Effect of Ribavirin on the Replication of Infectious Pancreatic Necrosis Virus in Fish Cell Cultures

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SUMMARY

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) at concentrations of 10 μg/ml or more, inhibited the replication of infectious pancreatic necrosis virus (IPNV) in both Chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG-2) cells. The drug was most effective when added just before or within 8 h p.i. Incorporation studies with radioactive precursors demonstrated that ribavirin suppressed cellular DNA and RNA synthesis within 2 to 3 h after addition of the drug. The inhibition of nucleic acid synthesis and the antiviral activity was gradually reversed within 3 to 5 days after removal of the drug from the infected cells. Polyacrylamide slab-gel electrophoresis combined with fluorography revealed that: (i) 0.5 μg/ml actinomycin D sufficiently inhibited host cell RNA synthesis thereby enabling the study of virus-specific RNA synthesis in infected cells and (ii) ribavirin inhibited the synthesis of all three virus RNA forms: the transcription intermediate, virus mRNA and progeny dsRNA.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is the causal agent of a contagious, high mortality disease of young, hatchery-reared salmonids. It can be grown in a variety of fish cell lines at temperatures below 24 °C, resulting in a characteristic cytopathogenic effect (Malsberger & Cerini, 1965). A single cycle of replication occurs within 16 to 22 h at 22 °C and a large proportion of progeny virus remains cell-associated (Kelly & Loh, 1972; Cohen et al. 1973). Electron microscopic observations of purified virus revealed structures of naked icosahedrons which were similar in size and shape to reovirus but lacked the characteristic double capsid of the latter (Cohen et al. 1973). Recent comparison of the biophysical properties of IPNV with those of reovirus indicated that the two viruses are completely different from one another and hence cannot be included in the same taxonomic group (Dobos et al. 1977).

The IPNV genome consists of two segments of dsRNA with mol. wt. of 2.5 × 10^6 and 2.3 × 10^6 (Dobos, 1976; Macdonald & Yamamoto, 1977). The mol. wt. of the structural proteins of the virion fall into three size classes: large (60000), medium (57000) and small (29000 and 27000) (Cohen et al. 1973; Dobos et al. 1977). Similar biophysical and biochemical properties are possessed by a number of unclassified viruses such as infectious bursal disease virus (IBDV) of chickens (Nick et al. 1976; Muller et al. 1979), Tellina virus (TV) of bivalve molluscs (Underwood et al. 1977) and Drosophila X virus (DXV; Teninges et al. 1979).

Attempts to treat and control IPNV epizootics in fish hatcheries using inorganic and
organic iodine compounds have been relatively unsuccessful (Snieszko et al. 1959; Economon, 1963). The only effective control at present is prevention of IPNV contamination. Recently, a new synthetic, non-toxic, broad-spectrum antiviral agent named ribavirin was shown to be active against a number of DNA and RNA viruses both in tissue culture and in laboratory animals (Sidwell et al. 1972; Lionel et al. 1973). The effect of the drug on the replication of dsRNA-containing viruses has not yet been reported. Therefore, it was of interest to determine the degree of antiviral activity of ribavirin against IPNV in fish cell cultures. This investigation hoped to lead to in vivo studies and perhaps practical application of the drug in hatcheries plagued by IPNV.

Ribavirin (l-β-d-ribofuranosyl-l,2,4-triazole-3-carboxamide) is a synthetic N-nucleoside (Poonian et al. 1976) which inhibits cellular nucleic acid synthesis, and also to a lesser degree, cellular protein synthesis (Larsson et al. 1978). It was shown to be non-toxic in test animals and ‘resting’ animal cells in tissue culture at the dosage used to inhibit the replication of a number of different viruses (Lionel et al. 1973). Ribavirin affects the guanoside monophosphate biosynthetic pathway, and its activity can be substantially reversed by guanosine or xanthosine and to a slightly lesser extent by inosine (Streeter et al. 1973). Reversal and enzyme inhibition studies have led to the conclusion that ribavirin exerts its antiviral activity as the 5'-monophosphate by inhibiting guanosine monophosphate synthesis at a step which involves the conversion of inosine monophosphate to xanthosine monophosphate (Streeter et al. 1973). The cytostatic effect of ribavirin is also reversed by removal of the drug from the cells (Larsson et al. 1978).

Virus attachment and penetration is unaffected by ribavirin but intracellular RNA and DNA synthesis (both cellular and viral) are greatly reduced by the drug (Sidwell et al. 1975; Katz et al. 1976).

It is important to distinguish between RNA synthesis of the host cell and RNA synthesis induced by the invading virus genome. To qualify as a true antiviral agent, ribavirin must exert a selective effect on virus-induced RNA synthesis so that virus growth is reduced without a corresponding reduction in the parallel metabolic processes of the host cell. Therefore experiments were designed to determine: (i) effect of ribavirin on rapidly growing trout cells (the drug has been used in vitro only on resting cells); (ii) cell toxicity level of the drug; (iii) effective antiviral dose; (iv) maximum delay period allowed between virus infection and application of the drug before protection of the cells from the virus ceases; (v) reversibility of ribavirin v. its protective effect (i.e., what happens to infected, ribavirin-treated cells after the drug is removed?); (vi) extent of inhibition of cell-specific and virus-specific RNA synthesis.

The results show that ribavirin reversibly inhibited IPNV replication in fish cell cultures, reducing the virus yield by 2 to 4 logs. This inhibition was due to inhibition of virus-specific single (ss) and double-stranded (ds) RNA synthesis in infected, drug-treated cells.

**METHODS**

**Tissue cultures.** Rainbow trout gonad (RTG-2) cells were obtained from the American Type Culture Collection (ATCC). Chinook salmon embryo cells (CHSE-214) were a kind gift from R. Macdonald, University of Calgary. Both cell lines were grown at 22 °C as monolayers in plastic tissue culture vessels (Corning Plastics Inc., New York, N.Y.), using Eagle’s minimum essential medium (MEM) with Earle’s salts, supplemented with 10% foetal calf serum and 50 μg/ml gentamicin.

**Virus.** IPNV (ATCC VR299) was propagated in CHSE-214 monolayers by infecting the cultures at a m.o.i. of 0.01, followed by incubation at 22 °C until extensive c.p.e. was observed, usually 2 to 3 days. After freezing and thawing to release cell-associated virus, the
culture fluid was assayed for infectivity by plaque titration and the virus was dispensed in 1 ml amounts and stored at -70 °C. Plaque titrations under atmospheric conditions were carried out according to the method of Wolf & Quimby (1973). The overlay consisted of Seaplaque agarose (Marine Colloids Inc., Rockland, Me.) and double-strength MEM buffered with 14 mm-N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES). Following incubation at 22 °C for 2 to 3 days, the tissue culture plates were covered with 10% neutral buffered formalin to fix the cells; the agarose was removed and the monolayers stained with 0.1% crystal violet. All titrations were performed in triplicate.

**Evaluation of the antiviral activity of ribavirin against IPNV.** Experiments testing the efficacy of ribavirin against IPNV were carried out in Linbro multi-vessel tissue culture trays (14 wells per tray). Cells were seeded in HEPES-buffered medium and then incubated at 22 °C for 24 h before virus exposure. Confluent monolayers were infected and virus was allowed to adsorb for 1 h. At this time, the inoculum was removed, the monolayers rinsed with medium and MEM (1 ml per well) containing ribavirin was added. The parameters which varied in these experiments were: (i) ribavirin concentration (0 to 80 µg/ml); (ii) time of application of ribavirin (pre- and post-infection); (iii) duration of exposure of cells to ribavirin; (iv) multiplicity of infection; (v) time of ribavirin removal from the cultures; (vi) times at which samples were harvested. Experiments were carried out in duplicate and samples were stored at -70 °C until titrated.

**Effect of ribavirin on cellular nucleic acid synthesis.** Cells were grown in HEPES-buffered MEM on the bottom of glass scintillation vials and were incubated at 22 °C to approx. 80% confluency. At this time the medium was replaced with fresh HEPES-buffered MEM without foetal calf serum, containing 10 µCi/ml of ³H-thymidine (sp. act. 18.5 Ci/mmole). The point at which label was added was designated as time zero. After 4 h of incubation, 10 µg/ml ribavirin were added to half of the vials, designated as test vials, while the rest of the vials remained as controls. At sampling times, incorporation of the isotope was stopped by replacing the medium from one of each control and test vials with 2 ml of ice-cold 10% trichloroacetic acid (TCA). These vials were stored in the cold until the end of the experiment and then all cells were rinsed twice with cold 10% TCA, twice with 95% ethanol and air dried. Five ml of scintillation fluid [0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) in toluene] were added to each vial and the acid-precipitable radioactivity counted in a liquid scintillation counter.

To test the reversibility of ribavirin, after 25 h exposure to ³H-thymidine, the drug-containing medium in the test vials was replaced with fresh medium containing the isotope but no ribavirin. Samples were again withdrawn at intervals and treated as above.

**Acrylamide gel electrophoresis of virus-specific RNA.** Confluent cell monolayers were infected with 0.2 ml of undiluted stock IPNV. After 1 h adsorption (designated as 0 h p.i.) both infected and uninfected cells were treated with: (a) 0.5 µg/ml actinomycin D (act D); (b) 10 µg/ml ribavirin; (c) combination of both drugs; (d) no drugs.

The cells were pulse-labelled for 2 h at successive 2 h intervals (up to 14 h p.i.) with 5 µCi/ml 5,6-³H-uridine. After each pulse the monolayers were dissolved in 0.1 ml of electrophoresis sample buffer [ESB: 0.05 M-tris-HCl pH 6.8, 1% (w/v) sodium dodecyl sulphate (SDS), 2 mM-EDTA, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and a trace of bromophenol blue to which was added 1 mg/ml proteinase K and a trace of naphthalene-1,5-disulphonic acid disodium salt]. The combination of proteinase K and SDS (a component of ESB) completely and irreversibly eliminated any ribonuclease activity (Hilz et al. 1975) and the danger of selective entrapment of RNA by proteins. The preparation was forced through a small hypodermic needle to shear the DNA and then 10 µl of each sample were applied on to a 1.5 cm² filter paper (Whatman no. 3) and left to
air dry. The dry filter paper squares were placed in cold 10% TCA and later immersed in cold methanol. After drying, the acid-precipitable radioactivity was counted in a liquid scintillation counter. The remaining samples were stored at room temperature overnight.

The RNA samples were analysed on composite agarose acrylamide slab-gels followed by fluorography (Sinclair & Mindich, 1976). Gels contained 2% acrylamide-0.1% bis (20:1), 0.5% agarose in 0.1 M-phosphate buffer containing 0.1% SDS. The 2% resolving gel was cast on top of a 5% acrylamide-0.5% agarose gel plug to prevent the resolving gel from slipping out during electrophoresis. Polymerization for both the resolving gel and the cushion was achieved with 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulphate. The electrophoresis buffer consisted of 0.1 M-phosphate buffer, pH 6.8, 0.1% SDS and 0.002 M-EDTA. The gels were pre-run at 50 V for 15 min. Electrophoresis of the samples was carried out at room temperature for 3 to 4 h at 50 V. After the run, the gels were dehydrated in methanol, soaked in a solution of 10% 2,5-diphenyloxazole (PPO) in methanol overnight, rinsed in water for 10 min, dried under vacuum and exposed to Kodak X-ray film at -70 °C for fluorography (Bonnet & Laskey, 1974). The films were exposed for 3 to 14 days, depending upon the amount of radioactivity in the RNA samples.

Media and reagents. Ribavirin was the generous gift of ICN Pharmaceuticals Inc. (Cleveland, Ohio). Foetal calf serum (virus and mycoplasma screened), MEM and Earle's balanced salt solution were obtained from Grand Island Biological Corp. (Grand Island, N.Y.). PPO, POPOP, methyl-3H-thymidine (18 Ci/mol) and 5,6-3H-uridine (40 to 50 Ci/mmol) were obtained from New England Nuclear Corp., (Boston, Mass.). Recrystallized acrylamide, bisacrylamide, TEMED and ammonium persulphate were obtained from Bio-Rad Laboratories Ltd. (Irvine, Calif.). Reagent grade tris, actinomycin D, bromophenol blue and agarose (electrophoresis grade) were obtained from Sigma (St. Louis, Mo.). HEPES, SDS and proteinase K were obtained from BDH (Poole, Dorset, U.K.). 2-Mercaptoethanol and naphthalene-1,5-disulphonic acid disodium salt were obtained from Eastman Kodak Co. (Rochester, N.Y.). Seaplaque agarose was obtained from Marine Colloids Inc.

RESULTS

Determination of cytotoxicity caused by ribavirin

Toxic levels of ribavirin in RTG-2 and CHSE-214 cells were determined by microscopic examination. For 5 days cells were graded by the degree of c.p.e. using a scale of 0 (normal cells) to 4 (virtually dead cells). With drug concentrations below 80 μg/ml, the cells showed no abnormalities, although ribavirin exhibited a cytostatic effect. When using drug concentrations greater than 100 μg/ml, most of the cells rounded up after 2 to 5 days, with progressive degeneration of the monolayers. The cells were killed in 2 days with ribavirin concentrations greater than 500 μg/ml.

The results indicated that in order to determine the antiviral effect of ribavirin in these cell lines, concentrations below 80 μg/ml should be used.

Effect of ribavirin on virus yield

Preliminary experiments showed that at an input m.o.i. of 5, every cell in a culture was infected and higher m.o.i. did not increase the virus yield. Therefore, in order to avoid possible interference by defective interfering (DI) particles, a m.o.i. of 5 was used in subsequent experiments unless otherwise stated.

Both infected CHSE-214 and RTG-2 cells were exposed to ribavirin concentrations ranging from 0 to 80 μg/ml after a 1 h virus adsorption period (designated as 0 h). All samples were harvested at the same time, that is, when infected cells without the drug
Effect of ribavirin on IPNV replication

Fig. 1. Inhibition of IPNV replication in CHSE-214 cells by ribavirin. Replicate cultures were infected, ribavirin added at different concentrations, virus harvested after 24 h p.i. and the infectivity titre determined by plaque titration on CHSE-214 cell monolayers.

Fig. 2. Effect of time of application of ribavirin on IPNV growth. The drug (10 μg/ml) was added to CHSE-214 cell cultures at various times before and after virus infection and the infectivity of virus progeny was determined after 24 h p.i. Abbreviations: -24R, pre-treatment with ribavirin for 24 h before virus infection followed by the removal of the drug after infection (at time zero); -24L, cells were exposed to the drug from 24 h before, until 24 h after infection; VC, virus control.

Exhibited extensive c.p.e. (usually 24 h p.i.). As shown in Fig. 1, there was approx. a 3 to 4 log reduction in virus yield in CHSE-214 cells exposed to ribavirin concentrations greater than 10 μg/ml. A similar experiment in RTG-2 cells resulted in a slightly greater than 1 log reduction in virus yield. The reason for the difference in the degree of inhibition of virus replication in the two cell lines was not clear and was not pursued further. For subsequent experiments, a drug concentration of 10 μg/ml was chosen since it was one of the lowest at which an appreciable reduction in virus titre was observed in CHSE-214 cells (Fig. 1). The minimal inhibitory concentration of ribavirin, defined as the concentration bringing about a 1 log reduction, was found to be 1.0 μg/ml (P < 0.01) in infected CHSE-214 cells and was at least as low as 10 μg/ml in infected RTG-2 cells (since lower concentrations were not tested).

When tested at higher m.o.i. (10 and 100), ribavirin at a concentration of 10 μg/ml effectively reduced virus titres by 3 to 4 log units in CHSE-214 cells indicating that increased m.o.i. did not alter the efficacy of the drug. Incubating drug-treated infected cells for periods longer than 24 h did not result in a higher virus titre which indicated that ribavirin effectively inhibited virus replication and did not merely slow it down.

Effect of time of application of ribavirin on virus replication

Ribavirin was added to confluent CHSE-214 cell monolayers at various times pre- and post-infection and the progeny virus was assayed at 24 h p.i. Pre-treatment of the cells and application of ribavirin early in the replicative cycle resulted in the greatest reduction of
Table 1. Effect of ribavirin on virus yield after removal of the drug

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virus yield (Fig. 2). The addition of the drug after 10 h brought about minimal inhibition, indicating that by this time most of the intracellular virus-specific RNA had already been made. In those cells that were pre-treated with the drug for 24 h before infection and ribavirin was removed at the time of infection, the replication of IPNV was inhibited to the same extent as in those cultures where the drug was not removed from the cells after infection (Fig. 2).

Reversibility of ribavirin

The effect of drug reversal on virus yield was studied by infecting a series of confluent CHSE-214 cell monolayers, treating them with ribavirin (10 μg/ml) at 0 h and replacing the medium containing the drug with fresh medium containing no ribavirin at 6, 12 and 14 h p.i. At the time of drug removal, another series of cell monolayers (designated virus controls) was infected and incubated for the same time period as those cells previously exposed to ribavirin (test samples). These virus controls were infected at the time of drug removal from the test samples so that the titres of the drug-treated reversed samples could be compared to titres of virus controls. Test samples were harvested at different times depending on when the corresponding virus controls showed extensive c.p.e.

The data presented in Table 1 show that the virus titres of the drug-treated samples increased with time after the drug was removed, while the titres of the virus controls remained relatively constant. The longer the infected cells were exposed to ribavirin, the longer was the recovery time for virus replication. In all cases the reversal of the antiviral effect of ribavirin was gradual, requiring several days; for example, even 4 days after reversal the virus yield was still reduced by more than 1 log in treated cells compared to untreated controls (Table 1). Nevertheless, in all cases the difference between the log titres of virus controls and log titres of ribavirin-treated cultures decreased with time after reversal indicating that after removal of the drug the cells were, once more, able to support virus replication.

Effect of ribavirin on cellular nucleic acid synthesis

The effect of ribavirin on cellular DNA and RNA synthesis was investigated in order to determine the length of time required for the drug to inhibit host nucleic acid synthesis. The incorporation of ³H-thymidine was measured in CHSE-214 cells which were grown on the bottom of glass scintillation vials. Incorporation of the isotope in both the presence and absence of ribavirin was followed for 73 h after which the acid-precipitable radioactivity in each sample was determined by liquid scintillation spectroscopy. The data in Fig. 3 show that within 2 to 4 h of the addition of ribavirin (time of 6 h in Fig. 3), cellular DNA synthesis was suppressed and upon removal of the drug (at 25 h in Fig. 3), there was an
Effect of ribavirin on IPNV replication

Fig. 3. Reversible inhibition of DNA synthesis in CHSE-214 cells by ribavirin monitored by the incorporation of $^3$H-thymidine. ○—○, Untreated control cells; ■—■, cells treated with 10 μg/ml ribavirin; ↓, time of drug addition; ↑, time of drug removal.

Fig. 4. Kinetics of $^3$H-uridine incorporation in CHSE-214 cells in the presence or absence of ribavirin. ○—○, Untreated control cells; ○—○, drug-treated cells; ↓, time of drug addition.

extended lag period (approx. 25 h) before the incorporation of $^3$H-thymidine into acid-insoluble molecules began to increase, thereby indicating that the inhibition of cellular DNA synthesis by ribavirin was reversible.

To determine the effect of ribavirin on cellular RNA synthesis, $^3$H-uridine was added to both drug-treated and control cultures of CHSE-214 cell monolayers which were grown on the bottom of scintillation vials and incubated at 22 °C. Portions from both control and test samples were withdrawn at different time intervals and the acid-precipitable radioactivity determined. The data in Fig. 4 show that within 3.5 to 4 h after the addition of ribavirin, RNA synthesis was completely inhibited. The recovery of RNA synthesis after removal of the drug was similar to that of DNA synthesis after drug removal (data not shown). Similar results were obtained in RTG-2 cell cultures (data not shown).

Polyacrylamide gel electrophoresis of virus-specific RNA in the presence and absence of ribavirin

In order to follow virus RNA synthesis it was necessary to inhibit background host RNA synthesis using act D. Preliminary experiments showed that 0.5 μg/ml of act D decreased host cell RNA synthesis by 84% within 2 to 4 h. This concentration of act D reduced virus yield by only 0.5 log, indicating that virus-specific RNA could be preferentially labelled with $^3$H-uridine in infected, act D-treated cells. Any reduction of RNA synthesis in these cultures after ribavirin treatment would demonstrate quantitatively the inhibitory effect of ribavirin on virus-specific RNA synthesis.

CHSE-214 cell monolayers were infected with undiluted stock virus, and after a 1 h adsorption period were treated in one of the following ways: (i) act D added at 0 h; (ii) act D
Fig. 5. Incorporation of $^3$H-uridine into acid-precipitable radioactivity in CHSE-214 cells pulse labelled at 2 h intervals. ○ --- ○, Uninfected cells treated with 0.5 μg/ml actinomycin D; ● --- ●, infected cells treated with 0.5 μg/ml of actinomycin D; △ --- △, infected cells treated with 0.5 μg/ml of actinomycin D and 10 μg/ml of ribavirin.

and ribavirin added at 0 h. Uninfected controls with identical drug combinations were also included. At sequential 2 h intervals, the cultures were pulsed with $^3$H-uridine (10 μCi/ml) for 120 min from 2 to 12 h p.i. At the end of the pulse, the medium was removed and aliquots from each sample were prepared for liquid scintillation spectroscopy. The data in Fig. 5 show that RNA synthesis in infected act D-treated cells increased up to 10 h p.i. followed by a rapid decrease. When ribavirin was added to infected act D-treated cultures, RNA synthesis rapidly ceased. However, these results did not indicate whether it was virus mRNA or virus dsRNA or both, that were inhibited by the drug. Therefore all samples were subjected to polyacrylamide gel electrophoresis followed by fluorography.

The data in Fig. 6(a) show that in the presence of act D or act D plus ribavirin (Fig. 6b), cellular RNA was not labelled, indicating complete inhibition of cellular RNA synthesis in the presence of these drugs. In act D-treated infected cells, three virus-specific RNA species were evident: (i) a diffuse band near the top of the gel, (ii) a sharp high mol. wt. double band co-migrating with virion dsRNA and (iii) two prominent RNA species migrating between the two rRNA markers. These virus-specific RNA species have been shown recently to represent: (i) a partially RNase sensitive LiCl-precipitable 14 to 16S transcription intermediate (TR.I), (ii) intracellular LiCl-soluble 14S progeny virus dsRNA and (iii) a RNase-sensitive, LiCl-precipitable, polysome-associated 24S RNA which could be hybridized to the virus RNA in vitro, and which represents virus mRNA (Somogyi & Dobos, 1980).

The band marked with X represents non-specific trapping of radioactivity, as shown previously (Somogyi & Dobos, 1980), and is present in all fluorograms.

The RNA gel pattern of ribavirin-treated infected cells showed that the synthesis of each of the three forms of virus-specific RNA was completely inhibited by the drug (Fig. 6b). These results correlated well with the 3 to 4 log virus yield reduction in ribavirin-treated cultures (Fig. 1). Similar RNA gel patterns were obtained when the experiment was repeated using RTG-2 cells.
Effect of ribavirin on IPNV replication

Fig. 6. (a) Fluorogram of virus-infected actinomycin D-treated (0.5 μg/ml) cells, pulse labelled with ³H-uridine at 2 h intervals. (b) Fluorogram of virus infected, actinomycin D-treated (0.5 μg/ml) and ribavirin-treated (10 μg/ml) cells, pulse labelled with ³H-uridine at 2 h intervals. Labelled cell lysates were analysed in 2% acrylamide-agarose slab gels. Abbreviations: 28S and 18S, cellular rRNA markers; TR.I, transcription intermediate; V, virion dsRNA; 24S, virion mRNA; X, non-specific trapping of radioactivity.
DISCUSSION

Ribavirin greatly inhibited IPNV replication in CHSE-214 cells at a concentration (10 μg/ml) which only prevented cell division but did not kill the cells. On the other hand the drug only moderately inhibited virus growth in RTG-2 cells. The dependence of the degree of activity of ribavirin on the cell type used conflicted with the report of Sidwell (1976) in which he stated that in tissue cultures the activity of the drug on the replication of DNA viruses greatly depended on the cell types used but that antiviral activity against RNA viruses was less dependent on cells. Since complete inhibition of cellular RNA synthesis by ribavirin in both cell lines required approx. 4 h, the difference in antiviral activity in these two cell lines could not be attributed to differences in the rate of ribavirin uptake or the size of nucleotide pools in these cells. Without further study of the mode of action, no conclusions can be made about the dependence of its antiviral activity on the cell types used.

The antiviral effect of ribavirin was maximal when the drug was added to cultures before or shortly after virus infection. These results were in agreement with those of Sidwell et al. (1975) who found maximal activity of ribavirin against Sendai virus when the drug was added just before virus infection or within 4 to 8 h p.i., indicating that in an in vivo situation, prophylactic or early therapeutic treatment would be most effective.

The reversibility of the action of ribavirin, by simply replacing the drug-containing MEM with fresh medium, was demonstrated in two experiments. (1) After 25 h of 3H-thymidine incorporation into uninfected CHSE-214 cells (Fig. 3), ribavirin was removed and 25 to 30 h later, DNA synthesis began to recover from the effects of the drug. These results confirmed the work of Larsson et al. (1978) who demonstrated that the effect of ribavirin on cellular DNA and RNA synthesis could be reversed by removing the drug from the cells. (2) In virus-infected cells, the antiviral effect of ribavirin was reversed in cultures that were exposed to the drug for various time intervals (6, 12, 24 h) at the beginning of virus infection (Table 1). The cells that were exposed to the drug the longest recovered the slowest, but the antiviral effect of ribavirin was still reversible since these cells, after a 4 to 5 day recovery time, produced nearly as much virus as those cells that were not treated with ribavirin. Therefore, both cellular and virus nucleic acid synthesis recovered from the effects of the drug at a rate dependent upon the duration of drug treatment. Acrylamide gel analysis of labelled RNAs from both infected and ribavirin-treated infected cells revealed that the synthesis of both mRNA and virus progeny dsRNA was inhibited by ribavirin.

Ribavirin has two major drawbacks as a potential antiviral agent against IPNV in fish hatcheries. Its action is reversible and therefore it would be expensive and impractical to pump ribavirin continuously into the waters of hatchery tanks containing infected fish. Ribavirin also does not exert a selective effect on virus-specific RNA synthesis but inhibits the synthesis of host cell nucleic acids as well, thereby preventing cell division. Due to this cytostatic effect, fish fries (which represent a rapidly growing cell population) would grow much more slowly (or in the case of removal of the drug from fish tanks, be killed by the virus). This suppression of growth by ribavirin would defeat the purpose of hatcheries, that is the rapid rearing of healthy trout and salmon.

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REFERENCES


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