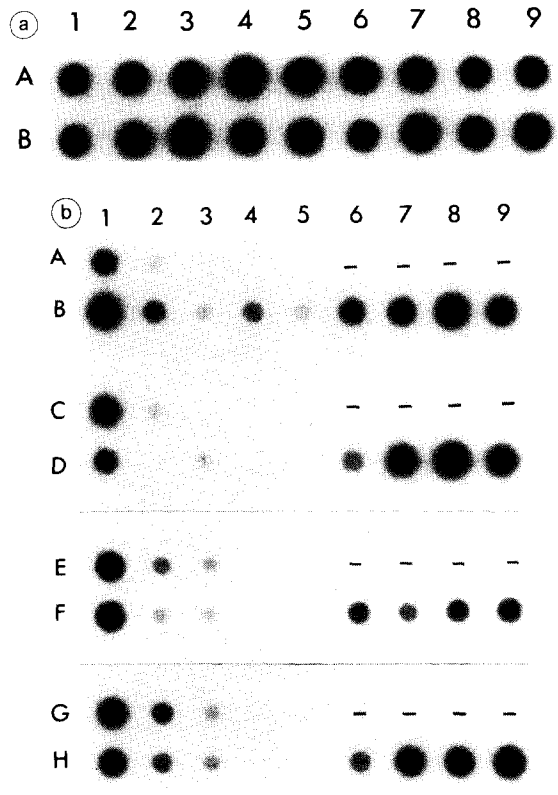

Fig. 1. (a) Serum DHBV DNA dot-blot hybridization from the two placebo-treated ducks (A and B). Lane 1 refers to the pre-treatment levels (week 5 of age), lanes 2–5 during placebo-treatment (weeks 6–9 of age) and lanes 6–9 the weekly follow-up serum samples collected after cessation of treatment. (b) Serum DHBV DNA dot-blot hybridization from the eight ducks treated with ampligen alone (A,B), combined with ganciclovir (C,D) or coumermycin A1 (E,F) or with all three (G,H). Lane 1 refers to pre-treatment (5 weeks of age) levels, lanes 2–3 during ampligen alone treatment, lanes 4–5 during combined treatments and lanes 6–9 the weekly follow-up serum samples after treatment had finished. Ducks A, C, E and G were sacrificed at the end of treatment and the livers removed whilst the ducks B, D, F and H were sacrificed 4 weeks later. (-) = Not tested.

DNA levels fell to below the limit of detection (G,H; lanes 4 and 5), but after the treatment finished, viral DNA levels gradually returned to that of pre-treatment.

(ii) Serum DHBsAg. As with the level of serum DHBV DNA, the sera of placebo-treated ducks showed little change in the amount of DHBsAg over the study period. This phenomenon was also observed for all ducks treated with the various antiviral regimes (results not shown) confirming previous observations (Wang et al., 1991).

(iii) Liver DHBV DNA. The level of viral DNA in the liver samples was

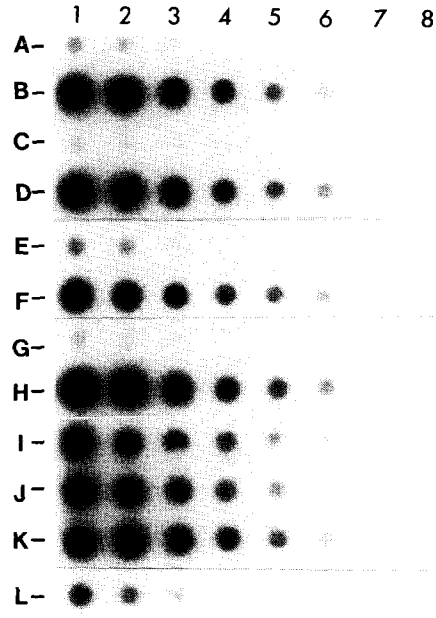

Fig. 2. Semi-quantitative dot blot hybridization analysis of the liver samples from the treated ducks (rows A-H) starting at 1.5 μg of total DNA (lane 1) with doubling dilutions out to 1:128 (lane 8). Row I is the 5 week (pre-treatment), row J is the 9 week (end of treatment) and row K the 13 week (follow-up) liver samples from the age-matched positive controls. The cloned DHBV DNA standards in row L are lOpg (lane 1), 5pg (lane 2), 1pg (lane 3), 0.1pg (lane 4) and O.O5pg (lane 5) pg of DHBV DNA. The sensitivity of the assay was between 0.05pg and 0.10pg of DHBV DNA detected per sample.

determined by dot blot analysis of doubling dilutions (Fig. 2). The amount of DHBV DNA per cell was then estimated by comparison with DHBV DNA standards (Table 1) using the method of Jilbert et al. (1988). For the age-matched positive control ducks (Fig. 2 and Table 1; I, J and K) the amount of intrahepatic DHBV DNA was consistently 360 viral genome equivalents (vge) per hepatocyte. All of the treatment regimes except ampligen and coumermycin A1 caused a greater than 80% reduction in DHBV liver DNA (Fig. 2 and Table 1; A,C,E and G). At the end of the follow-up period, viral DNA in the liver had rebounded in all treatment regimes (Fig. 2 and Table 1; B, D, F and H).

As total liver DNA dot blots do not distinguish the various replicative forms of the virus, the DNA was analysed by Southern blotting (Fig. 3). In the age-matched positive control ducks the relaxed circular (RC), double-stranded linear (L) and single-stranded (SS) forms of DHBV DNA were detected (Fig. 3;,I1, J1 and K1). When digested with *Eco*R1, the RC form was converted to L (Fig. 3; I2, J2 and K2), confirming the identity of these viral forms. At the completion of the therapeutic protocols, only low levels of the RC form were apparent (Fig. 3; A1, C1, E1 and G1). However, at the end of the follow-up period, the RC, L and SS forms all returned to similar levels to that of the controls (Fig. 3; compare B1, D1 and F1 to I1, J1 and K1) with the exception of

TABLE 1

Effect of ampligen therapy alone and in combination on the DHBV copy number in liver samples of treated compared to the age-matched control ducks

Duck*	VGE/ hepatocyte**	Percent reduction*** or increase ( )
A: Ampligen alone (end of treatment)	60	84%
B: Ampligen alone (follow-up)	360	0%
C: Ampligen plus ganciclovir (end of treatment)	<20	95%
D: Ampligen plus ganciclovir (follow-up)	>490	(135%)
E: Ampligen plus coumermycin A1 (end of treatment)	160	56%
F: Ampligen plus coumermycin A1 (follow-up)	410	(115%)
G: Ampligen plus ganciclovir plus coumermycin A1 (end of treatment)	40	89%
H: Ampligen plus ganciclovir plus coumermycin A1 (follow-up)	>490	(135%)
I: 5-week Positive Control (pre-treatment)	360	
J: 9-week Positive Control (end of treatment)	360	
K: 13-week Positive Control (follow-up)	360	

*Ducks A to H refer to the treated animals described in Fig. 1b and 2. Ducks I, J and K were the age-matched controls for pretreatment, end of treatment and follow-up comparisons.

**This data generated from Fig. 2. 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.

***Percent reduction was calculated from the vge value at the end of treatment and/or follow-up as compared to the positive age-matched untreated controls I, J and K.

Percent increase ( ) was calculated from the vge value in the follow-up for ducks D, F and H as compared to the positive age-matched untreated controls I, J and K.

the triple combination which appeared to rebound to a higher level (Fig. 3; H1).

Southern blot analysis was also performed on liver DNA that had been processed to enrich for the viral SC form (Fig. 4). Only treatments that included coumermycin A1 produced a reduction in the level of SC DNA (Fig. 4; E and G) and even here the levels returned at the end of the follow-up period (Fig. 4; F and H). With ampligen monotherapy there was an apparent rebound of DHBV SC DNA in the follow-up sample (Fig. 4; B).

(iv) Serum interferon-like activity. The level of interferon-like activity was measured in samples from all experimental birds (Table 2). Ducks obtained from a flock shown to be negative for DHBV showed similar levels of activity to that of congenitally-infected untreated positive control ducks, demonstrating

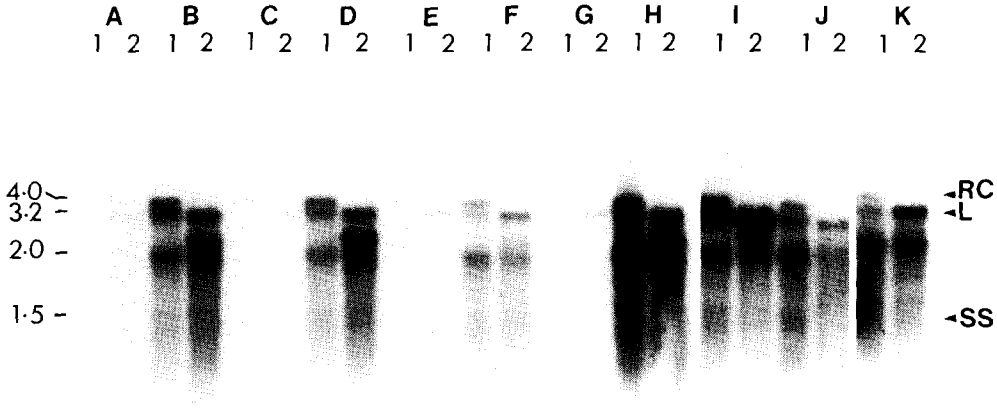

Fig. 3. Southern blot hybridization of the total DNA extracted from the liver tissue of the drug-treated ducks (A-H) and the age-matched positive control ducks at 5 weeks (I), 9 weeks (J) and 13 weeks (K). Each group of samples was run in pairs as uncut DNA (lane 1) and *Eco*RI cut DNA (lane 2). RC = relaxed circular; L = linear; SS = single-stranded DHBV DNA. Molecular weight (in kilobases) as estimated from markers are shown.

that DHBV infection per se does not affect the ability to induce interferon-like activity (results not shown).

During ampligen treatment of the ducks, five of eight had detectable serum levels of interferon-like activity (> 100 U/L) compared to the placebo-treated control animals. However, the time of the peak activity post-treatment was not consistent, occurring after 2 weeks of therapy in some birds (Table 2; A and B) and after 4 weeks in others (Table 2; C, E and F). Considering that all ducks

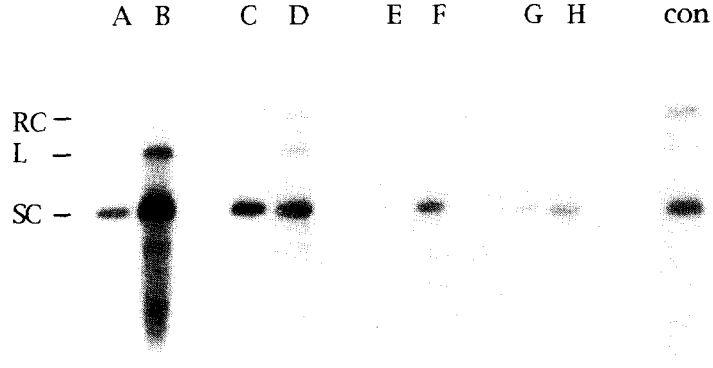

Fig. 4. Southern blot analysis of SC DNA extracted from liver tissue of treated and control ducks. Lanes A and B show ampligen monotherapy end of treatment and follow-up samples, respectively; lanes C and D, ampligen plus ganciclovir end of treatment and follow-up specimens; lanes E and F, ampligen plus coumermycin A1 end of treatment and follow-up samples; lanes G and H, ampligen plus coumermycin A1 with ganciclovir end of treatment and follow-up samples; lane CON is a sample from the 9-week-old positive control duck J. SC = supercoiled DHBV DNA.

TABLE 2

Titres of interferon-like activity (U/L) in sera from ducks treated with ampligen alone and in combinations with ganciclovir and/or coumermycin A1

Duck*	Pretreatment	Treatment				Follow-up
		week 1	week 2	week 3	week 4	
A	28.8	7.2	230.4	28.8	14.4	NT
B	14.4	14.4	230.4	57.6	230.4	14.4
C	14.4	7.2	7.2	7.2	230.4	NT
D	3.6	7.2	3.6	1.8	14.4	1.8
E	14.4	7.2	7.2	14.4	115.2	NT
F	7.2	7.2	7.2	57.6	115.2	57.6
G	14.4	14.4	14.4	7.2	3.6	NT
H	3.6	3.6	14.4	14.4	7.2	14.4
Placebo 1	7.2	14.4	14.4	14.4	28.8	7.2
Placebo 2	14.4	14.4	14.4	14.4	14.4	57.6

*The code corresponds to Table 1 for ducks A-H. Placebo 1 and placebo 2 refer to the two placebo treated birds described in the Materials and Methods section. NT = Not Tested.

(Table 2; A-H) received ampligen for a 2 week period before any other antiviral treatment was introduced, these differences may reflect idiosyncratic reactions to ampligen. Generally, there appeared to be no correlation between inhibition in the level of viraemia and the timing of the magnitude of the interferon-like response detected using this biological assay.

The interferon-like activity detected was completely abolished by heat treatment at 80°C whereas heating at 56°C had no effect. Treatment at either high or low pH reduced the activity by at least 50%. Trypsin digestion also completely abolished the interferon-like activity found in the ampligen-treated duck sera (results not shown).

Discussion

This is the first report describing an antiviral effect on hepadnaviral replication with the biological response modifier ampligen. Ducks treated with ampligen alone and in combination with ganciclovir and/or coumermycin A1 showed a reduction in circulating serum DHBV DNA and viral DNA in the liver. Ampligen monotherapy in all the eight ducks for the initial 2 weeks showed an antiviral effect before the other agents were introduced into the treatment regimes. The study is limited by the small numbers of animals treated, however it is clear that certain trends have emerged from the various treatment programs. Collectively, these results suggest that ampligen induces interferon-like activity in ducks. Little is known about duck interferons and their biological and antiviral effects. We used a cytopathic protection assay to detect interferon-like activity and demonstrated high titres in five of the eight

treated ducks. This activity was abolished by heat- and trypsin-treatment, supporting the suggestion that duck interferon was being produced. However, there was no obvious relationship between the level or time of peak interferon activity and reduction in serum and liver DHBV DNA levels. The reason for this is unclear but could reflect either the inadequacies of the assay system or individual variability in response to ampligen therapy.

Coumermycin A1 has been shown previously to inhibit DHBV SC DNA generation and processing in vitro (Civitico et al., 1990). In combination treatment with ampligen or with ampligen and ganciclovir in ducks, it did not display any additive inhibitory effect on overall circulating viral DNA or hepatic viral DNA markers. By the end of the treatment period, the viral load in terms of vge/hepatocyte was actually less when combined with ampligen compared to ampligen alone (see Table 1: 160 vs. 60). On cessation of treatment, viral replication returned to near the pre-treatment level or in some cases rebounded to greater than pre-treatment levels. All coumermycin A1 treatments were associated with some toxicity because the ducks developed abdominal ascites. The general toxicity of coumermycin A1 in its present form (Grundberg and Bennett, 1965) coupled with its limited availability and high cost would preclude its use generally as an antihepadnaviral agent.

In previous studies in man (Locarnini et al., 1989) and in congenitally-infected ducks (Wang et al., 1991) ganciclovir has been shown to dramatically reduce hepadnaviral replication. The addition of ganciclovir to the ampligen-treatment regime resulted in further reduction in serum and liver DHBV DNA concentrations, with the viral load, expressed in terms of vge/hepatocyte, being reduced in the combination compared to ampligen alone (see Table 1: <20 vs. 60). However, on cessation of treatment these markers rebounded, as was observed with some of the coumermycin A1 treatments. This rebound phenomenon was almost certainly due to the viral supercoiled DNA which acts as the transcriptional template (Tuttleman et al., 1986) and appeared to be relatively unaffected by the different treatments. Only when the treatment included coumermycin A1 was there any apparent effect on the SC DNA and this may have been due to associated toxicity. The inability to eliminate the transcriptional template of hepadnaviral replication may also partly explain the failure to reduce the DHBV surface antigenemia. The relatively long half-life of hepadnaviral surface antigen of 8 days (Frosner et al, 1982) would also be a factor in the maintenance of the circulating antigenemia.

Many carriers of HBV have evidence of a defective interferon system, with both production of and response to interferon blunted (Ikeda et al., 1986; Davis and Hoofnagle, 1986). The use of an interferon inducer such as ampligen might circumvent some of these defects and may overcome some of the problems associated with toxicity of exogenously administered α-interferon treatment. How ampligen exerts an antiviral activity against hepadnaviruses is unclear. Ampligen has been shown to induce interferons (Carter et al., 1972) and either directly or indirectly activate a number of interferon-induced enzymes (Montefiori and Mitchell, 1987). Using HBV transfected cell lines,

Hayashi and Koike (1989) found that interferon had a blocking effect on virion assembly by inhibiting the RNA-primed assembly of core particles. Furthermore, α-interferon has been shown to inhibit transcription of HBV RNA, probably mediated through an indirect effect on the HBV enhancer (Tur-Kaspa et al., 1990).

Ampligen has been evaluated either alone or in combination with zidovudine or dideoxyinosine against human immunodeficiency virus in vitro (Montefiori and Mitchell, 1987; Mitchell et al., 1987; Montefiori et al., 1989; O'Marro et al., 1992) and in vivo (Carter et al., 1987). Of the combination therapies, a synergistic interaction was demonstrated for ampligen and zidovudine but only an additive interaction for ampligen and dideoxyinosine. A synergistic combination chemotherapy has several clear advantages for the management of chronic hepatitis B. The HBV is not directly cytopathic to hepatocytes and the mechanism by which they are destroyed is believed to be immunologically-mediated (Thomas et al., 1986). Ganciclovir enhanced the antiviral effect achieved by ampligen in DHBV congenitally-infected ducks and longer term treatments with this and other ampligen combinations appear warranted. Future studies should be directed at defining the half-life of hepadnaviral SC DNA and determining the mechanism of persistence of serum DHBsAg. Such results may provide a rational basis for the use of combinations of antiviral and immunomodulatory regimes for the management of chronic hepatitis B infection in man.

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