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Inhibition of Bluetongue and Colorado Tick Fever Orbiviruses by Selected Antiviral Substances†

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The effects of four ribonucleic acid virus inhibitors were evaluated in cell cultures and in mice to determine inhibitory effects against bluetongue virus and Colorado tick fever virus (CTFV). Test compounds included 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin), 3-deazaguanine, 3-deazauridine, and 9-(*S*)-(2,3-dihydroxypropyl)adenine. Ribavirin-2′,3′,5′-triacetate (ribavirin triacetate) was evaluated in vivo against CTFV. Inhibition of cytopathic effect and plaque reduction were used to evaluate antiviral activity. In cytopathic effect inhibition studies, bluetongue virus was markedly inhibited by 3-deazaguanine and 3-deazauridine in Vero cells with moderate inhibition by the other agents. Ribavirin and 3-deazaguanine markedly inhibited CTFV in MA-104 cells, 3-deazauridine was slightly less active, and 9-(*S*)-(2,3-dihydroxypropyl)adenine was negative. Ribavirin was less effective in Vero cells against CTFV. When mice were inoculated intracerebrally with CTFV and treated by a single intracerebral injection with drug, ribavirin triacetate increased the number of survivors, 3-deazaguanine increased mean survival time, and ribavirin was negative. Intraperitoneal treatment of infected mice with ribavirin triacetate for 1 week significantly increased the number of survivors and mean survival time, providing strong evidence that the agent is active across the blood-brain barrier.

Bluetongue virus (BTV) and Colorado tick fever virus (CTFV) are members of the family *Reoviridae*, a group of viruses whose genomes consist of 10 to 12 segments of double-stranded ribonucleic acid (RNA) (11). These two and other related viruses were recently classified into a separate taxonomic group, the orbivirus genus (4). A large number of the orbiviruses isolated to date are insect viruses, and most isolates are nonpathogenic to humans and domestic animals (6, 8, 28).

The most important orbivirus pathogen of veterinary medical concern is BTV, which causes serious disease in sheep, cattle, and goats (28). The most pronounced effects seen with BTV are congenital malformations in lambs and calves (3, 15).

CTFV causes a febrile disease in humans that is usually not fatal. Chills, fever, headaches, muscle soreness, and vomiting are associated with the illness (2), and encephalitis may occur in children. Teratogenic effects (7) and encephalitis (13) have been induced by the virus in experimentally infected mice.

The effects of antiviral agents on infections caused by members of the orbivirus group have yet to be determined. The purpose of this study was to establish parameters for such evaluations and to determine the effect of some known inhibitors of other RNA viruses on these orbiviruses. Of the compounds selected for evaluation, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) has been the most thoroughly investigated against other viruses (22, 23). It has a broad spectrum of activity against deoxyribonucleic acid (DNA) and RNA viruses. 3-Deazaguanine (3-DG) and 9-(*S*)-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA] also inhibit both DNA and RNA viruses (1, 5), whereas 3-deazauridine (3-DU) is primarily an RNA virus inhibitor (12, 18). We recently reported the in vitro effects of these compounds against several rotaviruses, which are also of the *Reoviridae* family; all exhibited a moderate virus-inhibitory effect (23a). A derivative of ribavirin, ribavirin-2′,3′,5′-triacetate (ribavirin triacetate) was also examined in vivo in the present study against CTFV. In recent reports this compound, probably acting mechanistically like ribavirin, was shown to have potential as an in vivo inhibitor of influenza (25) and arenaviruses (24).

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MATERIALS AND METHODS

Antiviral compounds. Ribavirin, ribavirin triacetate, 3-DG, and 3-DU were obtained from ICN Pharmaceuticals, Inc. (Covina, Calif.). (*S*)-DHPA was provided by Eric DeClercq, Rega Institute of the Catholic University of Leuven (Leuven, Belgium). Each was dissolved in cell culture medium at an initial concentration of 2,000 μg/ml for use in these studies. Drug-containing medium was stored at 4°C for 2 to 3 months with no loss of antiviral activity.

Cells. Embryonic rhesus monkey kidney (MA-104) cells, obtained from Mary Estes, Baylor College of Medicine (Houston, Tex.), and African green monkey kidney (Vero) cells and baby hamster kidney (BHK-21; clone 13) cells, both purchased from Flow Laboratories (Inglewood, Calif.), were used. The MA-104 cells originally came from Microbiological Associates (Bethesda, Md.). Growth medium for all cells was Eagle minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 0.19% $NaHCO_3$, and 50 μg of gentamicin per ml. For antiviral tests the fetal bovine serum was reduced to 5%.

Viruses. BTV (strain 8, serotype 10) was obtained from Lynn Barber, U.S. Department of Agriculture Research Laboratory (Denver, Colo.). Pools of virus were prepared from BHK-21 cells (10) and stored in 0.002 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.8) at 4°C. CTFV (Florio strain) was obtained from the American Type Culture Collection (Rockville, Md.). A pool of CTFV for in vivo studies was made from infected brains of mice. Virus was further passaged in Vero cells for in vitro experiments. CTFV was extracted by homogenization of infected tissues or cells in cell culture medium containing 20% Freon 113. After separating the Freon phase by centrifugation, the virus-containing supernatant fluids were stored in 5% sorbitol (26) at −90°C. Since an objective of this research was to determine the response of various members of the *Reoviridae* family of viruses to these antiviral substances, we had some concern about whether BTV was indeed similar to CTFV, although both have been provisionally placed in the orbivirus group. To further investigate the relationship of these two viruses, viral genomic RNAs were extracted from virions (27) and electrophoresed into 10% polyacrylamide gels prepared by the method of Laemmli (14). Reovirus type 3 (Abney strain) was run in parallel for comparison. The identity of CTFV was further established by serum neutralization with anti-CTFV hamster serum. The anti-CTFV serum and reovirus were obtained from the Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley.

In vitro antiviral experiments. The methods used to assess antiviral activity included inhibition of viral cytopathic effect (CPE) and plaque reduction. The CPE studies were conducted in 96-well disposable microplates (Vangard International, Neptune, N.J.) by the method of Sidwell and Huffman (21). Plaque reduction studies were performed with Vero cell monolayers in 60-mm petri dishes (Falcon Plastics, Oxnard, Calif.). Plaquing medium contained the supplemented MEM described above with 100 μg of diethylaminoethyl dextran per ml, 0.8% agarose, 0.003% neutral red (for second overlay), and drug. Virus was allowed to absorb on the cells for 1.5 h in the presence of drug before the first overlay was applied. The second overlay was applied at 72 h, and plaques were counted at 96 h. Seven concentrations of each test compound varying by one-half $\log_{10}$, plus drug-free controls, were used for all studies.

Antiviral activity was expressed as minimum inhibitory concentration, in which ≥10% inhibition of CPE or ≥20% reduction in plaque numbers was considered indicative of positive antiviral effect, and by virus rating. The virus rating is a numerical expression we have described previously (19, 21) of the overall inhibition by a chemical agent of the virus-induced CPE, taking into account discernible cytotoxicity of the agent. Since previous biochemical studies have shown that these agents affect MA-104 cell macromolecular uptake or synthesis (23a), we used those data for defining cytotoxicity in calculating virus ratings. Essentially, all dilutions of the test compounds which caused viral inhibition were considered as also having cell-inhibitory effects; the resultant virus ratings were lower than comparable ratings calculated by using minimum cytostatic doses determined by microscopic examination of cells. Therefore, the interpretation of virus rating was modified as follows: ≤0.3, weak antiviral effect; 0.4 to 0.7, moderate antiviral effect; ≥0.8, marked antiviral effect.

In vivo experiments. Swiss Webster female mice (Simonson Labs, Gilroy, Calif.) weighing 17 to 18 g were used. Infected mice received $10^{5.5}$ plaque-forming units of CTFV per ml intracerebrally (i.c.) in a 0.02-ml volume of Eagle MEM. Ribavirin and 3-DG were administered only i.c. Ribavirin triacetate was given both i.c. and intraperitoneally. The i.c. injections were given 3 h before or 3 h after virus inoculation in doses of 15 or 7.5 mg/kg. Ribavirin triacetate was inoculated intraperitoneally twice a day for 7 days in doses of 500 and 250 mg/kg per day. Drugs and placebo were prepared in sterile 0.85% NaCl. Both the i.c. and intraperitoneal drug studies were run in parallel and included accompanying toxicity control experiments.

RESULTS

Figure 1 shows double-stranded RNA band patterns for BTV, CTFV, and reovirus. BTV and reovirus both possessed 10 RNA segments characteristic of their species. In contrast, CTFV was found to contain 12 double-stranded RNA segments.

The in vitro antiviral effects of the four test agents against BTV and CTFV are shown in Table 1. 3-DG had the greatest inhibitory effects against both viruses, followed by moderate to marked activity by 3-DU. Ribavirin showed strong inhibition of CTFV in MA-104 cells, although the inhibitory effect against BTV in Vero cells was only moderate. The inhibition of BTV by (*S*)-DHPA was also moderate; the agent was

completely negative against CTFV. Ribavirin triacetate was inactive against these viruses in vitro.

In comparing CTFV plaque reduction with CPE inhibition in Vero cells (Table 2), the data show one peculiarity. Ribavirin was apparently inactive in inhibiting CPE development but did retard plaque formation. The other agents showed approximately the same inhibition under both assay conditions. Although (*S*)-DHPA caused no reduction of plaque numbers, the compound did reduce plaque sizes by 50% at a dose of 1,000 μg/ml.

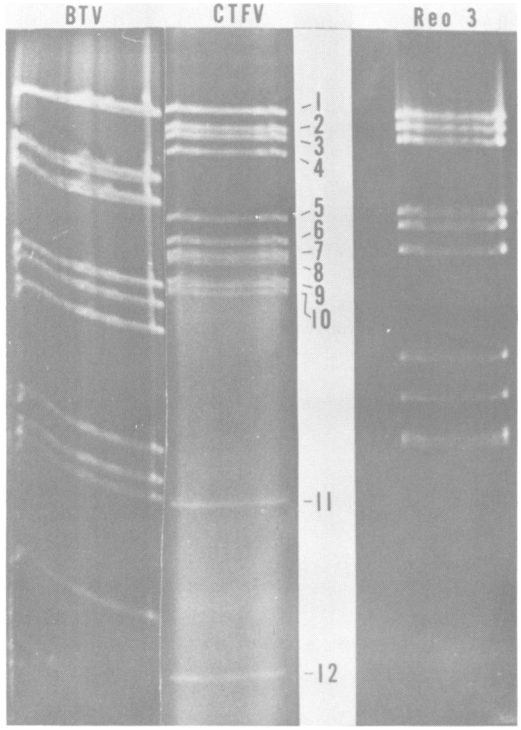

FIG. 1. *RNA band patterns of BTV, CTFV, and reovirus type 3 (Reo 3).*

In the mouse studies with CTFV, in which the drug was administered i.c. (Table 3), ribavirin had no appreciable effect on the infection. The 15-mg/kg dose appeared to be moderately toxic, with deaths noted in toxicity control mice. Treatment with 3-DG significantly increased mean survival time at both doses used when administered 3 h before the virus. An increase in the number of survivors was observed for the group of mice treated i.c. with 15 mg of ribavirin triacetate per kg 3 h before virus inoculation. When ribavirin triacetate was given intraperitoneally for 7 days to infected mice (Table 4), statistically significant survivor and mean survival time increases were evident. The drug appeared to be well tolerated at these high concentrations, although no weight gains were evident in toxicity control mice until 3 to 4 days after treatment ended.

DISCUSSION

In the cell culture studies reported here, all four agents had moderate to marked inhibitory effects against BTV, with 3-DG and 3-DU being most active. Only (*S*)-DHPA was of no apparent value in reducing CTFV CPE; the other agents were very active inhibitors. There appeared to be definite cell-related differences in antiviral effects of ribavirin against CTFV in MA-104 (Table 1) compared to Vero (Table 2) cells. Previous reports have shown ribavirin to be less effective against viruses in Vero cells compared to other cell lines (9), as confirmed in this report. Research with ^{14}C-labeled ribavirin indicates that Vero cells take up the drug at a slower rate than BHK-21 cells and that the plateau level of ribavirin in Vero cells is also low (Peter Canonico, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Md., personal communication). This may account for the moderate activity against both BTV and CTFV seen

TABLE 1. *Effects of antiviral substances on BTV infections in Vero cells and CTFV infections in MA-104 cells*[a]

Virus	Virus titer[b]	Virus-inhibitory effect of:							
		Ribavirin		3-DG		3-DU		(*S*)-DHPA	
		VR[c]	MIC[d]	VR	MIC	VR	MIC	VR	MIC
BTV	$10^{4.5}$ (0.003)[e]	0.5	32	1.1	3.2	0.9	10	0.5	32
CTFV	$10^{4.5}$ (0.003)	1.0	3.2	0.9	3.2	0.7	32	0	>1,000

[a] Determined by CPE inhibition parameter; determinations were based on 100% CPE in virus controls.
[b] Plaque-forming units per 0.1 ml.
[c] Virus rating.
[d] Minimum inhibitory concentration (μg/ml).
[e] Multiplicity of infection.

TABLE 2. *Anti-CTFV[a] activity as determined by CPE inhibition and plaque reduction (PR) in Vero cells*

Drug concn (μg/ml)	% of control with:							
	Ribavirin		3-DG		3-DU		(S)-DHPA	
	CPE	PR	CPE	PR	CPE	PR	CPE	PR
1,000	100	0	0	0	0	0	100	101
320	100	0	0	0	0	0	100	100
100	100	2	23	0	26	11	100	92
32	100	64	42	0	50	100	100	96
10	100	96	53	0	80	103	100	ND[b]
3.2	100	105	80	56	100	98	100	ND
1.0	100	99	100	99	100	101	100	ND
0	100	100[c]	100	100[c]	100	100[c]	100	100[c]
Virus rating	0	0.7	0.8	1.1	0.7	0.6	0	0
Minimum inhibitory concn (μg/ml)	>1,000	32	3.2	3.2	10	100	>1,000	>1,000

[a] Virus was used at $10^{3.5}$ plaque-forming units per 0.1 ml for CPE inhibition and $10^{2.2}$ plaque-forming units per ml for PR.
[b] ND, Not determined.
[c] Approximately 150 plaque-forming units per petri dish.

TABLE 3. *Effect of a single i.c. administration of antiviral substances on CTFV-induced encephalitis*

Compound	Dose (mg/kg)	Treatment time[a] (h)	Toxicity control (survivors/total)	Infected, treated (survivors/total)	Survivor increase[b]	Infected, treated mean survival time (days)	Mean survival time increase[c]
Saline	0	3 pre	5/5	2/12		8.9	
	0	3 post		1/12		8.1	
Ribavirin	15	3 pre	3/5	1/5[d]		7.5	
	15	3 post		0/7[e]		7.3	
	7.5	3 pre	5/5	1/10		8.4	
	7.5	3 post		0/10		7.9	
Ribavirin triacetate	15	3 pre	5/5	5/10	$P < 0.1$	9.0	
	15	3 post		2/10		8.5	
	7.5	3 pre	5/5	2/10		9.1	
	7.5	3 post		1/10		8.8	
3-DG	15	3 pre	5/5	2/10		9.6	$P < 0.01$
	15	3 post		0/10		7.7	
	7.5	3 pre	5/5	2/10		9.9	$P < 0.001$
	7.5	3 post		0/10		8.1	

[a] Hours before and after infecting with virus.
[b] Probability (chi-square analysis with Yate's correction).
[c] Probability (Student *t* test).
[d] Of 14, 9 died within 1 h due to drug toxicity.
[e] Of 14, 7 died within 1 h due to drug toxicity.

in this cell line. It was particularly interesting that ribavirin was ineffective against CTFV-induced CPE in Vero cells, although the drug was actually antiviral when assayed by plaque reduction. Development of CTFV CPE was relatively slow (4.5 days to reach 100%) and was not easily discernible, which may partially explain this variation in results. The CPE assay also required a 10-fold greater virus concentration to cause the CPE than was required in MA-104 cells. Uninfected Vero cells were markedly sensitive to 3-DG and 3-DU, with cytotoxic or cytostatic effects readily discernible at antiviral levels. This may account for the positive inhibition of CTFV CPE caused by these agents in Vero cells. It was not surprising that ribavirin triacetate was virtually inactive in vitro, since the acetyl groups are not readily cleared in cell culture, as we have discussed previously (19).

Previous results of biochemical assays for de-

TABLE 4. *Effect of intraperitoneal administration[a] of ribavirin triacetate on CTFV-induced encephalitis*

Compound	Dose (mg/kg per day)	Toxicity control (survivors/total)	Infected, treated (survivors/total)	Survivor increase[b]	Infected, treated mean survival time (days)	Mean survival time increase[c]
Saline	0	5/5	0/12		7.7	
Ribavirin triacetate	250	5/5[d]	4/10	$P < 0.02$	10.3[e]	$P < 0.05$
	500	5/5[d]	5/10	$P < 0.01$	9.4	$P < 0.01$

[a] Treatment was twice a day for 7 days, starting 2 h preinfection.
[b] Probability (chi-square analysis with Yate's correction).
[c] Probability (Student *t* test).
[d] No weight gains were observed until 3 to 4 days after final treatment.
[e] Data are skewed due to one mouse dying of encephalitis on day 18.

termining cytotoxicity in MA-104 cells revealed that each drug inhibits uptake and incorporation of ^{3}H-labeled amino acids, uridine, or thymidine (23a). Although implying a lack of specificity of antiviral action, these data may not correlate directly to toxic effects of drug in vivo. Ribavirin and (*S*)-DHPA appear to be well tolerated in animals (5, 23), whereas 3-DG and 3-DU have relatively greater toxic properties in vivo (1, 17).

It is not unusual that these two provisional members of the orbivirus genus of viruses responded somewhat differently to these drugs, since the comparative RNA banding patterns run indicated that the CTFV had 12, rather than the usual 10, double-stranded RNA segments. To our knowledge, this is the first published report of RNA banding patterns run with CTFV, and the results suggest that further consideration should be given as to the appropriate classification of this virus.

Conditions of the in vivo studies were designed, in part, to evaluate the ability of ribavirin triacetate to cross the blood-brain barrier and to evaluate the other positive agents i.c. (*S*)-DHPA, which may also have the potential of crossing the blood-brain barrier, was inactive against the virus in vitro, so it was not evaluated in vivo. Sufficient quantities of 3-DU were not available to test that compound in vivo. Ribavirin and 3-DG were previously shown to be ineffective against central nervous system infections unless given i.c. (1, 20).

It is believed that the increase in mean survival time caused by 3-DG may be due to the drug persisting in the brain for a long period of time. The compound is relatively water insoluble (1) and therefore was administered as a particulate suspension. Treatment of infected mice with ribavirin triacetate caused a marginally significant increase in the number of survivors when the compound was given i.c. This effect may be due, in part, to a slow clearance of drug from the injection site and the persistence of the compound in the blood (19). Because ribavirin is highly water soluble and rapidly excreted from the body (16), the compound may not have remained in the target organ long enough to be efficacious.

The in vivo study provides tangible evidence that ribavirin triacetate may be able to cross the blood-brain barrier in sufficient concentration to inhibit viral replication. Significant increases in mean survival time and in the number of survivors for intraperitoneally treated mice, observed at two drug concentrations, support this conclusion. Ribavirin triacetate may have a higher partition coefficient than ribavirin (Roland Robins, Department of Chemistry, Brigham Young University, personal communication), which would allow it to penetrate into the brain more readily.

Whether these or other antiviral agents could be efficiently used to treat animal diseases such as bluetongue remains to be seen. There may be instances, such as with valuable breeding animals and as the cost of livestock continues to rise, where the antiviral therapy would be justifiable over existing, less expensive treatments. An antiviral agent would have potential use if the drug could clear the animal of infectious virus, so that the semen or animal could be shipped internationally.

Results of these studies provide further evidence of the usefulness of ribavirin triacetate in treating certain viral infections. Additional research will be required to determine whether the agent is efficacious against other virus-induced encephalitis diseases when the drug is administered other than i.c.

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