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In Vitro and In Vivo *Phlebovirus* Inhibition by Ribavirin

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Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was markedly inhibitory in vitro to Adames and Balliet strains of Punta Toro virus (PTV), a *Phlebovirus* related to Rift Valley fever and sandfly fever viruses. By using inhibition of viral cytopathic effect in LLC-MK$_2$ cells with both virus strains, the 50% effective dose was 4 to 10 μg/ml and the virus rating was 1.3. The Adames strain of PTV infection in mice was established for evaluation of the in vivo antiviral efficacy of ribavirin. The drug was administered subcutaneously (s.c.) twice daily for 5 to 7 days beginning 4 h pre-virus inoculation, 24 h post-virus inoculation, or 36 h post-virus inoculation, with increased survivors, reduced hepatic icterus, reduction of serum glutamic oxalic acid transaminase and serum glutamic pyruvic acid transaminase, and inhibition of infectious virus from sera and livers of infected mice. The minimum effective dose was 4.7 mg/kg per day, with a maximum tolerated dose of 75 mg/kg per day. When the same treatment schedule beginning 4 h pre-virus inoculation, 4 h post-virus inoculation, or 24 h post-virus inoculation was used, orally administered ribavirin was effective at doses as low as 6.3 mg/kg per day. Single s.c. ribavirin treatments at doses of 175 to 700 mg/kg administered from 4 to 48 h post-virus inoculation were also effective. No effect was seen when ribavirin was administered s.c. to mice infected intracerebrally with the PTV strain Balliet, even though treatment was begun 36 h before virus exposure.

Phlebovirus infections have long been recognized as major diseases in humans (13). Although there are 37 known viruses in this genus (15), which is in the *Bunyaviridae* family, the principal agents causing serious disease in humans are phlebotomus, or sandfly, fever and Rift Valley fever viruses (26). Over 12,000 members of the Allied armed forces in Italy and Sicily during World War II were hospitalized with infections caused by phlebotomus fever virus. Severe epizootics and epidemics of Rift Valley fever have been reported since 1930 which have resulted in the deaths of thousands of sheep and cattle (14); a recent outbreak of Rift Valley fever in Egypt killed approximately 600 humans (11). In addition to these viruses from Europe, Africa, and the Middle East, at least four phleboviruses from Central or South America have been reported which appear to cause a disease similar to phlebotomus fever. These are Chagres, Alenquer, Candiru, and Punta Toro viruses (10, 14, 23, 25). To date, no drugs are available to treat these diseases.

The Punta Toro virus (PTV), Adames strain, has recently been reported by Pifat and Smith (D. Y. Pifat and J. F. Smith, Microb. Pathogen., in press) to induce a nonencephalitic, lethal infection in mice that is characterized particularly by fulminant hepatocellular necrosis following peripheral inoculation of the virus. The disease induced in the mouse appears similar in many respects to the disease induced by PTV in humans and by other phleboviruses in both humans and livestock. This is particularly significant since these viruses are transmitted in nature by sandflies or mosquitoes, the bite of which resembles the subcutaneous inoculation used in mice. We used this animal model to study in depth the effects of the broad-spectrum antiviral agent (20) 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) on this virus infection, as well as on the encephalitic disease in mice induced by a neurotropic strain of PTV.

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MATERIALS AND METHODS

Viruses. The Adames strain of PTV was provided by the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID). The virus was initially isolated from the serum of a patient in Panama in 1972. It was passed twice in African green monkey kidney (Vero) cells at USAMRIID and then twice through rhesus monkey kidney (LLC-MK$_2$) cells by us; the virus was plaque isolated each time from LLC-MK$_2$ cells. A large virus pool was prepared from the last plaque isolate; the virus identity of this pool was confirmed by serum neutralization, as described by Hsiung (6), by using specific antisera. The Balliet strain of PTV was obtained from the American Type Culture Collection (Rockville, Md.). The virus was originally isolated from a patient from Panama in 1966. This virus was plaque purified twice as described above, its identity was confirmed, and a pool was subsequently made. Both virus pools were placed in ampoules and stored at −70 C.

Cells. The cells used included LLC-MK$_2$ and two lines of African green monkey kidney cells (MA-104 and Vero). The LLC-MK$_2$ and MA-104 cells were maintained in minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 0.1% $NaHCO_3$ without antibiotics. The Vero cells were grown in Medium 199 (GIBCO) with fetal bovine serum and buffer, as described above. All cells were determined to be free of mycoplasma.

Animals. Female C57BL 6 mice (weight, 10 to 12 g) were obtained from Simonsen Laboratories (Gilroy, Calif.). All animals were quarantined 24 h prior to use.

Test compound. Ribavirin was provided by USAMRIID via Technassociates, Inc. (Rockville, Md.) in dry powder form. The compound was prepared in minimum essential medium for in vitro studies and in saline or water for in vivo experiments.

In vitro antiviral experiment. Antiviral activity was determined against 320 cell culture 50% infectious doses of PTV

by using the degree of inhibition of viral cytopathic effect (CPE) that developed in 5 to 7 days in a 96-well microplate test system described previously (15). The experiments with the Adames strain of PTV were run in parallel in LLC-MK_2, MA-104, and Vero cells. In all experiments, the compound was added 15 min after virus exposure. Later experiments with the Balliet strain of PTV were run with LLC-MK_2 cells only. Confirming experiments were run by using CPE inhibition and virus titer reduction at the maximum tolerated dose (MTD) of ribavirin. In initial studies, the MTD was determined as the highest dose that did not cause visually discernible cytotoxic effects in concurrently run toxicity controls. As a follow-up determination in LLC-MK_2 cells, the MTD was further defined as the highest dose in which viable cell increase occurred after 6 days of exposure to the drug. Antiviral activity was expressed as virus rating, as described previously (18), and by 50% effective dose, as ascertained by plotting the dose versus the CPE inhibition on a semilogarithmic chart. Therapeutic indices (TIs) were calculated as the MTD divided by the 50% effective dose. Virus yield was determined by endpoint assay of multiple 10-fold dilutions of eluates from frozen and thawed plates. Titers of the eluates were determined in LLC-MK_2 cells, with virus quantified by determining the CPE induced in triplicate microplate cups.

Development of virus infection. An experiment was run to further characterize the PTV infection in C57BL/6 mice, since it is important in antiviral studies to correlate the development of infectious virus in sera and livers with the degree of hepatic icterus and with elevations in serum glutamic oxalic acid transaminase (SGOT), serum glutamic pyruvic acid transaminase (SGPT), and total bilirubin (BN).

Pifat (D. Y. Pifat, Ph.D. dissertation, University of Maryland, Baltimore, 1985) has shown that the PTV infection is highly dependent upon the age and strain of mice, with 3-week-old C57BL/6 mice being generally considered most suitable. Titrations with PTV indicated that a window of acceptable lethality at a particular virus dilution is seen, while higher or lower concentrations from the ideal viral inoculum result in less lethality in mice. We presume that this is due to interference by defective virus particles. In this experiment, a group of 200 female mice (age, 3 weeks; weight, 10 to 12 g) was infected subcutaneously (s.c.) with an approximate 75% lethal dose of the Adames strain of PTV (10^5 PFU). Five mice were randomly selected and killed daily from days 1 through 10 and on days 13, 16, and 19 post-virus inoculation. Each animal was bled, the serum was separated, and the livers were removed. Hepatic icterus was assigned a score of 0 to 4, according to the degree of discoloration. Each liver was homogenized to a 10% (wt/vol) suspension prepared in minimum essential medium and assayed for virus by diluting each homogenate 10-fold to a titer of 10^{-5}; portions of 0.2 ml were added to triplicate cups of LLC-MK_2 cell monolayers in 96-well microplates. Viral CPE was determined after 5 days of incubation at 37°C, and 50% endpoints were determined (12). Each serum sample was assayed in a similar manner for infectious virus. Titration of SGOT, SGPT, and BN was accomplished by using colorimetric kits from Sigma Chemical Co. (St. Louis, Mo.). Spectrophotometric readings for these colorimetric assays were performed in duplicate by using a microplate autoreader (EL309; Bio-Tek Instruments, Inc., Winooski, Vt.).

***In vivo* antiviral experiments.** The *in vivo* antiviral experiments varied somewhat according to the objectives of the study, but the general design was as follows. A total of 20 mice infected s.c. with PTV were treated with each drug dose, and 40 infected mice were treated with saline as virus controls. There were 10 infected animals in each cage. A total of 5 sham-infected mice at each drug dosage served as toxicity controls, and 5 or 10 additional animals were used as normal controls. All uninfected animals were held in rooms that were separate from those in which the infected mice were held. The uninfected animals were weighed immediately prior to the initial treatment and again 18 h following the final treatment. On infection day 3 or 5, mice from one cage of each group which had been infected and drug treated, mice from two cages of virus controls, and mice from one cage of normal controls were killed and bled and their livers were removed. Hepatic icterus was assigned a score, and the livers were photographed and then frozen at −70°C until they were assayed for infectious virus. The serum was also frozen and later thawed and assayed for SGOT and SGPT levels. If an infected animal died of obvious infection before it was sacrificed the livers were assigned a score of 4 and SGOT, SGPT, and virus titers were assigned maximal values. The remaining animals which were not killed were examined daily and deaths were recorded through day 21, at which time the experiment was terminated.

The ribavirin doses used varied by twofold dilutions, with the highest dosage being the approximate MTD, as determined in preliminary toxicity assays.

Statistical evaluations. Increases in survivors were analyzed by using chi-square analysis with the Yates correction. Increases in mean survival time of animals that died on or before day 21 and reductions in SGOT and SGPT and PTV levels in the liver were evaluated by using the Student *t* test. Ranked sum analysis (Wilcoxon test) was used to compare inhibition of mean liver scores.

RESULTS

In vitro antiviral activity. The in vitro effects of ribavirin on both strains of PTV are summarized in Table 1. Against the Adames strain of PTV, inhibitory effects (virus rating and TI) were maximal in LLC-MK_2 cells. In LLC-MK_2 cells, reductions of 2.7 $\log_{10}$ units in the Adames virus strain yield and 1.0 $\log_{10}$ units in the Balliet virus strain yield were demonstrated at the MTD. The MTDs, as determined by microscopic examination of the cells, varied from 3.2 to 32 μg/ml, depending on the cell line. A follow-up MTD, deter-

TABLE 1. Inhibitory effects of ribavirin against PTV in vitro

PTV virus strain and cells	VR[a]	ED_{50} (μg ml)[b]	MTD (μg ml)[c]	TI[d]	VTR[e] at MTD
Adames					
MA-104	1.0	23	3.2	0.14	ND[f]
LLC-MK_2	1.3, 1.3	9.8, 4	32, 10 (32[g])	3.3, 2.5	2.7
Vero	0.9	78	32	0.4	ND
Balliet					
LLC-MK_2	1.3	5	10 (32[g])	2	1.0

[a] VR, Virus rating.
[b] ED_{50}, 50% effective dose.
[c] Determined as the maximum dose causing no microscopically discernible cytotoxic effects.
[d] TI = MTD/50% effective dose.
[e] VTR, Virus titer reduction; determined by assaying triplicate samples of supernatants from MTD-treated infected cells in LLC-MK_2 cells.
[f] ND, Not done.
[g] MTD, as determined by viable cell count 6 days after drug exposure.

mined in LLC-MK_2 cells by using viable cell count, was 32 μg/ml.

Development of PTV infection in mice. Virus titers developed relatively rapidly, reaching maximum titers by day 2 post-virus inoculation (Fig. 1). The virus titers in serum were higher than those in the liver, with maximum serum dilutions containing infectious virus titers of $>10^{-6.7}$, compared with maximum infectious dilutions of liver homogenate of $10^{-5.0}$. In the antiviral experiments, however, the liver virus titers often exceeded this level. Hepatic icterus or jaundice achieved peak discoloration scores of >3.5 by infection day 4. The SGOT, SGPT, and BN levels, all of which usually provide evidence of severe liver damage when suitably elevated (3), also reached maximal levels by days 3 to 4, declining rather precipitously after this time (Fig. 1). The BN levels appeared to peak on day 4 after virus exposure, and were so variable (standard error, ±2 to 3 mg/100 ml) that the mean BN levels appeared to rise again toward the 19-day postinfection period.

The PTV infection was lethal to 60% of concurrently infected mice that were held for the duration of the study; there was a mean survival time of 4.6 days in those mice. Thus, mice sampled after approximately day 8 may have been taken from a selected population of survivors, and the data at these later sampling times may not be reflective of the true picture of the disease.

Effects of twice daily s.c. treatments. When ribavirin was administered twice daily for 5 days, beginning 4 h pre-virus inoculation (Table 2), all infected animals treated with the four ribavirin dosages survived the experiment; there was 60% survival among the virus controls. Anti-PTV activity was also seen as inhibition of mean liver score and reductions in SGOT and SGPT levels and virus titer in the liver. The experiment was repeated, but treatment was delayed until 24 or 36 h post-virus inoculation. All ribavirin-treated animals survived the infection, and significant inhibition of hepatic icterus and reductions in SGOT and SGPT levels were again seen (data not shown). The delayed therapy did not affect the mean PTV titers in the livers of the mice, with one exception. At the 75-mg/kg per day dose, which was begun 36 h post-virus inoculation, a 1.9 $\log_{10}$ ($P < 0.01$) reduction was seen. Results of this experiment indicate that ribavirin administered s.c. is effective when administered prophylactically as well as therapeutically to mice infected with the Adames strain of PTV.

TI determination. The TI of ribavirin administered s.c. was determined by treating PTV-infected mice with ribavirin twice daily for 7 days beginning 4 h pre-virus inoculation. Ribavirin doses varied twofold, from 150 to 0.6 mg/kg per day. In this experiment, 10 infected mice were used for each drug dose, and 20 animals were used as virus controls; increases in the number of survivors and in the mean survival time were used as parameters of antiviral activity. Toxicity and normal controls were again run in parallel. The results (Table 3) indicated that the compound was effective at levels as low as 4.7 mg/kg per day. Since the 150-mg/kg per day dose level was lethally toxic, 75 mg/kg per day was considered the MTD, although some inhibition of weight gain was seen at lower levels. By using the MTD as it was defined, a TI of 16 was determined. The PTV challenge in this experiment was somewhat more severe than that seen in the s.c. treatment experiments described above, with 95% of the virus control animals dying during the experiment.

Effect of single s.c. ribavirin treatments. An experiment was run in which 700, 350, or 125 mg of ribavirin per kg was administered s.c. in single treatments to PTV-infected mice. In separate but parallel experiments, the dose was administered 4, 12, 24, 48, 72, or 96 h post-virus inoculation. The

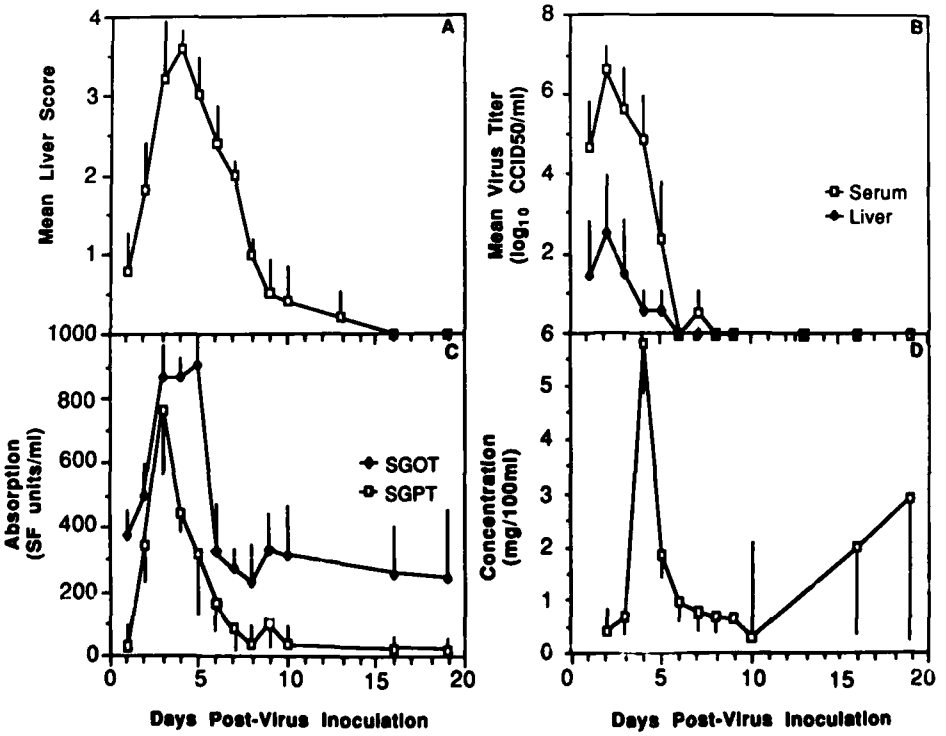

FIG. 1. Development of PTV infection in mice (± standard error). (A) Liver icterus; (B) virus titers in serum and liver; (C) SGOT and SGPT; (D) serum total BN. Abbreviations: SF, Sigma-Frankel; $CCID_{50}$, cell culture 50% infectious dose.

TABLE 2. Effect of s.c. administered ribavirin on PTV infections in mice[a]

Dose (mg/kg per day)	Toxicity control		Infected, treated					
	No. of survivors/total no.	Host wt change (g)[b]	No. of survivors/total no.	Mean survival time (days)[c]	Mean liver score[d]	Mean SGOT[e]	Mean SGPT[e]	Mean liver virus titer (log_{10})
75	5/5	−0.4	10/10[f]	>21.0[g]	0.9[g]	215[g]	48[g]	0.0[g]
37.5	5/5	0.2	8/10	9.0	0.9[g]	248[g]	49[g]	0.6[f]
18.8	5/5	0.6	10/10[f]	>21.0[g]	1.0[g]	271[g]	47[g]	0.3[g]
9.4	5/5	1.0	10/10[f]	>21.0[g]	1.4[g]	728[f]	282	0.4[g]
0			12/20	6.4	2.7	7,022	5,459	1.7
Normal	5/5	0.9			0.6	234	21	0.0

[a] Twice daily for 5 days, beginning 4 h pre-virus inoculation.
[b] Difference between initial weight prior to treatment and weight at 18 h following final treatment.
[c] Mice that died before day 21. Scores of 0 (normal) to 4 (maximum jaundice) were assigned to livers.
[d] Mean liver score of mice killed on infection day 5.
[e] Sigma-Fraenkel units per milliliter in serum taken on day 5.
[f] $P < 0.05$.
[g] $P < 0.01$.

results showed that ribavirin is highly effective (100% survivors) at all doses used, when administered as late as 24 h post-virus inoculation. Treatment at 48 h post-virus inoculation was effective at 350- and 175-mg/kg doses, but the later single treatments resulted in essentially no significant increase in survivor number or in mean survival time.

Effect of oral ribavirin treatment. The effect of per os (p.o.) ribavirin administration to PTV-infected mice was determined (Table 4). Ribavirin was administered twice daily for 5 days and began 4 h pre-virus inoculation, 4 h post-virus inoculation, or 24 h post-virus inoculation; and doses were given in twofold increments from 3.2 to 100 mg/kg per day to determine the TI for each treatment period. Ribavirin was highly effective when used p.o. at each treatment time; the MTD was considered to be 100 mg/kg per day, and the minimum effective dose was approximately 6.3 mg/kg per day at each treatment initiation time. The TI for this p.o. treatment route was thus calculated to be approximately 16, which was the same as that for the s.c. treatment route.

Effect against intracerebral infections. Two experiments were run to determine whether ribavirin administered s.c. twice daily for 9 days beginning 36 h pre-virus inoculation would affect the Balliet strain of PTV infection induced by intracerebral (i.c.) inoculation into 5-week-old mice (weight, 15 to 18 g). Two virus inocula, 1 and 10 50% lethal doses (LD_{50}s), were used in the experiments. At the 1 LD_{50} virus inoculum, 9 of 20 (45%) of the virus control mice survived the infection, with a mean survival time of 12.6 days. At the higher virus dose, 1 of 20 (5%) of the virus control mice survived, with a mean survival time of 11.9 days. Brains removed from the virus controls infected with 1 LD_{50} on infection day 6 had a mean titer of $10^{3.6}$ infectious particles per ml; the brains from mice that received the higher virus inoculum had a mean titer of $10^{5.7}$ infectious particles per ml. Ribavirin treatment had no perceivable inhibitory effect on the infection of animals that received either virus inoculum. The 9-day treatment at the 75-mg/kg per day ribavirin dose resulted in a 0.5-g weight loss during the treatment period, compared with a 0.5-g weight gain in normal controls, indicating that the dose was the approximate MTD.

Influence of PTV dose on antiviral activity of ribavirin. To determine the influence of virus dose on the in vivo anti-PTV activity of ribavirin, ribavirin used at doses of 75, 37.5, 18.8, and 9.4 mg/kg per day was administered twice a day for 7 days, beginning 4 h pre-virus inoculation to four separate groups of mice. Each group received 10-fold increasing doses of the Adames strain of PTV administered s.c. Effects of treatment on the total number of survivors and on the mean survival time were determined for each group. At ribavirin doses of 18.8 to 75 mg/kg per day, marked efficacy was seen, with 80 to 100% survivors in the ribavirin-treated groups compared with 5 to 20% survivors in each of the placebo-treated controls (survivors in virus controls were as follows: 0.1 LD_{50}, 15%; 1 LD_{50}, 15%; 10 LD_{50}, 5%; 100 LD_{50}, 20%). However, at a ribavirin dose of 9.4 mg/kg per day, which was considered to approach the minimum effective dose level, the ribavirin activity declined with increasing virus dose (Fig. 2), with no significant increase in survivors occurring at the 100 LD_{50} PTV dose.

DISCUSSION

Ribavirin was previously found to be inhibitory to the related Rift Valley fever virus infection in vitro and in mice (24). Huggins et al. (8) initially reported preliminary studies on the effect of ribavirin on PTV infections in hamsters. They found that 100 mg of the drug per kg per day, administered by an undefined route from days 0 through 4, resulted in an 80% increase in survivors. In that study,

TABLE 3. TI determination of s.c. ribavirin treatment on PTV infections[a]

Dose (mg/kg per day)	Toxicity control mice		Infected treated mice	
	No. of survivors/total no.	Host wt change (g)[b]	No. of survivors/total no.	Mean survival time (days)[c]
150	1/5	−1.5	10/10[d]	>21.0[d]
75	5/5	1.4	10/10[d]	>21.0[d]
37.5	5/5	1.1	10/10[d]	>21.0[d]
18.8	5/5	1.4	9/10[d]	20.0
9.4	5/5	1.6	4/10[e]	5.3
4.7	5/5	1.1	7/10[d]	6.0
2.3	5/5	1.8	0/10	5.8
1.2	5/5	2.3	3/10	5.6
0.6	5/5	2.4	1/10	4.9
0			1/20	4.8

[a] Twice daily for 7 days, beginning 4 h pre-virus inoculation.
[b] Difference between initial weight and weight at 18 h following final treatment (normal control weight change, 2.7 g).
[c] Mean survival time of mice that died before day 21.
[d] $P < 0.01$.
[e] $P < 0.05$.

TABLE 4. Effect of orally administered ribavirin on PTV infections in mice[a]

Dose (mg/kg per day)	Treatment beginning:					
	4 h pre-virus inoculation		4 h post-virus inoculation		24 h post-virus inoculation	
	No. of survivors/total no.	Mean liver score	No. of survivors/total no.	Mean liver score	No. of survivors/total no.	Mean liver score
100[b]	10/10[c]	0.2[c]	10/10[c]	0.4[c]	10/10[c]	0.8[c]
50	10/10[c]	0.3[c]	10/10[c]	0.3[c]	10/10[c]	3.0
25	10/10[c]	1.2[c]	10/10[c]	1.2[c]	7/10[d]	0.8[c]
12.5	7/10[c]	2.8	6/10[d]	1.2[c]	5/10	3.1
6.3	2/10	2.8	9/10[c]	2.7	7/10[d]	3.3
3.2	6/10[d]	3.1	4/10	3.9	5/10	3.2
0	5/20	3.2	5/20	3.2	5/20	3.2

[a] Treatments were twice daily for 5 days.
[b] Toxicity control mice at the 100-mg/kg per day dose survived but gained only 1.8 g compared with a 3.4-g weight gain in normal controls.
[c] $P < 0.01$.
[d] $P < 0.05$.

ribavirin, used at 25 mg/kg per day, the only other dose used, was essentially inactive. Results of the present study extend and amplify the findings of Huggins et al. (8), which show that ribavirin is effective against PTV both in vitro and in experimentally infected mice. The in vitro data also confirm an earlier observation (7) that the antiviral activity of ribavirin may be somewhat cell dependent.

Activity against PTV was seen when the drug was administered p.o. as well as s.c. This activity correlated well with that observed in other studies, in which ribavirin was well absorbed orally (2) and reached high levels in the liver (4), which appears to be the principal target of the Adames strain of PTV. The drug was wholly ineffective against the i.c. administered Balliet strain of PTV, a result that again correlates with observations in which minimal amounts of ribavirin were found in the brains of treated animals (4). The compound has been reported to be similarly ineffective against i.c. infections in mice induced by herpesvirus types 1 and 2 (17), Semliki Forest virus, Western equine encephalitis virus, or neurotropic influenza virus (21).

We attempted to increase levels of the drug in the brain by beginning treatment of the i.c. infected animals 36 h pre-virus inoculation. This pretreatment was not effective, however. Two doses of the Balliet strain of PTV were used, one being the approximate LD_{50}, to determine whether reduction of the initial viral load would improve the efficacy of ribavirin. No activity was seen at either virus level, which we conclude was a result of the failure of the drug to achieve adequate concentrations in the brains of the animals, especially since the Balliet strain of PTV was markedly sensitive to ribavirin in vitro. This failure to inhibit the infection induced by the i.c. inoculated virus is probably of minor importance, since human infections with phleboviruses do not usually result in encephalitis, except in rare instances with Rift Valley fever (9) and, possibly, Toscana viruses (25).

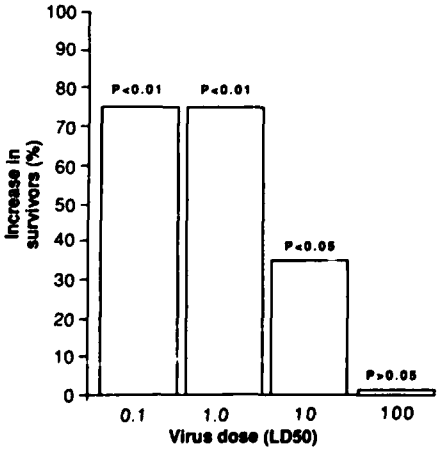

FIG. 2. Influence of PTV dose on in vivo antiviral efficacy of ribavirin (9.4 mg/kg per day, administered s.c. twice daily for 7 days beginning 4 h pre-virus inoculation).

The peripheral infection of susceptible strains of mice with the Adames strain of PTV has been reported previously (Pifat and Smith, in press; Pifat Ph.D. dissertation) to cause severe necrotizing hepatitis, with viral antigen restricted almost exclusively to the liver. Viremia developed to maximal levels between 24 and 48 h postinfection and began to decline by 4 days postinfection. The disease development shown in the present study correlates well with these earlier findings and extends observations of the disease to include development of high SGOT, SGPT, and BN levels in the sera of infected mice. Elevations in transaminase activities are usually associated with necrosis of hepatic parenchymal cells, as well as destruction of certain other tissues, resulting in leakage of the enzymes into the blood (3). Increased amounts of BN in the blood reflects a lack of clearance of this bile pigment from the liver due to hepatic dysfunction or excessive erythrocyte hemolysis (3). The wide BN variation seen in this study may have been a result of the excessive erythrocyte hemolysis that occurred in the process of removing and separating the blood.

The activity of ribavirin against this principally hepatic disease that is induced by an RNA virus matches closely previous reports of the inhibitory effects of the compound against hepatic diseases in mice induced by murine hepatitis (19), Rift Valley fever (24), and Friend leukemia (16) viruses. It is also significant that ribavirin has proven effective against type A (infectious) hepatitis in a number of controlled clinical trials (21).

The mechanism of action of ribavirin against PTV has not yet been determined. The effects of the compound have often been considered multifaceted (22); the 5′-monophosphate metabolite of ribavirin is inhibitory to IMP dehydrogenase, leading to inhibition of viral nucleic acid synthesis (for a review, see reference 21). When Venezuelan equine encephalitis replicates in the presence of ribavirin, the virus appears to lack an isolable cap structure, and the viral

mRNA translates at a very inefficient rate (1). The compound may also interfere with mRNA translation, possibly by being incorporated into the mRNA cap (5). Pifat and Smith (in press) have shown that interferon plays a pivotal role in the control of PTV infections of mice: high titers of interferon in serum were shown in PTV-challenged mice by days 2 to 3 after infection. Ribavirin, in conjunction with interferon, could delay viral synthesis in the animal for a sufficient period of time to enable other immune mechanisms to clear the virus from the animal.

The significant anti-PTV effects of ribavirin, which were seen even when drug administration was delayed until the infection was well established, when treatment was by p.o. as well as by s.c. routes, and with a relatively high TI, suggest that this triazole nucleoside has possible practical use as a therapeutic agent for treating severe *Phlebovirus* infections.

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LITERATURE CITED

1. **Canonico, P. G., J. S. Little, P. B. Jahrling, and E. L. Stephen.** 1980. Molecular aspects of the antiviral activity of ribavirin on Venezuelan equine encephalomyelitis virus, p. 1370–1372. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. 2. American Society for Microbiology, Washington, D.C.
2. **Catlin, D. H., R. A. Smith, and A. I. Samuels.** 1980. C^{14}-ribavirin: distribution and pharmacokinetic studies in rats, baboons and man, p. 83–98. *In* R. A. Smith and W. Kirkpatrick (ed.), Ribavirin: a broad spectrum antiviral agent. Academic Press, Inc., New York.
3. **Cornelius, C. E.** 1980. Liver function, p. 201–257. *In* J. J. Kaneko (ed.), Clinical biochemistry of domestic animals. Academic Press, Inc., New York.
4. **Ferrara, E. A., J. S. Oishi, R. W. Wannemacher, Jr., and E. L. Stephen.** 1981. Plasma disappearance, urine excretion and tissue distribution of ribavirin in rats and rhesus monkeys. Antimicrob. Agents Chemother. **19**:1042–1049.
5. **Goswami, B. B., E. Borek, O. K. Sharma, J. Fujitaki, and R. A. Smith.** 1979. The broad-spectrum antiviral agent ribavirin inhibits capping of messenger RNA. Biochem. Biophys. Res. Commun. **89**:830–836.
6. **Hsiung, C. D.** 1982. Virus assay and neutralization test, p. 25–35. *In* C. D. Hsiung (ed.), Diagnostic virology. Yale University Press, New Haven, Conn.
7. **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.
8. **Huggins, J. W., P. Jahrling, M. Kende, and P. G. Canonico.** 1984. Efficacy of ribavirin against virulent RNA virus infections, p. 49–63. *In* R. A. Smith, V. Knight, and J. A. D. Smith (ed.), Clincal application of ribavirin. Academic Press, Inc., New York.
9. **Maar, S. A., R. Swanepol, and M. Gelfand.** 1979. Rift Valley fever encephalitis. A description of a case. Cent. Afr. J. Med. **25**:8–11.
10. **McCloskey, R. V., and A. Shelokov.** 1965. Further characterization and serologic identification of Chagres virus, a new human isolate. Am. J. Trop. Med. Hyg. **14**:152–155.
11. **Meegan, J. M., R. H. Watten, and L. W. Laughlin.** 1981. Clinical experience with Rift Valley fever in humans during the 1977 Egyptian epizootic. Conf. Epidemiol. Biostat. **3**:114–123.
12. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
13. **Sabin, A. B.** 1948. Phlebotomus fever, p. 454–461. *In* T. M. Rivers (ed.), Viral and rickettsial infections of man. Lippincott, Philadelphia.
14. **Sather, G. E.** 1970. Catalogue of arthropod borne viruses of the world. Am. J. Trop. Med. Hyg. **19**:1103–1104.
15. **Shope, R. E.** 1985. Bunyaviruses, p. 1055–1082. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roisman, and R. E. Shope (ed.), Virology. Raven Press, New York.
16. **Sidwell, R. W., L. B. Allen, J. H. Huffman, J. T. Witkowski, and L. N. Simon.** 1975. Effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) on Friend leukemia virus infections in mice. Proc. Soc. Exp. Biol. Med. **148**:854–858.
17. **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.
18. **Sidwell, R. W., and J. H. Huffman.** 1971. Use of disposable micro tissue culture plates for antiviral and interferon induction studies. Appl. Microbiol. **22**:797–801.
19. **Sidwell, R. W., J. H. Huffman, N. Campbell, and L. B. Allen.** 1977. Effect of ribavirin on viral hepatitis in laboratory animals. Ann. N.Y. Acad. Sci. **284**:239–246.
20. **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.
21. **Sidwell, R. W., G. R. Revankar, and R. K. Robins.** 1985. Ribavirin: review of a broad-spectrum antiviral agent, p. 49–108. *In* D. Shugar (ed.), Viral chemotherapy, vol. 2. International encyclopedia of pharmacology and therapeutics, section 116. Pergamon Press, Inc., Elmsford, N.Y.
22. **Smith, R. A., R. W. Sidwell, and R. K. Robins.** 1980. Antiviral mechanisms of action. Annu. Rev. Pharmacol. Toxicol. **20**:259–284.
23. **Srihougse, S., and K. M. Johnson.** 1974. Human infections with Chagres virus in Panama. Am. J. Trop. Med. Hyg. **23**:690–693.
24. **Stephen, E. L., D. E. Jones, C. J. Peters, G. A. Eddy, P. S. Loinzeaux, and P. B. Jahrling.** 1980. Ribavirin treatment of toga-, arena- and bunyavirus infections in subhuman primates and other laboratory animal species, p. 169–183. *In* R. A. Smith and W. Kirkpatrick (ed.), Ribavirin: a broad spectrum antiviral agent. Academic Press, Inc., New York.
25. **Travassos da Rosa, A. P. A., R. B. Tesh, F. P. Pinheiro, J. F. S. Travassos da Rosa, and N. E. Peterson.** 1983. Characterization of eight new phlebotomus fever serogroup arboviruses (Bunyaviridae: *Phlebovirus*) from the Amazon region of Brazil. Am. J. Trop. Med. Hyg. **32**:1164–1171.
26. **Verani, P., M. C. Lopes, L. Nicoletti, and M. Balducci.** 1980. Studies on phlebotomus-transmitted viruses in Italy. I. Isolation and characterization of a sandfly fever Naples-like virus, p. 195–201. *In* J. Vasenjak-Hirjan (ed.), Arboviruses in the Mediterranean countries. Gustav Fisher Verlag, Stuttgart, Federal Republic of Germany.