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Efficacy of high dose–short duration ribavirin aerosol in the treatment of respiratory syncytial virus infected cotton rats and influenza B virus infected mice

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Summary

Fifteen to 20 mg/ml ribavirin administered as a small particle aerosol for 10 – 18 h per day is currently the regimen generally used to treat experimental or naturally-occurring respiratory syncytial (RS) or influenza virus infections in humans. To determine if such prolonged treatment schedules could be reduced, cotton rats and mice were inoculated with RS or influenza B virus, respectively, and then treated with different concentrations of ribavirin small particle aerosols. Aerosols generated from reservoirs containing 60 mg/ml ribavirin given 2 h twice daily, protected cotton rats from RS virus and mice from influenza B virus as well as aerosols generated from reservoirs containing 20 mg/ml ribavirin given 11 h daily. Aerosols generated from reservoirs containing 40 or 20 mg/ml given 2 h daily were less efficacious. There was no evidence of intolerance or pulmonary histopathology in infected or uninfected animals exposed to any of the doses of ribavirin tested. These studies indicate that use of aerosols containing higher concentrations of ribavirin than generally used to treat respiratory virus diseases may permit significantly shorter treatment schedules without loss of efficacy or increase in toxicity.

Ribavirin; Aerosol treatment; Influenza; Rsv

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Introduction

Ribavirin, 1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide, is an antiviral that has been used successfully in small particle aerosol in clinical trials to treat respiratory syncytial (RS) [1,5,11] and influenza [2,4,6,12] virus infections of children and adults. This drug has recently been licensed for use as a small particle aerosol for the treatment of serious RS virus infections in children. Presently, the general use of long treatment schedules (10–18 h daily) discourage the use of ribavirin for other than severe illness. If treatment schedules could be significantly reduced without loss of efficacy, the breadth of use of this modality of treatment could be increased.

We have previously shown that by increasing the concentration of ribavirin in the delivery reservoir, mice inoculated with influenza A virus could be protected from lethal disease using two 2 h daily exposures to ribavirin aerosols as well as mice exposed to continuous 11 h daily regimens [17]. No untoward effects were observed in mice exposed to the higher concentrations of ribavirin aerosol. In this study, we measured the effects of increasing ribavirin concentrations and shortening duration of daily treatments on RS virus infection of cotton rats and influenza B virus infection of mice.

Materials and Methods

Animals

Three- to 6-wk-old cotton rats (*Sigmoden hispidus*) of either sex were used in all experiments. These animals were obtained from a colony maintained by us and each was a descendent of 2 pairs of cotton rats obtained from the Small Animal Section, Veterinary Research Branch, Division of Research Service, National Institutes of Health.

Mice used in the experiments were 6- to 8-wk-old CD-1 male mice obtained from Charles River Breeding Laboratories (Wilmington, MA). All animals were housed in cages covered with barrier filters and fed mouse chow and water ad libitum.

Virus

Seed RS (Long strain) and influenza B/Hong Kong/72 viruses were obtained from the American Type Culture Collection (Rockville, MD). A stock RS virus pool was prepared in HEp-2 tissue monolayers as described previously [10]. The influenza B/Hong Kong/72 virus stock used was a mouse lung suspension prepared as has been described in detail previously [14]. In the present study animals lightly anesthetized with ether were inoculated intranasally (i.n.) with either 100 median infectious doses (MID_{50}) of RS virus or four median lethal doses (MLD_{50}) of virulent sixth passage influenza virus. Control animals in experiments to evaluate histologic changes were inoculated with either HEp-2 or normal lung lysates.

Collection and processing of lungs

Cotton rats and mice to be tested for pulmonary virus titers were killed by cervical dislocation on day four after virus inoculation. In the case of mice which received 4 days of aerosol treatment, this was done prior to the initiation of the day 4 aerosolization. Lungs were removed and one lobe from each was placed in formalin for histologic studies. The remaining lobes were homogenized using glass tissue homogenizers (cat. no. K886600; Kontes, Vineland, N.J.) and a Wheaton overhead stirrer (model 903475; Millville, N.J.). The homogenates were clarified by centrifugation (100 × *g*) and tested immediately for virus.

Virus quantification

Lung homogenates derived from animals inoculated with influenza B virus were serially diluted using minimal essential medium and tested in Madin Darby canine kidney (MDCK) tissue cells (Flow Laboratories, McLean, VA) using trypsin containing medium as described previously [14]. After incubation for 5 days at 37°C, 0.05 ml of a 0.5% suspension of chicken erythrocytes was added to each well. Wells exhibiting hemagglutination were considered to be infected with influenza virus.

Each dilution of homogenate derived from animals infected with RS virus was tested in HEp-2 cells and incubated at 37°C. Wells in plates used to detect RS virus were observed for syncytia formation on day 5 after inoculation. Wells exhibiting syncytia were considered to be infected with RS virus.

Histological methods and evaluations

After fixation in buffered formalin for a minimum of 24 h, all lung tissues were embedded in low-melting point paraffin, sectioned at 5 μm thickness, and stained with hematoxylin eosin. The stained sections were coded by number and given to Drs. Donald Greenberg and Toshiaki Kawai, Department of Pathology, Baylor College of Medicine, Houston TX, for blinded evaluation of histopathology.

Aerosol treatments

Aerosol machines using Collison nebulizers modeled after the design of K.R. May [3] were used in these experiments. These machines and their use to deliver antivirals have been described in detail previously [13,14,16]. Ribavirin was obtained from ICN Pharmaceuticals, Costa Mesa, CA. Suspensions of this drug were prepared in water containing 20, 40, or 60 mg/ml. Aerosol was delivered at 12.5 l/min to animals kept in plastic cages covered with plastic tops. (The 40 mg/ml ribavirin was delivered only to mice, because of the limited availability of cotton rats.) Placebo animals receiving aerosols containing water only were not routinely included as extensive preliminary testing showed water aerosol alone to have no effect on replication of virus or the disease induced by either RS or influenza B virus. Cotton rats were treated with ribavirin aerosols on days +1 through +3 after virus inoculation; mice were treated with drugs on days +1 through +4.

Ribavirin quantification

Quantification of ribavirin deposited in lungs of cotton rats exposed to contin-

uous small particle aerosol was performed using a Waters (Danvers, MA) high performance liquid chromatography (HPLC) system as described previously [8]. Briefly, lungs were removed from animals exposed for 2 h of continuous aerosol, rinsed of surface blood and homogenized in 2.0 ml of water. The resulting homogenates were centrifuged at 13000 × *g* to remove large debris and then deproteinized by ultrafiltration through CF25 ultrafiltration cones. The ultrafiltrates were added to a phenyl boronate affinity column and ribavirin was eluted with 0.1 M formic acid. Each eluate was lyophilized, resuspended and assayed by reverse phase HPLC.

To determine if residual ribavirin was present in lung homogenates from animals being tested for virus, portions of representative samples were irradiated with ultraviolet (UV) light (germacidal bulb at a distance of 30 cm for 7 min) to kill any virus present. These samples and homogenates from uninfected lungs were then serially diluted (two-fold dilutions) and compared with medium controls for their ability to inhibit added (approximately 10 $TCID_{50}$) influenza or RS virus.

Statistics

The statistical significance betweeen the geometric mean titer (GMT) of virus in the untreated control group and different experimental groups was determined using the Wilcoxon nonpaired rank sum test [9]. The statistical significance of the final mortality in each experimental group and the control group was determined using chi-square 2 × 2 contingency tables [9].

Results

RS virus studies

As indicated in Table 1, all cotton rats exposed to small particle aerosols of ribavirin had reduced virus titers in their lungs as compared to virus titers observed

Table 1

Comparison of virus titers in lungs of cotton rats inoculated with respiratory syncytial virus and treated with different doses of rivabirin aerosol

Group	Ribavirin administration schedule[a] (h)	Ribarivin reservoir concentration (mg/ml)	Pulmonary RS virus titer (log_{10}/g of lung)[b]	
			Expt. 1	Expt. 2
1	(No treatment)	–	4.4 ± 0.5	4.6 ± 0.4
2	1 × 11	20	3.4 + 0.8	3.1 + 0.8
3	2 × 2	20	3.9 ± 0.6	4.2 ± 0.5
4	2 × 2	60	3.2 + 1.0	3.1 + 0.8

[a] Number of exposures to small particle aerosol per day × the duration of each exposure. Treatments were given on day +1, +2 and +3.

[b] Geometric mean titer ± standard deviation; underlined value indicate statistical significance of $p<0.01$ compared to group 1 (untreated, virus controls) using the Wilcoxon nonpaired rank sum test and 10 degrees of freedom. Number of animals/group in experiment 1 = 16, in experiment 2 = 6.

Table 2

Comparison of virus titers and deaths in mice inoculated with influenza B/HK/72 virus and treated with different doses of ribavirin aerosol.

Group	Ribavirin administration schedule[a] (h)	Ribavirin reservoir concentration (mg/ml)	Pulmonary influenza virus titer (log_{10}/g of lung)[b]		Deaths[c] no. dead
			Expt. 1	Expt. 2	no. inoc.
1	(No treatment)	–	6.1 ± 1.1	6.6 ± 0.4	8/11
2	1 × 11	20	4.8 + 0.9	5.0 + 0.8	0/10
3	2 × 2	20	5.4 ± 0.5	5.9 ± 0.4	3/10
4	2 × 2	40	5.0 ± 0.4	5.3 + 0.5	0/10
5	2 × 2	60	4.5 + 0.8	5.1 + 0.7	0/10

[a] Number of exposures to small particle aerosol per day × the duration of each exposure. Treatments were given on day +1 through +4. Animals collected on day +4 for determination of virus titers were sacrificed prior to the start of treatment on this day.

[b] Geometric mean titer ± standard deviation; underlined values indicate statistical significance of $p<0.05$ compared to group 1 (untreated, virus controls) using Students t-test and 10 degrees of freedom.

[c] Cumulative number of deaths/group on day 10 after virus inoculation (inoc); underlined entries indicate statistical significance with $p<0.05$ compared to untreated virus controls using 2 × 2 chi-square contigency tables and one degree of freedom.

in lungs of virus inoculated, untreated control cotton rats (group 1). In both experiments statistically significant and comparable reductions in pulmonary virus titer were observed in groups 2 (1.0 log_{10} reduction in experiment 1 and 1.5 log_{10} in experiment 2) and 4 (1.2 log_{10} reduction in experiment 1 and 1.5 log_{10} in experiment 2), the groups which received 20 mg/ml of ribavirin 11 h/day and 60 mg/ml of ribavirin 2 h twice daily, respectively. Cotton rats receiving 20 mg/ml of drug 2 h twice daily had no significant reduction in virus titer as compared to virus controls in either experiment.

Influenza virus studies

All groups of animals inoculated with influenza virus that were exposed to ribavirin aerosols also had reduced pulmonary virus titers when compared to virus titers in the untreated control group (Table 2). Highly significant ($p<0.01$) and similar reductions in titers occurred in groups 2 and 5 in both experiments. However, significant protection ($p<0.05$ compared to virus controls) was observed in group 4, the group exposed to 40 mg/ml ribavirin twice daily for 2 h, only in experiment 2. No significant reductions in pulmonary virus titers were observed in either experiment in the group given 20 mg/ml ribavirin 2 h twice daily.

As indicated in the last colomn in Table 2, all groups of mice exposed to small particle aerosols of ribavirin had fewer deaths than group 1, the untreated virus control group. Significant protection ($\chi^2 = 8.9$, $p<0.01$ compared to controls) was observed in the group treated with 20 mg/ml of ribavirin 11 h/day for 4 days (group 2), and in the groups of mice treated with 40 and 60 mg/ml 2 h twice daily for 4 days (groups 4 and 5, respectively). In contrast, mice receiving 20 mg/ml of drug 2 h twice daily were not significantly protected ($p>0.05$) from lethal disease.

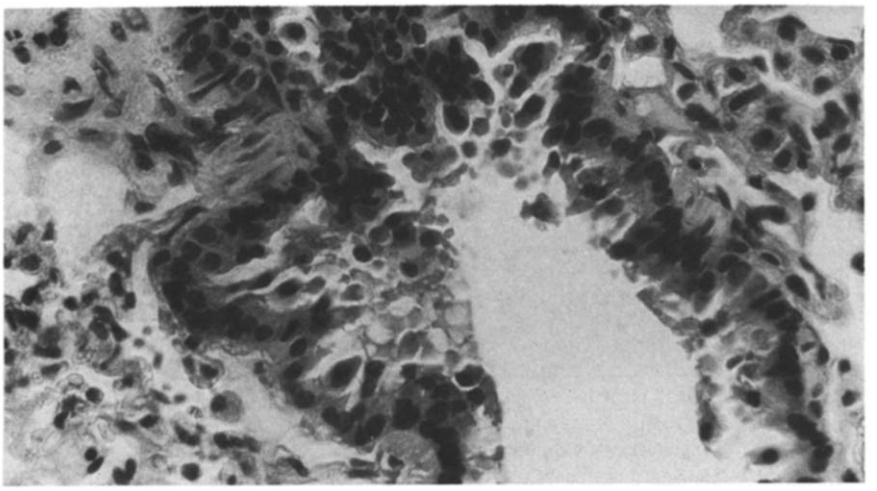

Fig. 1. Bronchiolitis, focal pneumonia and necrosis in lungs from an untreated, respiratory syncytial (RS) virus infected cotton rat, day +4, 100×; stained with hematoxylin and eosin (HαE).

Histologic findings

Examination of sections of lung from untreated RS virus infected control cotton rats collected on day +4 after inoculation of virus revealed evidence of bronchiolitis, focal pneumonia and necrosis of the broncholar epithelium in nearly all microscopic fields (Fig. 1). In contrast, sections of lung collected on day +4 from infected cotton rats treated with 20 mg/ml of ribavirin 11 h/day (not shown) or 60 mg/ml twice daily (Fig. 2) exhibited only focal bronchiolitis and occasional areas of peribronchiolar pneumonia. Bronchiolitis and moderate focal pneumonia were observed in most microscopic fields in sections of lung from infected cotton rats

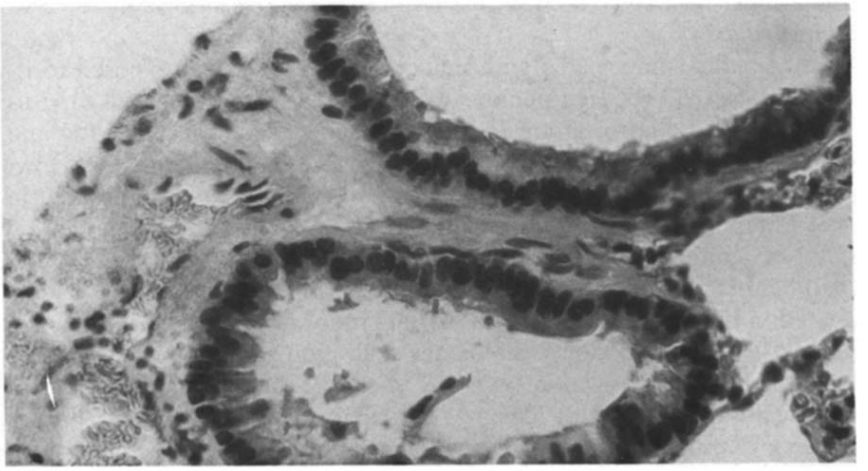

Fig. 2. Reduced bronchiolitis and pneumonia in lungs from a RS virus infected cotton rat treated twice daily on days +1 through +3 for 2 h with 60 mg/ml ribavirin aerosol, day +4; 100×, stained with HαE.

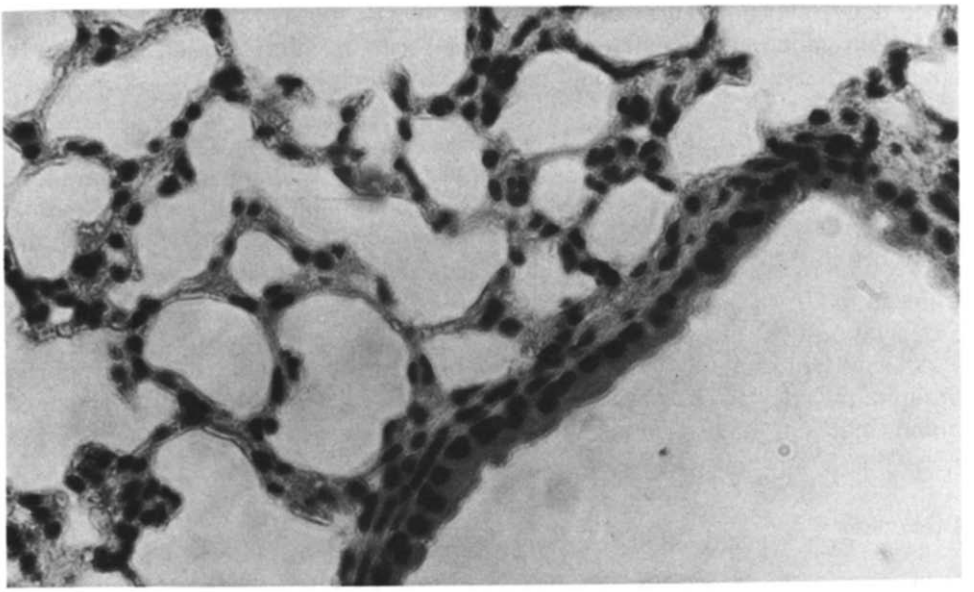

Fig. 3. Lung from an uninfected cotton rat exposed twice daily on days +1 through +3 for 2 h to 60 mg/ml ribavirin aerosol, day +4; no apparent histopathologic changes are apparent; 100×; HαE stain.

treated with 20 mg/ml of drug intermittently (not shown). No evident histopathology was observed in sections of lung from untreated cotton rats inoculated with HEp-2 lysate or uninfected cotton rats given 60 mg/ml of ribavirin twice daily (Fig. 3).

Similar histologic findings were found in lungs of mice given influenza B virus and treated with the different doses of ribavirin aerosols; mice treated with 20 mg/ml ribavirin daily for 11 h or with 60 mg/ml twice daily for 2 h had the least evidence of histopathologic changes. Infected mice given 20 mg/ml ribavirin 2 h

Table 3

Concentration of ribavirin in lungs of cotton rats and mice exposed for 2 h to continuous small particle aerosols of ribavirin[a].

Ribavirin reservoir concentration (mg/ml)	Pulmonary ribavirin concentration (nanomoles/g of lung)	
	Cotton rat	Mouse
0	0	0
20	20.4 ± 4.1	66.4 ± 8.5
40	N.D.[b]	82.8 ± 16
60	43.2 ± 13.3	167.2 ± 35.2

[a] Mean ± standard deviation determined by high performance liquid chromatography; 4–6 animals/group.

[b] Not done.

twice daily, and untreated animals had the most extensive histopathologic findings. Untreated mice inoculated mouse lung lysate or uninfected mice exposed to 60 mg/ml ribavirin twice daily had no evident histopathology.

Pulmonary drug deposition

As shown in Table 3, mean (± standard deviation) pulmonary concentrations of ribavirin in cotton rats exposed for 2 h to continuous small particle aerosols generated from reservoirs containing 20 or 60 mg/ml of drug were 20.4 ± 4.1 and 43.2 ± 13.3 nmole/g of lung, respectively. Concentrations of drug in mice similarly exposed to 20, 40 or 60 mg/ml of drug were 66.4 ± 8.5, 82.8 ± 16.0 and 167.2 ± 35.2 ng/g of lung, respectively.

A significant antiviral activity was not observed in lung homogenates from any group of mice tested in assays looking for residual ribavirin (i.e., in UV irradiated homogenates of lungs from mice being tested for virus titers).

Discussion

The results of these studies show that the same level of protection against RS and lethal influenza B virus infections in cotton rats and mice can be achieved with intermittent 4 h treatment regimens of ribavirin aerosols as can be achieved with continuous 11 h daily regimens, if the concentration of ribavirin in the aerosol generator reservoir is increased from 20 mg/ml to 60 mg/ml. Aerosols generated from reservoirs containing 40 mg/ml given 2 h twice daily also provided significant protection against lethal influenza disease, although in some experiments (e.g., experiment 1 in Table 2) the degree of protection against pulmonary virus replication was less than that seen with the higher ribavirin concentrations. (The 40 mg/ml concentration was not tested against RS virus infection.) In contrast, the 20 mg/ml concentration of ribavirin given 2 h twice daily did not significantly reduce pulmonary virus titers in any experiment or significantly reduce mortality in mice given lethal doses of influenza B virus.

In these experiments both cotton rats and mice exposed to 60 mg/ml concentrations of ribavirin exhibited 2-fold greater accumulations of drug in their lungs after 2 h of continuous treatment than animals exposed to 20 mg/ml concentration of drug (Table 3). (In the earlier study [17] up to 4-fold differences between the two groups were noted.) However, no histologic evidence of pulmonary toxicity or obvious untoward effects were observed in infected or uninfected mice or cotton rats treated with 60 mg/ml or lesser doses of ribavirin. Kinetic studies of pulmonary levels [17] suggest that one factor that may help reduce toxicity is the rapid decline in pulmonary levels which occurs when aerosol delivery is stopped.

As shown in Table 3, cotton rats exposed to the continuous small particle ribavirin aerosols accumulated much lower levels of ribavirin than mice similarly exposed. This species difference in pulmonary drug accumulation may be a result of the lower minute ventilation per unit body weight of cotton rats as compared to mice [6]. Humans have still lower minute ventilation per unit body weight values

than either the cotton rats or mice [6] and may be expected to accumulate lower levels of ribavirin than either rodent species. However, humans exposed to higher drug concentrations would still be expected to have increased levels of ribavirin in their lungs than people exposed to lower aerosol concentrations of this drug.

The equivalent efficacy of higher concentrations of ribavirin administered for shorter delivery intervals as more conventional and longer treatments has now been seen with 3 viruses (RS, influenza B and influenza A) and in two rodent species, (e.g., mouse and cotton rat). Together the data suggest that it may be that total dosage and not duration of treatment, that is the primary factor determining the efficacy of ribavirin.

The importance of these findings lies in the fact that if successful treatment of respiratory virus disease with small particle aerosols of ribavirin can be achieved with shortened treatment periods and without loss of efficacy or increase in toxicity, this mode could be used to treat moderate RS, influenza and other respiratory diseases. The data presented suggests that high dose–short duration ribavirin aerosol may be successful in the treatment of respiratory virus infections in humans.

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