Effect of Ribavirin on Murine Cytomegalovirus Infection

JOHN N. DOWLING, BOSKO POSTIC, AND LUCILLE O. GUEVARRA

Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received for publication 1 July 1976

Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a new synthetic nucleoside, inhibited murine cytomegalovirus in cell culture. This was shown by the inhibition of viral cytopathic effect and plaque formation, as well as reduction in the yield of virus. Despite this *in vitro* antiviral effect, ribavirin did not protect mice from mortality produced by a high inoculum of cytomegalovirus. When a lower inoculum was used to initiate a chronic infection, the administration of ribavirin for 9 days had no effect on the titer of cytomegalovirus in the salivary gland, kidney, liver, and spleen. Thus, ribavirin was ineffective in the treatment of both acute and chronic murine cytomegalovirus infections.

Cytomegalovirus causes a broad spectrum of clinical illnesses including congenital infection, the mononucleosis syndrome, and involvement of various organs in immunosuppressed hosts (18). Human cytomegaloviruses (HCMV) are inhibited by the nucleoside analogues adenine arabinoside, cytosine arabinoside, and 5-iodo-2'-deoxyuridine in cell culture (4). In clinical trials, these antiviral agents may transiently suppress urinary excretion of HCMV, but generally fail to eradicate the virus or alter the natural course of disease (2, 3, 8, 9, 13). The search for antiviral agents capable of inhibiting HCMV should therefore be continued.

Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a new synthetic nucleoside which is active in cell culture against numerous deoxyribonucleic acid and ribonucleic acid viruses (5). The *in vitro* inhibition of herpesviruses, including murine cytomegalovirus (MCMV), by ribavirin has compared favorably with other antiviral compounds (5, 14, 19). In animal systems, ribavirin demonstrated activity against influenza A and B (7), vaccinia (15), and herpes simplex (15) infections. The infection in mice produced by MCMV is well characterized (10, 11, 20) and has been used as a model to evaluate the effectiveness of chemotherapeutic agents (6). This work examines the activity of ribavirin against MCMV in cell culture and in mice. The antiviral effect in cultured cells was first determined by three methods. Then, ribavirin was evaluated in both acute, lethal and chronic, subclinical murine infections.

MATERIALS AND METHODS

Animals. Swiss-Webster CD-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, Mass.

Virus. MCMV of the Smith strain was main-

tained by animal passage. Weanling mice were inoculated intraperitoneally (i.p.) with approximately 10^5 plaque-forming units (PFU) of MCMV. Four to five weeks later, the mice were sacrificed and their salivary glands were removed. Stock virus consisted of a 10% (wt/vol) salivary gland homogenate in medium 199 containing 25% sorbitol.

Cell culture. MCMV was grown in secondary mouse embryo tissue culture (METC) maintained with medium 199 supplemented with 2% newborn calf serum, 0.2% NaHCO_3 , penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and nystatin (50 U/ml). Titration was carried out in METC by the plaquing method using tragacanth overlay (12). After 7 days, the tragacanth was removed and the cell sheets were stained with a dye-fixer solution containing 0.5% methylrosaniline chloride, 5% formalin (vol/vol), 50% ethanol (vol/vol), and 0.85% NaCl in distilled water (1). Plaques were enumerated with the aid of a dissecting microscope.

Ribavirin. Ribavirin was kindly supplied by Robert W. Sidwell (ICN Nucleic Acid Research Institute, Irvine, Calif.).

CPE inhibition. MCMV was added to METC in tube cultures. After virus adsorption, ribavirin was added in the appropriate concentration in the maintenance medium. Controls consisted of tubes to which medium without drug was added. Maintenance medium was not changed during the course of an experiment. Tubes were examined daily and scored for cytopathic effect (CPE) on a scale of 0 to 4+. Protection was defined as less than 1+ (<25%) CPE in the test cell sheet at the time when virus control monolayers first showed 4+ (100%) CPE.

Yield reduction. MCMV was grown in tube cultures with or without drug in the maintenance medium, as for the examination of CPE inhibition. At intervals, the medium was removed and titered for MCMV (supernatant virus). After addition of 2.0 ml of fresh medium, the cell cultures were frozen and thawed three times and clarified by low-speed centrifugation, and the infectivity of the supernatant was determined by the plaque method (cell virus).

Plaque reduction. After adsorption of virus onto

METC, varying concentrations of ribavirin were added to the tragacanth overlay. Control cultures received overlay without drug. Plaques were stained and counted as for MCMV titration. The counts on treated and untreated monolayers were contrasted. In addition, plaque sizes were measured using a calibrated ocular in a microscope at $\times 10$ magnification. Ten randomly chosen plaques at each drug concentration were measured, the largest and smallest diameters of a plaque were averaged, and the area was calculated.

Acute MCMV infection. The MCMV suspension was inoculated i.p. with 0.2 ml of medium 199 as carrier. Control animals received medium alone. Ribavirin was administered for a total of 9 days, beginning the day preceding virus inoculation. The total daily drug dose was calculated for a particular group of mice based on their mean weight and given as twice-daily i.p. injections of ribavirin in 0.2 to 0.3 ml of physiological saline. Control animals received saline only.

Chronic MCMV infection. Four-week-old mice were given approximately 10^6 PFU of MCMV, i.p. After 5 weeks, surviving animals were divided into three groups of 17 mice. Two groups were given ribavirin, 50 mg/kg per day and 100 mg/kg per day, i.p. in twice-daily injections for 9 days. The control group received saline twice daily. On the day after the last drug dose, the mice were killed by cervical dislocation, and the organs of each mouse were dissected out. The spleens, kidneys, livers, and salivary glands from each treatment group were pooled, weighed, and ground in a mortar and pestle with sterile sand, and medium 199 was added to make a final 10% organ concentration. These suspensions were centrifuged, and the supernatant was titered for MCMV.

RESULTS

To examine the ability of ribavirin to inhibit CPE, tube cultures of METC were inoculated with 10-fold dilutions of MCMV from 3×10^6 to 3×10^3 PFU. After virus adsorption, between 1 and 1,000 μ g of ribavirin per ml was added in the maintenance medium. At each virus concentration tested, 25 μ g of ribavirin per ml protected the cell sheet from CPE when control monolayers showed evidence of diffuse CPE. In these experiments, no visible cytotoxicity was produced by 50 μ g of ribavirin per ml; concentrations of 100 μ g/ml or more regularly produced cell destruction.

Yield reduction was examined in cells infected with 3×10^5 PFU of MCMV and with 25 μ g of ribavirin per ml in the medium. Cells and supernatant were harvested from drug and control tubes at intervals from 1 to 96 h and then were titered. The results are shown in Fig. 1. The titer of MCMV in control cells and supernatant began to rise by 31 h after infection and reached 1.4×10^5 and 1.3×10^6 PFU/0.1 ml, respectively, at 96 h. In contrast, ribavirin in-

hibited productive infection through 72 h. At 96 h, the cell suspension contained only 6.2×10^1 PFU/0.1 ml and the supernatant contained 6.5×10^2 PFU/0.1 ml.

To examine the effect of ribavirin on plaque production, the drug was incorporated into the overlay at varying concentrations after inoculation of cell cultures with 10-fold dilutions of the virus suspension. The effect was measured by the MCMV titer (Table 1). At a concentration of 10 μ g/ml, ribavirin reduced the number of plaques 9-fold, and at 25 μ g/ml, 19-fold. In addition, there was a marked reduction noted in the size of the plaques that formed with increasing concentrations of ribavirin in the overlay. This obvious difference in plaque size was quantitated by measuring 10 randomly chosen plaques at each drug concentration and calculating their average area (Table 1). The difference in plaque size between 0 and 10 μ g/ml is highly significant ($t = 9.98$, $P < 0.001$), as is the difference between 10 and 25 μ g/ml ($t = 5.92$, $P < 0.001$). The plaques at 50 and 100 μ g of ribavirin per ml were so tiny that they could not be accurately enumerated. Therefore, it was not possible to measure titer reduction at drug concentrations above 25 μ g/ml.

The reduction in plaque counts was examined further with a lower inoculum of virus,

FIG. 1. Reduction in yield of MCMV produced by ribavirin in mouse embryo cell culture. The cells were inoculated with 3×10^5 PFU of MCMV. Drug-treated cultures contained 25 μ g of ribavirin per ml in the maintenance medium.

TABLE 1. Inhibition of MCMV plaque formation by ribavirin

Ribavirin (μ g/ml)	Titer (PFU)	Plaque size ^a (mm ²)
0	3.3×10^8	0.90
10	3.7×10^7	0.33
25	1.7×10^7	0.13

^a Average area of 10 plaques measured at 10^{-5} dilution.

approximately 100 PFU, at ribavirin concentrations ranging from 1.0 to 100 $\mu\text{g/ml}$. For each experiment, a dose-response curve was constructed on a probit graph from which the ribavirin concentration required to inhibit plaque number by 50% was determined. The results are summarized in Table 2. Some variation between experiments, especially in the drug concentration required to completely inhibit plaque formation, is apparent. Nevertheless, concentrations of ribavirin between 9 and 23 $\mu\text{g/ml}$ reduced the plaque number by 50%. Between 25 and 100 μg of ribavirin per ml was required to completely inhibit plaque production.

The effect of ribavirin on acute, lethal MCMV infection was assessed in adult mice weighing about 30 g. In the first experiment, the two inocula of virus chosen, 1.7×10^6 and 2.5×10^6 PFU, killed 25 and 58% of mice, respectively (Table 3). The administration of

ribavirin, 50 or 100 mg/kg per day for 9 days, did not protect the animals from death at either virus level. There was no mortality among mice treated with these dosages of ribavirin alone, but the mice were observed to have mild diarrhea. This drug toxicity was reflected in the failure of mice receiving ribavirin to gain weight at the rate of saline-treated controls during the course of the experiment.

Drug toxicity, as manifested by weight loss and mortality, was more striking at higher doses (Table 3, experiment 2). The administration of 150 and 200 mg of ribavirin/kg per day alone for 9 days killed 17 and 45% of mice, respectively. These doses did not protect against mortality due to MCMV. In fact, the toxicity of ribavirin appeared to be additive with the effects of the infection, so that, at each level of virus, mortality increased with increasing drug dose.

To evaluate the effect of ribavirin on chronic, clinically latent MCMV infection, mice infected 5 weeks previously were treated with the drug for 9 days. The titers found in the pooled spleens, livers, kidneys, and salivary glands of these animals are given in Table 4. Some variation in titer between groups is apparent, but there was no consistent reduction in titer related to the dose of ribavirin received.

DISCUSSION

The evaluation of the in vitro activity of antiviral agents is not standardized. Three techniques were used to assess the effect of ribavi-

TABLE 2. Ribavirin concentration required for 50 and 100% reduction of plaques produced by a small inoculum of MCMV^a

Expt	Ribavirin ($\mu\text{g/ml}$)	
	ED ₅₀	ED ₁₀₀
1	8.9	50
2	11.5	>25
3	12.3	25
4	23.0	100

^a Approximately 100 PFU of MCMV per monolayer.

TABLE 3. Effect of ribavirin on acute MCMV infection in mice

Expt	MCMV (PFU/mouse)	Ribavirin (mg/kg per day)	No.	Mortality		Maximum Δ weight (%)
				No.	%	
1	None	None	6	0	0	+9.8
		50	13	0	0	+5.5
		100	14	0	0	-1.8
	1.7×10^6	None	12	3	25	-14.4
		50	11	5	45	-11.3
		100	12	6	50	-18.7
	2.5×10^6	None	12	7	58	-22.0
		50	12	10	83	-20.9
		100	10	4	40	-20.1
2	None	None	9	0	0	0.0
		150	12	2	17	-17.3
		200	12	5	45	-20.8
	1.7×10^6	None	12	1	8	-9.4
		150	12	4	33	-25.8
		200	12	11	92	-29.3
	2.5×10^6	None	10	5	50	-17.3
		150	12	8	67	-23.6
		200	12	12	100	-30.0

TABLE 4. *Effect of ribavirin on chronic MCMV infection in mice*

Ribavirin (mg/kg per day)	MCMV titer (log PFU/0.1 g)			
	Spleen	Liver	Kidney	Salivary gland
None	4.90	0.85	1.84	6.58
50	4.18	1.43	0.59	6.86
100	3.48	1.53	2.58	6.93

rin against MCMV. Ribavirin demonstrated considerable activity whether measured by protection from CPE, reduction in yield, or inhibition of plaque formation. The drug was effective in cell culture against levels of virus equivalent to and exceeding those used to produce lethal infections in mice. The approximate mean lethal dose (LD_{50}) of MCMV in adult animals was 2.5×10^6 PFU (Table 3). Ribavirin at a concentration of 25 μ g/ml protected against CPE in cells inoculated with 3×10^6 PFU, or 1.2 LD_{50} , of MCMV and reduced by 19-fold the number of plaques produced by 3.3×10^6 PFU (132 LD_{50}) of MCMV (Table 1).

Despite this activity in cell culture, ribavirin appeared to have little antiviral effect on either acute or chronic MCMV infections in the intact animal. There are a number of considerations that might account for this failure. It is possible that combinations using younger mice or other dosage schedules would have yielded different results. Since resistance to the lethal effects of MCMV infection increases with the age of the animal (12), adult mice were used in these studies in an attempt to maximize any impact of ribavirin. For the same reason, the drug was given for a day preceding the administration of virus. The dosage schedules originally chosen to treat acute MCMV infection were similar to those that appeared to be effective against influenza infection in mice (7). When the given doses produced no protection, larger amounts were tried, but resulted in excessive toxicity. Administration of 200 mg of ribavirin/kg per day for 9 days produced 42% mortality, a figure consistent with the LD_{50} observed in mice by others (7).

Ribavirin had no effect on the titer of MCMV in the organs of chronically infected mice. This suggests that mortality was not favorably influenced because ribavirin failed to exert significant antiviral activity in vivo. Perhaps the drug failed to reach the sites of virus multiplication, or inhibitory concentrations of ribavirin-5'-phosphate, the active form of the drug (17), were not achieved in infected cells. Huffman et al. (5) showed that the activity of ribavirin in cell culture against a particular virus can

vary markedly, depending on the cell type used. The efficacy of ribavirin in treating influenza infections (7) indicates that antiviral concentrations are achieved in the lung. The failure of parenterally administered ribavirin to influence the outcome of encephalitis due to herpes simplex or vaccinia virus in mice suggested that the compound did not cross the blood-brain barrier (15). No attempt was made in the present investigation to determine if biologically active drug accumulated in the spleen, liver, kidney, and salivary gland, the target organs of MCMV infection.

The possibility that ribavirin exerted an immunosuppressive effect in mice which interfered with virus elimination must be considered. In a controlled, double-blind study of cytosine arabinoside in patients with disseminated herpes zoster, Stevens et al. (16) found that the duration of disseminated disease was prolonged by drug therapy, especially in patients with stage III and IV lymphoma. This adverse effect was ascribed to depression of host response to zoster produced by cytosine arabinoside.

Recently, Kelsey et al. (6) examined the effects of cytosine arabinoside and 5-iodo-2'-deoxyuridine on MCMV. Both drugs inhibited MCMV in cell culture, but neither affected the lethal outcome of disseminated MCMV infection in suckling mice, even at doses that were lethal for a proportion of drug-treated controls. It is not surprising that nucleoside analogues, which interfere with both host cell and viral nucleic acid synthesis, might fail to have significant antiviral activity at subtoxic doses. Although it would be hazardous to extrapolate our results with MCMV to HCMV infections, it may prove difficult to achieve therapeutic activity with ribavirin in man without incurring unreasonable toxicity.

ACKNOWLEDGMENTS

This study was supported by a special grant from the Monsour Medical Foundation, Jeannette, Pa., and in part by the Veterans Administration.

LITERATURE CITED

1. Armstrong, J. A. 1971. Semi-micro, dye-binding assay for rabbit interferon. *Appl. Microbiol.* 21:723-725.
2. Barton, B. E., and J. O'H. Tobin. 1970. The effect of idoxuridine on the excretion of cytomegalovirus in congenital infection. *Ann. N.Y. Acad. Sci.* 172:90-95.
3. Ch'ien, L. T., N. J. Cannon, R. J. Whitley, A. G. Diethelm, W. E. Dismukes, C. W. Scott, R. A. Buchanan, and C. A. Alford, Jr. 1974. Effect of adenine arabinoside on cytomegalovirus infections. *J. Infect. Dis.* 130:32-39.
4. Fiala, M., A. W. Chow, K. Miyasaki, and L. B. Guze. 1974. Susceptibility of herpesviruses to three nucleoside analogues and their combinations and enhancement of the antiviral effect at acid pH. *J. Infect. Dis.* 129:82-85.

5. Huffman, J. H., R. W. Sidwell, G. P. Khare, J. T. Witkowski, L. B. Allen, and R. K. Robins. 1973. In vitro effect of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. *Antimicrob. Agents Chemother.* 3:235-241.
6. Kelsey, D. K., E. R. Kern, J. C. Overall, and L. A. Glasgow. 1976. Effect of cytosine arabinoside and 5-iodo-2'-deoxyuridine on a cytomegalovirus infection in newborn mice. *Antimicrob. Agents Chemother.* 9:458-464.
7. Khare, G. P., R. W. Sidwell, J. T. Witkowski, L. N. Simon, and R. K. Robins. 1973. Suppression by 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) of influenza virus-induced infections in mice. *Antimicrob. Agents Chemother.* 3:517-522.
8. Kraybill, E. N., J. L. Sever, G. B. Avery, and N. Movasaghi. 1972. Experimental use of cytosine arabinoside in congenital cytomegalovirus infection. *J. Pediatr.* 80:485-487.
9. McCracken, G. H., and J. P. Luby. 1972. Cytosine arabinoside in the treatment of congenital cytomegalic inclusion disease. *J. Pediatr.* 80:488-495.
10. Mannini, A., and D. N. Medearis, Jr. 1961. Mouse salivary gland virus infections. *Am. J. Hyg.* 73:329-343.
11. Medearis, D. N., Jr. 1964. Mouse cytomegalovirus infection. II. Observations during prolonged infections. *Am. J. Hyg.* 80:103-112.
12. Osborn, J. E., and D. L. Walker. 1970. Virulence and attenuation of murine cytomegalovirus. *Infect. Immun.* 3:228-236.
13. Rytel, M. W., and H. M. Kauffman. 1976. Clinical efficacy of adenine arabinoside in therapy of cytomegalovirus infections in renal allograft recipients. *J. Infect. Dis.* 133:202-205.
14. Sidwell, R. W., J. H. Huffman, G. P. Khare, L. B. Allen, J. T. Witkowski, and R. K. Robins. 1972. Broad-spectrum antiviral activity of Virazole: 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 177:705-706.
15. Sidwell, R. W., L. B. Allen, G. P. Khare, J. H. Huffman, J. T. Witkowski, L. N. Simon, and R. K. Robins. 1973. Effect of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on herpes and vaccinia keratitis and encephalitis in laboratory animals. *Antimicrob. Agents Chemother.* 3:242-246.
16. Stevens, D. A., G. W. Jordan, T. F. Waddell, and T. C. Merigan. 1973. Adverse effect of cytosine arabinoside on disseminated zoster in a controlled trial. *N. Engl. J. Med.* 289:873-878.
17. Streeter, D. G., J. T. Witkowski, G. P. Khare, R. W. Sidwell, R. J. Bauer, R. K. Robins, and L. N. Simon. 1973. Mechanism of action of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. *Proc. Natl. Acad. Sci. U.S.A.* 70:1174-1178.
18. Weller, T. H. 1971. The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. *N. Engl. J. Med.* 285:203-214, 267-274.
19. Witkowski, J. T., R. K. Robins, R. W. Sidwell, and L. N. Simon. 1972. Design, synthesis, and broad-spectrum antiviral activity of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J. Med. Chem.* 15:1150-1154.
20. Wu, B. C., J. N. Dowling, J. A. Armstrong, and M. Ho. 1975. Enhancement of mouse cytomegalovirus infection during host-versus-graft reaction. *Science* 190:56-58.