COMPARISON OF THE DEVELOPMENT OF RESISTANT STRAINS OF TYPE 1 HERPES SIMPLEX VIRUS TO *IN VITRO* ANTIVIRAL ACTIVITY OF 5-iodo-2'-DEOXYURIDINE OR RIBAVIRIN.

J. H. Huffman, L. B. Allen, and R. W. Sidwell

*ICN Nucleic Acid Research Institute
ICN Pharmaceuticals, Inc.
Irvine, California 92715*

INTRODUCTION

Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was first reported as a broad-spectrum antiviral compound in 1972.^{1,2} We are unaware of any published reports of development of resistance to its antiviral action.

We have noted⁴ that treatment of Type 1 herpes simplex virus (HSV/1) in HSV/1-infected KB cells with concentrations of ribavirin as low as 3.2 μ g/ml resulted in virus titer reductions of over 1 log₁₀ while markedly inhibiting the development of viral cytopathic effects (CPE) in the cells. In the same studies we found that amounts of infectious virus, inversely proportional to the concentration of drug applied, could still be recovered after 72 hours incubation of infected cells in the presence of ribavirin.

In describing the rapid development of resistance to the antiviral activity of 5-iodo-2'-deoxyuridine (IDU) by HSV/1 in cell culture, Buthala³ noted that HSV/1 replication proceeded even at high concentrations of IDU.

These somewhat similar observations of the *in vitro* activity of IDU, to which viral resistance is readily established, and the newer antiviral drug, ribavirin, suggested a study comparing the ease with which resistance of HSV/1 to each drug could be demonstrated. We also recognize that viral resistance should be considered in the overall evaluation of any antiviral drug. It is pertinent that Herrmann and Herrmann⁵ have suggested that an antiviral drug may not be considered to have specific antiviral activity unless virus mutants that are resistant to the action of the drug are observed.

The present report describes our studies on the development of drug resistance of HSV/1 when it is passed concurrently in IDU, ribavirin, or drug-free medium.

MATERIALS AND METHODS

Cell Cultures

Monolayer cultures of human carcinoma of the nasopharynx (KB) and African green monkey kidney (Vero) cells were grown in Eagle minimal essential medium with Earle's salts (EMEM; Grand Island Biological Company, Santa Clara, Calif.), 0.1% NaHCO₃, supplemented with 10% fetal bovine serum (FBS), and lacking antibiotics. Stock cultures were prepared in 32-oz. glass prescription bottles.

Virus. Type 1 herpes simplex virus (HSV/1) strain HF was obtained from the American Type Culture Collection (Rockville, Md.).

Preparation of Virus Pools

Stock cultures of KB or Vero cells were seeded in growth medium (3 ml/dish) in Multi-dishes (Model FB-6-TC, Linbro Scientific Company, Inc., New Haven, Ct.) and allowed to grow to confluency at 37° C in a moist 5% CO₂, 95% air atmosphere. The cell growth medium was then removed and HSV/1, diluted in test medium (EMEM containing 5% FBS, 0.25% NaHCO₃, and gentamicin at 50 µg/ml), was placed on the cells at a multiplicity of infection of approximately 1. The virus was allowed to adsorb at 37° C for 1 hour prior to the addition of 3 ml of test medium without drug, test medium containing 10 µg ribavirin/ml, or test medium containing 10 or 1.0 µg IDU/ml. The cells were then returned to the incubator until the CPE due to herpes virus appeared in all cells in the test medium without drug. The dishes were frozen and thawed one time, and virus pools were prepared from each set of dishes containing similar medium. Four to five passages of each virus were done in a similar manner prior to testing for drug resistance.

Determination of Drug Resistance

The virus pools that had been prepared in KB cells were titrated in KB cells in Micro Test II tissue culture plates (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) to obtain the cell culture infective dose, 50% endpoint (CCID₅₀) of each pool. The various pools of virus were then diluted in test medium to equivalent titers and examined for their relative sensitivity to ribavirin or IDU in Micro Test II plates as previously described.⁶ The CPE value in the plates was read microscopically approximately 72 hours following infection with virus. The CPE at each concentration of drug is expressed as a percentage of the average CPE reading obtained for the virus control of the particular virus pool being examined. Virus ratings were calculated as previously described.⁶

FIGURE 1. Effect of ribavirin on IDU-resistant and sensitive HSV/1 in KB cells.

FIGURE 2. Development of resistance to IDU antiviral action by HSV/1 in KB cells.

Those virus pools that had been prepared in Vero cells were titrated by use of a plaqueing technique as follows. Vero cells were grown as monolayers in Multi-dishes in EMEM without antibiotics. After growth medium had been removed, replicate monolayers of cells were each infected with 0.1 ml of serial $\log_{10}$ dilutions of virus in test medium without antiviral drug. The virus was allowed to adsorb for 1 hour at 37°C with the dishes being rocked every 15 minutes during the adsorption period. The cells of each dish were then overlayed with 4 ml of a mixture of equal volumes of 1.8% Seakem agarose (MCI Biomedical, Division of Marine Colloids, Inc., Rockland, Me.), prepared in distilled water, and 2×(EMEM, 5% FBS, 0.25% NaHCO_3 , 50 μg gentamicin/ml). After 3 days at 37°C one ml of 1:5000 neutral red, in physiological saline solution, was added to each dish for 1½ hours. The excess neutral red was aspirated from the surface of the agarose and the dishes were returned to the CO_2 incubator for approximately 6 hours before the resulting plaques were counted. The respective virus pools were then diluted to obtain equal concentrations of plaque-forming units and replicate dishes of confluent Vero cells were infected with 0.1 ml of each virus. After virus adsorption for 1 hour, the cells received overlays the same as in the plaque titration except that some of the overlays contained ribavirin or IDU in varying concentrations. After the 3-day incubation period, plaques were counted and the average number of plaques at each drug concentration was expressed as a percentage of plaques obtained in dishes without antiviral drug.

RESULTS

HSV/1 grown in KB cells in 10 μg ribavirin/ml for four straight passages developed no resistance to the antiviral activity of ribavirin as shown in FIGURE 1. When the virus was passed in parallel in IDU, however, virus resistance to the antiviral activity of IDU appeared in both the 10- and 1.0- μg /ml-passed pools (FIGURE 2). The ribavirin-passed virus retained its sensitivity to IDU (FIGURE 2), but the

TABLE 1
VIRUS RATING AND MINIMUM INHIBITORY CONCENTRATION OF RIBAVIRIN OR IDU VS
HSV/1 VIRUS PASSED IN MEM, RIBAVIRIN, OR IDU IN KB CELLS

Test Compound	Virus*	Virus Rating (VR)	Minimum Inhibitory Concentration (MIC)
Ribavirin	H/MEM 4×	>1.3	<1.0
Ribavirin	H/R (10) 4×	>1.3	<1.0
Ribavirin	H/IDU (10) 4×	0.8	10.0
IDU	H/MEM 4×	>1.2	<1.0
IDU	H/R (10) 4×	>1.1	<1.0
IDU	H/IDU (10) 4×	0.8	3.2

*HSV/1 passed four times in drug-free medium (MEM), ribavirin (10 $\mu\text{g/ml}$), or IDU (10 $\mu\text{g/ml}$), respectively.

IDU-resistant virus was also resistant to the activity of ribavirin (FIGURE 1). The calculated virus ratings (VR) and minimum inhibitory concentrations of these drugs versus the three different virus pools (TABLE 1) show the same pattern of resistance and sensitivity to the drugs as shown in the figures.

Since we have demonstrated a variation in the antiviral activity of ribavirin against the same virus in different cell lines⁴ and even in the "same" cell lines obtained from different suppliers (unpublished observations), we also determined to examine the development of resistance to these compounds by HSV/1 in Vero cells. After five passages in Vero cells, the ribavirin-grown HSV/1 is as sensitive to ribavirin activity as is the virus passed in drug-free medium (FIGURE 3). There was no antiviral activity shown by ribavirin at 10 $\mu\text{g/ml}$ on either of these virus pools in the Vero cell plaquing experiments even though we have seen some activity at this ribavirin level in these cells without the agarose overlay. The HSV/1 grown for 5 passages in these cells exposed to IDU at 10 $\mu\text{g/ml}$ shows a marked resistance to the antiviral activity of IDU (FIGURE 4).

FIGURE 3. Lack of development of resistance to the antiviral action of ribavirin by HSV/1 in Vero cells.

DISCUSSION

Resistance of the HF strain of HSV/1 to the antiviral action of ribavirin does not develop, nor does IDU cross-resistance develop by passage of the virus in ribavirin under the conditions of these experiments. However, IDU resistance does develop rapidly when the virus is passed in low levels of IDU, and the IDU-resistant virus also attains cross-resistance to ribavirin activity in KB cells. The investigation has not proceeded sufficiently far to determine if this cross-resistance is also seen in Vero cells. However, this resistance would indicate, by resistance development criteria,⁵ that ribavirin is acting as a virus specific antiviral compound. Additional studies to further elucidate this concept are planned.

The increased plaque formation by the IDU-resistant HSV/1 in Vero cells may indicate a partial drug dependence, although this possibility has not yet been investigated.

FIGURE 4. Development of IDU resistance by HSV/1 in Vero cells.

The stability of the resistance characteristics of these virus pools has not been determined beyond one passage without drug.

SUMMARY

Exposure of HSV/1 to low concentrations of ribavirin during 4-5 passages does not produce ribavirin-resistant virus. IDU resistance was developed by HSV/1 while it was being passed simultaneously. This resistance was seen to develop in both KB and Vero cells. The IDU-resistant virus is also resistant to ribavirin in KB cells.

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EFFECT OF RIBAVIRIN ON VIRAL HEPATITIS IN LABORATORY ANIMALS

Robert W. Sidwell, John H. Huffman, Nancy Campbell,
and Lois B. Allen

*ICN Pharmaceuticals, Inc.
Nucleic Acid Research Institute,
Irvine, California 92715*

Human viral hepatitis has continued to be of major concern to public health authorities because of its relative high rate of incidence throughout the world.¹ The disease is generally regarded as being at least two entities, Type A (infectious) hepatitis and Type B (serum) hepatitis, caused by separate, as yet not fully characterized, viruses. It is unfortunate that despite the severe public health problem of these diseases, experimental systems that could be used to evaluate potential chemotherapeutic agents for treatment of hepatitis are virtually nonexistent.

The broad-spectrum antiviral activity²⁻⁶ of the triazole nucleoside 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) suggested possible utility against viruses causing hepatitis. This potential efficacy was further enhanced by the finding that the orally administered drug can be recovered in significant quantities, > 40% and > 5%, in liver and spleen, respectively, of mice and rats within 30 to 60 minutes of treatment.⁷ Experiments were therefore undertaken to attempt to demonstrate ribavirin's efficacy against experimental infections induced by two widely differing viruses that cause hepatitis in laboratory animals. The results of these experiments are described in this report.

MATERIALS AND METHODS

Viruses

Viruses used in these studies were an RNA coronavirus, the Friend-Braunsteiner strain of murine hepatitis⁸ (MHV) obtained from the American Type Culture Collection (Rockville, Md.), and a DNA herpesvirus, equine abortion virus^{9,10} (EAV) provided by Dr. P. E. Came, Schering Corp. (Bloomfield, N.J.). Both viruses were prepared as liver homogenates after two intraperitoneal (i.p.) passages in the pertinent host animals.

Drug

Ribavirin was synthesized in these laboratories,³ and used in a sterile physiological saline solution.

Animals

Male Swiss Webster mice (Hilltop Lab Animals, Inc., Chatsworth, Calif.) weighing 15-18 g, and female Syrian golden hamsters (Lakeview Hamster Colony, New-

field, N.J.) weighing 40–50 g were used in the studies with MHV and EAV, respectively.

EAV Studies

Individually caged hamsters were inoculated i.p. with approximately 10 times a 50% lethal dose (LD_{50}) of EAV. Ribavirin or saline only was administered i.p. 1 hr before virus inoculation and continued twice daily for 4 days. The animals were observed daily for death. Two dosages of the drug, 200 and 100 mg/kg/day, were used. This experiment was later repeated using 400 and 300 mg/kg/day. In another experiment, a single i.p. injection of 500 mg/kg of ribavirin was given within 10 min after virus inoculation. Ten infected hamsters were used in each group receiving ribavirin, and 20 infected animals received saline as virus controls. Five uninfected hamsters were similarly treated to serve as toxicity controls.

MHV Studies

Mice were inoculated i.p. with approximately 10 LD_{50} of MHV for this series of experiments. In the first study, ribavirin in doses of 75, 37.5, and 18.8 mg/kg/day was administered i.p. to the animals twice daily for 9 days, beginning 2 hr before virus inoculation. The experiment was later repeated using drug dosages of 18.8, 9.4, and 4.7 mg/kg/day. As with the EAV studies, ten infected animals were used in each drug treatment group, with 20 used as virus controls and five for each toxicity control group.

Friend *et al.*¹¹ have reported that, in MHV-infected mice, virus replication resulted in liver necrosis and increases in serum glutamic oxaloacetic transaminase (SGOT). An experiment was therefore run to determine the effect of ribavirin treatment on this parameter, as well as on serum bilirubin, on the concentration of virus recoverable from the liver, and on the observable necrosis caused by the infection in the liver. For this experiment, large groups of mice were treated i.p. with saline or ribavirin (20 mg/kg/day) twice daily for 9 days, beginning 2 hr before virus inoculation. On days 1, 3, and 5, five mice per group were removed for collection of blood and livers. SGOT activity was determined colorimetrically using the kit commercially available from Sigma Chemical Co. (St. Louis, Mo.).¹² Serum bilirubin (direct) was likewise measured using the modified Nosslin technique¹³ employing American Monitor Corp. reagents (Indianapolis, Ind.). Virus levels in the liver were determined using death endpoints in hamsters injected i.p. with varying dilutions of the liver homogenates.

RESULTS

EAV Studies

Hamsters infected with EAV and treated with saline began dying on day 3, and by day 7 all had died with a mean survival time of 4.2 days (FIGURE 1). Ribavirin at a dosage of 200 mg/kg/day caused an increase in mean survival time of 2.2 days, which was statistically significant ($p < 0.05$, t -test). Of these ribavirin-treated hamsters, 20% survived the infection. No toxicity was seen at this dose. The lower ribavirin dosage of 100 mg/kg/day was not effective. When the experiment was repeated using

higher ribavirin levels, the 400 mg/kg/day dosage was lethally toxic to the control animals, and the 300 mg/kg/day dosage was approximately 25% lethally toxic. Each dose caused significant ($p < 0.02$) increases in mean survival time in the infected animals. When ribavirin was administered in a single i.p. injection at the time of virus inoculation, a 1.1-day increase in mean survival time was seen, but such an increase was insignificant.

MHV Studies

Ribavirin at the three higher levels was effective in preventing death in the mice (TABLE 1). When the experiment was repeated using altered doses, significant anti-

FIGURE 1. Effect of ribavirin treatment on equine abortion virus induced hepatitis in hamsters.

viral effects were again seen, although at the two lowest doses the effect was in the form of significant mean survival time increase only (TABLE 1).

Virus control SGOT levels rose to a high level by day 3 and remained elevated through day 5 (FIGURE 2). By day 7, too many of the animals had died to continue taking samples. Ribavirin treatment delayed the increase of this enzyme, although by day 5, a moderate increase had occurred. Later studies indicated this level soon declined to normal values. Toxicity control animals exhibited no significant alteration in SGOT. Serum bilirubin levels were likewise elevated in the virus control animals through the 5th and last day of the study (FIGURE 3). Ribavirin treatment in both infected and uninfected mice caused an initial moderate rise in bilirubin which then declined by day 3, although in infected, treated mice a rise was seen again on day 5.

Concentrations of MHV in the liver of virus control mice were at relatively high

TABLE I
EFFECT OF RIBAVIRIN TREATMENT* ON MURINE HEPATITIS VIRUS INFECTIONS

Drug Dosage (mg/kg/day)	Tox. Control Surv./Total	Infected, Treated Surv./Total	Surv. Incr. p†	Infected, Treated Mean Surv.‡ Time (Days)	Surv. Time Increase p§
<i>Expt 1</i>					
75	5/5	5/10	0.008	7.2	< 0.001
37.5	5/5	6/10	0.002	6.8	< 0.001
18.8	5/5	7/10	< 0.001	5.7	< 0.05
0		1/20		4.4	
<i>Expt 2</i>					
18.8	5/5	10/10	< 0.001	> 21.0	< 0.001
9.4	5/5	4/10	0.17	6.8	< 0.001
4.7	5/5	3/10	0.28	6.4	< 0.01
0		4/20		4.8	

**b.i.d.* for 9 days, beginning 2 hr before virus inoculation.

†Fisher's exact test.

‡Animals dying on or before day 21.

§Student's *t*-test.

levels by day 1, increasing to a peak concentration on day 3, but dropping by day 5 (FIGURE 4). This latter decline may be a result of a selecting process, since the majority of the animals had died by day 5, and those remaining alive may have had a lesser initial infection. The maximum virus level attained preceded the mean day of death by about 1.5 days. Ribavirin treatment markedly slowed the production of recoverable virus, although a relatively high titer was seen on day 5.

By days 3 and 5, marked damage was plainly visible in the virus control livers; this

FIGURE 2. Effect of ribavirin treatment on serum glutamic oxaloacetic transaminase levels in mice infected with murine hepatitis virus.

FIGURE 3. Effect of ribavirin treatment on serum bilirubin (direct) levels in mice infected with murine hepatitis virus.

infection was notably less apparent in the ribavirin-treated animals. Scores applied to the degree of liver damage in these mice are summarized in TABLE 2. Liver samples from infected, untreated mice examined histopathologically were found to have moderate hepatitis evidenced by cytoplasmic vacuolation in the form of cloudy swelling and lipidosis. Focal necrosis and hemorrhage were also in evidence.

DISCUSSION

These studies offer considerable evidence that parenterally administered ribavirin has a marked effect on hepatitis in mice induced by MHV, an RNA virus, but that

FIGURE 4. Effect of ribavirin treatment on the concentration of hepatitis virus in livers of mice infected with murine hepatitis virus.

the drug, at least by the treatment regimens used in these experiments, has only a marginal influence on hepatitis in hamsters induced by EAV, a DNA virus.

Preliminary results suggest that ketoaldehydes derived from steroid compounds¹⁴ and certain substituted morpholinium quaternary salts¹⁵ may have a moderate efficacy against MHV infections; other drugs showing action against MHV infection include the antihistamine drug Benadryl and the antibiotic Streptothricin. In the study with the antihistamine, the effect was to prevent necrosis of liver cells, rather than to inhibit viral replication. Streptothricin was thought more to exert its effect as a direct action on the virus than to be a therapeutic effect.¹⁷ The action of ribavirin indicates a probable therapeutic and antiviral effect, although additional studies using later initiation of treatment are needed.

Drugs reported to be most effective against EAV infections in hamsters include 9- β -D-arabinofuranosyladenine¹⁸ and 9- β -D-arabinofuranosylhypoxanthine monophosphate.¹⁹

Both MHV and EAV infections were rapidly fulminating to a lethal endpoint; such an infection does not mimic the more slowly progressing, less fatal human hepatitis infections, so it is difficult to ascertain the predictability of either system to the human situation.

TABLE 2
EFFECT OF RIBAVIRIN TREATMENT ON MURINE HEPATITIS VIRUS-INDUCED LIVER DAMAGE
IN INFECTED MICE

Treatment	Mean Liver Damage Score*		
	Day		
	1	3	5
Ribavirin (20 mg/kg/day)	0.5	1	1.2
Saline	2.0	3.1	3.7

*Scores of 0 to 4 assigned by visual examination of degree of damage, with 0 = normal liver and 4 = total involvement of liver.

Biochemical studies have indicated that ribavirin may exert its antiviral effect as the 5'-monophosphate by inhibiting enzymes involved in guanosine monophosphate synthesis.²⁰ The drug is readily converted to the 5'-phosphate in the liver and spleen, presumably by adenosine kinase. Such an active involvement of the liver and spleen in the drug's metabolism suggests its possible utility for therapy of hepatitis, since these organs are of primary importance in the early replication of murine hepatitis viruses.¹¹ Oxford²¹ has reported evidence for ribavirin's inhibition of structural and nonstructural polypeptides and antigens of influenza virus, which may be an alternative mechanism of action for the drug against MHV as well.

At present, ribavirin is undergoing clinical trial against acute Types A and B hepatitis and is also being used in an attempt to eliminate Australian antigen positives from chronic human carriers. Early reports suggest definite efficacy against the Type A infections,²² and possibly success in the antigen studies.^{23,24}

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