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Short Communication

Inhibition of hepatitis delta virus RNA replication in primary woodchuck hepatocytes

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Summary

Cell cultures of primary woodchuck hepatocytes can be infected with hepatitis delta virus (HDV) as demonstrated by the appearance of genomic HDV RNA 7 days after inoculation. This tissue culture system was used to study the effect of antiviral substances. Ribavirin inhibited HDV replication at a concentration of 10 μg/ml, if added up to three days post infection. Suramin had an inhibitory effect only when added simultaneously with the virus, at a concentration of 200 μg/ml. This concentration had no toxic effect on primary woodchuck hepatocytes. α-Amanitin showed a weak inhibitory effect only at the highest nontoxic concentration of 0.1 μg/ml. Acyclovir had no effect.

Hepatitis delta virus (HDV); Replication; Primary hepatocyte culture; Ribavirin; Suramin; Acyclovir; α-Amanitin

Superinfection of chronic Hepatitis B Virus (HBV) carriers with hepatitis delta virus (HDV) results in a severe acute liver disease and even fatal outcome (Smedile et al., 1982; Rizzetto et al., 1983; Ponzetto et al., 1987). HDV is a defective virus which requires the coat of HBV or woodchuck hepatitis virus (WHV) for formation of infectious particles (Ponzetto et al., 1985). The genome of HDV is a single-stranded circular RNA of 1,7 kb and has properties similar to viroids and related satellite RNAs of plants (Kos et al., 1986; Wang et al., 1986; Wu and Lai, 1989; Taylor et al., 1987). The high degree of intramolecular base-pairing due to

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homologous sequences results in a rodlike secondary structure of the HDV genome. A recent report on an UV-sensitive site within the HDV RNA (Branch et al., 1989), which has previously been found in potato spindle tuber viroid RNA, suggests a secondary structure similar to viroids. Autocleavage of HDV RNA in the presence of Mg^{2+} and subsequent re-ligation similar to viroids has been observed (Sharmeen et al., 1988; Wu and Lai, 1989). HDV RNA is thought to replicate by a rolling circle mechanism since multimeric forms of genomic and antigenomic strands have been detected in HDV-infected liver tissue (Chen et al., 1986).

Inhibition of HDV replication in chronic HDV infection has been attempted. Up to now only clinical studies with α-interferon (INF-α) have been reported (Thomas et al., 1987; Rosina et al., 1987; Hoofnagle et al., 1987). Preliminary results indicate that there is some inhibition of HDV replication, but no eradication of HDV infection. Hoofnagle et al. (1987), for example, found in two patients a return of HDV antigen (HDAg) in liver to pretreatment levels after a 12-month course of INF-α. Foscarnet, an inhibitor of viral DNA polymerases which is also effective on HBV, has been used for the treatment of three patients with fulminant hepatitis due to a co-infection with HDV and HBV (Hedin et al., 1987). Some positive effect was reported. A controlled study with a larger number of patients is needed.

Recently, primary hepatocyte cultures have been established (Taylor et al., 1987; Choi et al., 1988) which can be employed to study inhibitors of HDV replication in vitro. In the present study, we selected four different antiviral substances: ribavirin, a synthetic nucleoside analogue with broad-spectrum virustatic properties against RNA and DNA viruses (Sidwell et al., 1972; Witkowski et al., 1972; Sidwell et al., 1979). Ribavirin has been reported to alleviate the symptoms of the acute form of hepatitis B (Galvao et al., 1975, 1977; Huggins et al., 1977; Hess et al., 1981), but is not effective against chronic hepatitis B (Ellenhorn et al., 1978).

Suramin was chosen because of its inhibitory effect on different retroviruses, including HIV (De Clercq, 1979; Mitsuya et al., 1984; Petcu et al., 1988). The broad-spectrum enzyme-inhibiting properties of suramin (De Clercq, 1987) seemed to be a good rationale for trying the compound against HDV.

α-Amanitin is an inhibitor of the replication of plant viroids, infectious single stranded circular RNA molecules which depend on plant cell DNA dependent RNA polymerases for their replication (Mühlbach et al., 1979). α-Amanitin was tested mainly because the RNA structure of these viroids is similar to that of HDV RNA (Branch et al., 1989; Wu et al., 1989). Acyclovir, a guanosine derivative, was chosen because in vivo inhibition of HBV replication has been demonstrated in patients with chronic hepatitis B (Weller et al., 1983; Alexander et al., 1986). In addition, inhibition of duck hepatitis B virus polymerase and WHV polymerase has been demonstrated with the triphosphate of acyclovir (Hantz et al., 1984; Fourel et al., 1987).

The woodchucks used for liver perfusion (MW 523, MW 564, MW 566) were born in our colony and had no markers of previous WHV or HDV infection. Hepatocytes were obtained by a modified liver perfusion method (Choi et al., 1988)

originally described by Berry and Friend (1968) and Seglen (1976). The liver was perfused in situ through the portal vein and vena cava inferior with 1 mg/ml of collagenase type I (Seromed, Berlin, F.R.G.) at 37°C in a recirculation system using a modified reflux condenser with a pressure of 200 mm H_2O. All solutions were saturated with CO_2/O_2 (5%/95%). After the perfusion, about 5×10^5 hepatocytes were seeded per 25 cm^2 culture flask (Nunc, Denmark) coated with 0.1 mg collagen (SERVA, Heidelberg, F.R.G.) dissolved in 0.1% acetic acid. The medium was Dulbecco's (Gibco) supplemented with 6 mM $NaHCO_3$ (pH 7.4), 1% non-essential amino acids, 5 μg insulin per ml (Sigma), 0.01 mM hydrocortisone (Sigma), 400 IU penicillin, 200 μg of streptomycin per ml, 25 μg of gentamycin per ml, 1.25 μg polymyxin per ml and 10% fetal calf serum (FCS). After attachment of the cells (approx. 2–4 h) the medium was changed. Cultures were kept for 12 h at 37°C with 5% CO_2. Cultures were further incubated at 37°C in closed flasks without CO_2. The medium was changed every day. Hepatocytes were characterized by a typical formation in arrays resembling liver plates. In addition, most of the cells were binucleate.

The hepatocytes were inoculated with acute phase serum of a woodchuck after HDV superinfection. The inoculum for the hepatocytes was the acute phase serum of Woodchuck DW57 after HDV superinfection (6th passage of HDV in Woodchuck) and has been described in detail in a previous publication (Choi et al., 1988).

Prior to infection, flasks of primary woodchuck hepatocytes were washed three times with phosphate-buffered saline (PBS) one day after plating and inoculated with 7 μl infectious serum adjusted to a final volume of 1 ml with serum-free Dulbecco's medium. One hour after adsorption of virus suspension at room temperature, 4 ml of Dulbecco's medium containing 10% FCS were added to the cells.

Antiviral substances were diluted in bidistilled sterile water, stored in aliquots at −20°C and diluted immediately in culture medium before adding them to the cell cultures. The stock solution of ribavirin (Sigma, St. Louis, MO) was 50 mg/ml, of suramin (Germanin, Bayer 205, Bayer, Leverkusen, F.R.G.) 100 mg/ml, of acyclovir (acycloguanosine, Sigma, St. Louis, MO) 10 mg/ml and of α-amanitin (Sigma, St. Louis, MO) 200 μg/ml. Final concentrations in cell culture were 1, 10, 50 and 100 μg/ml for ribavirin, 2, 20 and 200 μg/ml for suramin, 1 and 10 μg/ml for acyclovir and 0.1 μg/ml for α-amanitin. Toxicity of compounds was judged by morphological changes of cells compared to controls or by detachment of cells from culture flasks.

For HDV RNA extraction, cells were lysed by vigorous shaking after addition of 5 ml of NGM solution (4 M guanidine rhodanide, Fluka 50990, 0.5 M mercaptoethanol, 50 mM Na-acetate pH 5.5) to the cultures. The lysate was layered on a CsCl cushion of 1.8 g/cm^3 in TEA buffer (10 mM triethanolamine, pH 7.4, 1 mM ethylenediamine-tetraacetic acid (EDTA)) and centrifuged at 35 000 rpm, 15°C for 16 h. Supernatant (cell lysate and CsCl) was removed carefully, RNA pellets were redissolved in TEA buffer and transferred to new tubes. RNA was extracted with 1 vol chloroform (96% chloroform/4% isoamylalcohol) and precipitated with 2.5 vol of cold abs. ethanol at −20°C.

Redissolved RNA (3 μl) was denatured at 65°C for 20 min in 10 μl of 50%

formamide (deionised), 16% formaldehyde, 10% 1× MOPS (20 mM 3-morpholino-propane-sulfonic acid; 50 mM sodium acetate; 1 mM EDTA, adjusted to pH 7.0 with NaOH), and 24% water. After addition of 2 μl loading-buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol, 0.4% xylenecyanol), the RNA was loaded onto the gel and electrophoresed in a 1% agarose gel in 1 × MOPS, 2.2 M formaldehyde at 120 V for 3 h with constant cross-circulation of the electrophoresis buffer and transferred by capillary blotting to a nylon membrane (Pall, New York, NY) overnight. After heating at 80°C for 2 h, the filter was prehybridized at 50°C overnight in a solution containing 50% formamide, 5 × SSPE buffer (20 × SSPE: 3.6 M NaCl, 0.2 M sodium phosphate pH 7.7, 2 mM EDTA), 5 × Denhardt's solution (Ficoll 400 10 mg/ml, polyvinylpyrrolidone 10 mg/ml, bovine serum albumin 10 mg/ml), 500 μg/ml of tRNA, and hybridized at 58°C overnight in the same solution (50 $\mu l/cm^2$ filter area), containing 5×10^6 cpm ^{32}P-UTP-labelled HDV RNA/ml (specific activity: 2×10^9 cpm/μg RNA. The [^{32}P]UTP HDV RNA (Riboprobe) was prepared as recommended by the manufacturer (Promega Biotec, Madison, Wisconsin). The template for the SP6 reaction was a 650 bp cDNA fragment of the HDV RNA, cloned into pGem-2 at the *Eco*RI site. The cDNA subcloned into pGem-2 originated from a 650 bp cDNA cloned into M 13 (kindly provided by Dr. J. Taylor, Fox Chase Cancer Center, Philadelphia, PA). After hybridisation, the nylon membrane was washed at 68°C in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate,) containing 0.1% sodium dodecyl sulfate (SDS). Molecular weight markers for the RNA gels were 16S and 23S ribosomal RNAs from *E. coli* (chain length for 16S RNA: 1700 nucleotides, for 23S RNA: 3500 nucleotides) (Boehringer Mannheim, F.R.G.). Autoradiography was done at −70°C overnight and again for 4 days.

In our initial experiments, we inoculated the hepatocytes one day after plating with 7 μl of HDV-positive serum. Simultaneously, we added the antiviral compounds to the cell culture medium at different concentrations. In a parallel experiment, we added the antiviral compounds to the tissue culture medium three days after the inoculation of hepatocytes. The tissue culture medium containing antiviral substances was changed every day. Hepatocytes were harvested 14 days after inoculation: total HDV RNA was extracted and tested for progeny HDV RNA by Northern blot and nucleic acid hybridization.

The concentrations of the antiviral drugs, which were not toxic for hepatocytes (no detachment of cells) are given in Table 1.

Ribavirin and suramin inhibited HDV replication in primary hepatocytes. Ribavirin completely inhibited the replication of HDV RNA at a concentration of 10 μg/ml when present immediately after inoculation, as could be shown by Northern blot of extracted RNA (Fig. 1). In repeated experiments, the inhibitory effect of ribavirin was seen beginning at a concentration-range between 5 to 10 μg/ml (data not shown). This inhibition of HDV RNA replication was also observed when ribavirin at a concentration of 10 μg/ml was added up to 3 days after inoculation of hepatocytes (data not shown). Suramin was added either simultaneously with the virus or at day 3 after inoculation and re-added daily for 2 weeks (Fig. 2). HDV RNA synthesis was inhibited by suramin at 200 μg/ml, added at the

TABLE 1

Substances used in vitro against HDV

Substance	Concentration	Inhib.	Toxic concentration[a]	Therapeutic serum concentration[b]
Ribavirin	10 μg/ml	+	>1000 μg/ml	15 μg/ml
Suramin	200 μg/ml	+	>200 μg/ml	100 μg/ml
Acyclovir	10 μg/ml	−	>100 μg/ml	25 μg/ml
α-Amanitin	0.1 μg/ml	−	0.5 μg/ml	-

[a]For ribavirin, suramin and acyclovir: the given concentration is the highest concentration tested which did not lead to morphological changes or detachment of cells.
[b]Values given in the literature for clinical trials.

time of infection. Suramin added 3 days post-infection had no effect. Concentrations of suramin lower than 200 μg/ml did not inhibit HDV RNA replication (Fig. 2). The toxic dose for suramin was not established; however, the concentration of 200 μg/ml was not toxic to hepatocytes. Replication of HDV RNA in the presence of suramin at 200 μg/ml was comparable to that in non-treated control cells, when suramin was added daily from the 3rd till the 14th day after virus inoculation (Fig. 2).

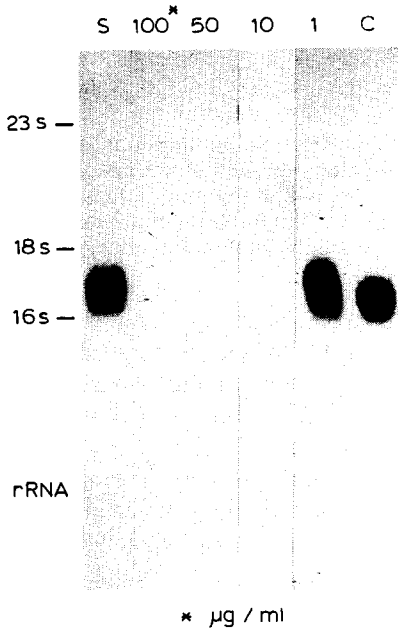

Fig. 1. Northern blot of HDV RNA extracted from ribavirin-treated cells 2 weeks after inoculation. Ribavirin was added simultaneously with HDV. Ribavirin concentrations ranged from 1 to 100 μg/ml. Complete inhibition was present in cells treated with ribavirin at a concentration of 10 μg/ml. S, HDV RNA from serum of an experimentally infected woodchuck; C, HDV RNA from an infected, but not treated, cell culture.

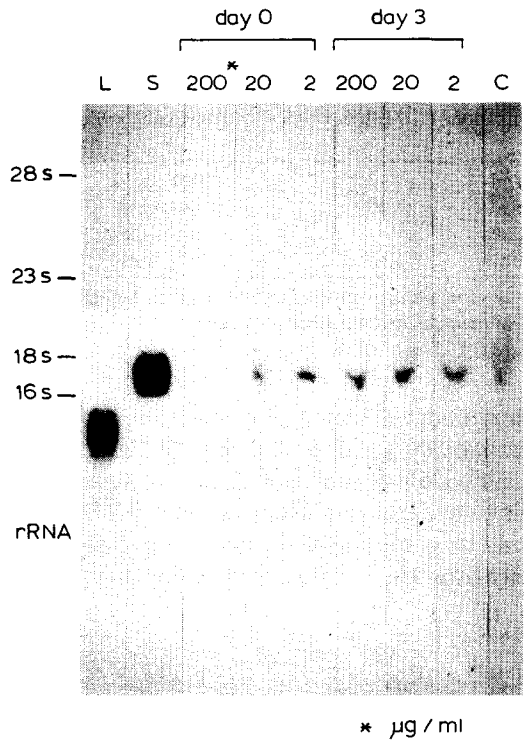

Fig. 2. Northern blot of HDV RNA extracted from suramin-treated cells. Suramin was added either simultaneously with HDV or 3 days after infection. In both cases, 3 different concentrations of suramin, ranging from 2 to 200 µg/ml, were used. L, HDV RNA from liver of an experimentally infected woodchuck; S, HDV RNA from serum of an experimentally infected woodchuck; C, HDV RNA from an infected, but not treated, cell culture.

Acyclovir at a concentration of 1 or 10 µg/ml added simultaneously with the infection to cell cultures and given for 14 days had no inhibitory effect on HDV RNA replication (Fig. 3). Surprisingly, an even higher amount of HDV RNA could be extracted from acyclovir-treated cells than from control cells in repeated experiments. The effect of α-amanitin on HDV RNA replication could only be evaluated at a concentration of 0.1 µg/ml. At a concentration of 0.5 µg/ml α-amanitin was toxic to the cells, resulting in a viability of about 60% after one day of treatment. Under these circumstances, it was not clear whether the weak antiviral effect at 0.1 µg/ml was specific or due to a beginning toxicity for the hepatocytes (Fig. 3).

Ribavirin has been shown to inhibit the replication of a variety of viruses in vivo, e.g. influenza A (Knight et al., 1981), RSV (Hruska et al., 1980) and Lassa fever virus (Stephen et al., 1979). It also inhibits HDV RNA replication in vitro. In our experiments, no HDV replication was observed in primary woodchuck hepatocytes when ribavirin was added to the cells at a concentration of 10 µg/ml. Even when administration began three days after infection, ribavirin effectively inhib-

ited HDV RNA replication. Ribavirin acts by decreasing intracellular GMP pools (Smith et al., 1974) and inhibiting mRNA-guanylyltransferase, which is involved in the 5'-capping of mRNAs (Goswami et al., 1979). Both mechanisms might also be involved in the inhibitory effect of ribavirin on the replication of HDV RNA.

Suramin at a concentration of 200 μg/ml was only active when given simultaneously with the virus. This result suggests that suramin has no inhibitory effect on HDV RNA replication once infection has taken place. It is possible that the major mode of action of suramin is based upon the blocking of adsorption or penetration of HDV. Suramin has been shown to inhibit the adsorption or penetration of viruses (De Clercq, 1987; Petcu et al., 1988), but since it is inhibitory to a wide variety of enzymes, several mechanisms may be responsible for the antiviral effects of suramin (De Clercq, 1987).

Acyclovir had no inhibitory effect on HDV replication. In the literature, inhibition of HBV and WHV polymerase by acyclovir triphosphate has been described (Hantz et al., 1984). The mechanism of inhibition of HBV by acyclovir is

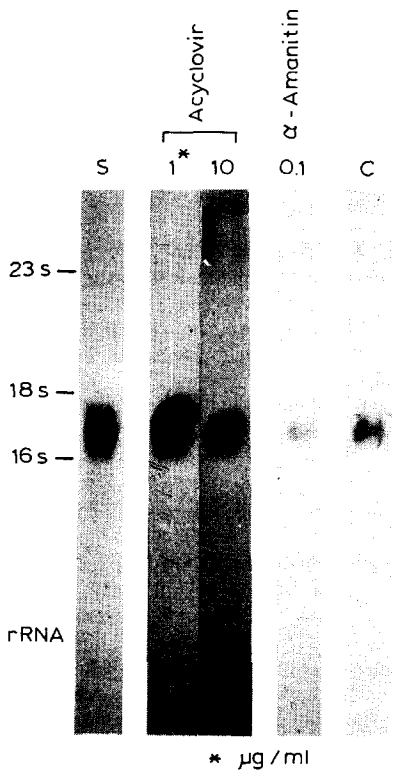

Fig. 3. Northern blot of HDV RNA extracted from acyclovir- and α-amanitin-treated cells. Note the higher yield of HDV RNA from acyclovir-treated cells than from control cells. S, HDV RNA from serum of an experimentally infected woodchuck; C, HDV RNA from an infected, but not treated, cell culture.

not yet clear. Since HBV does not encode for its own thymidine kinase, which is known to phosphorylate acyclovir selectively in the herpes simplex virus system to its active form, the triphosphate, one cannot expect a similar mode of action against HBV replication. Acyclovir has no suppressive effect on HDV RNA replication. In fact, slightly more HDV RNA was found in acyclovir-treated cells than in control cells. We have no explanation for this finding.

It has been shown that α-amanitin selectively inhibits the eucaryotic DNA-dependent RNA polymerases II and III, thereby suppressing the replication of viroids in plant cell cultures (Mühlbach et al., 1979). α-amanitin had a slight inhibitory effect on the replication of HDV RNA at a concentration of 0.1 μg/ml. However, at 0.5 μg/ml it was definitely toxic. Considering the extended application time of the compound, a cytotoxic effect at 0.1 μg/ml could not be excluded, although such an effect was not microscopically obvious.

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