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Further studies with short duration ribavirin aerosol for the treatment of influenza virus infection in mice and respiratory syncytial virus infection in cotton rats

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Summary

Ribavirin aerosol administration has been shown to be effective in the treatment of respiratory syncytial virus (RSV) infections in infants and in influenza A and B virus infections in young adults. Long treatment schedules and potential for environmental contamination have stimulated the search for alternative dosing schedules. Thus, we attempted to determine the length of time of ribavirin aerosol necessary for effective treatment of influenza and RSV. In RSV-infected cotton rats, aerosolization for just 30 min with high-dose ribavirin (HDR:60 mg ribavirin/ml in reservoir), 3 times daily, reduced viral lung titers/gm of tissue by 1.1 $\log_{10}$. In influenza virus-infected mice, 15 min of aerosolized HDR, 3 times daily, was effective in reducing both mortality and pulmonary virus titers (1.1 $\log_{10}$ reduction). When the intervals between aerosol administration each day were equally divided (i.e., q.8 h), the treatments were most effective. Treatment for 45 min, once daily, was not as effective as divided doses. Calculations of ribavirin concentrations in respiratory secretions following 15 min treatment in mice with HDR indicated that drug levels dropped below the ED_{50} for influenza viruses after about 9 h. A daily dosage of ribavirin, estimated to be 8–15 mg/kg, was effective for the treatment of influenza and RSV infections.

Ribavirin; Aerosol; Respiratory syncytial virus; Influenza; Animal

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Introduction

Ribavirin aerosol administration has been shown to be effective in the treatment of influenza A and B virus infections in young adults (Gilbert et al., 1985; Knight et al., 1981; McClung et al., 1983; Wilson et al., 1984) and in respiratory syncytial virus (RSV) infections in infants (Taber et al., 1983; Hall et al., 1983; Hall et al., 1985). Long treatment schedules and the potential for environmental contamination have stimulated a search for alternative dosing schedules. In previous animal studies, high dose ribavirin (HDR) (60 mg of ribavirin/ml in the reservoir) administered for 2 h, twice daily was as effective as the standard (20 mg of ribavirin/ml in the reservoir), 11–18 h daily regimen in the treatment of influenza A and B virus infections in mice (Wyde et al., 1986; Wyde et al., 1987) and of RSV infections in cotton rats (Wyde et al., 1987).

In a recent clinical trial of RSV in infants (Englund et al., 1990), 9 children received HDR aerosol treatment for 2 h, 3 times daily for up to 5 days. Mean peak ribavirin concentrations in respiratory secretions obtained from these children equalled 1725 μM and virus titers were reduced by more than 98% on day 3 of therapy. HDR administration was well tolerated by all patients. Although this was not an efficacy study, the data suggest that HDR treatment was as good as the standard treatment and was without toxicity associated with the more concentrated ribavirin in the aerosol (600 vs. 200 μg/l of aerosol). In this clinical trial and the above animal studies, the time of aerosol administration was reduced, but with the increase in ribavirin concentration, the total ribavirin dosage was similar between the HDR and the standard regimen.

In this report, the duration of HDR aerosol necessary for effective treatment was determined for influenza and RSV infections in mice and cotton rats, respectively. Establishment of short treatment periods should help determine safe and effective regimens for the administration of ribavirin to humans while reducing exposure of hospital personnel and others to potentially dangerous aerosols (Fackler et al., 1990; Gladu and Ecobichon, 1989).

Materials and Methods

Materials

Ribavirin was provided by Viratek (Costa Mesa, CA).

Animals

Six- to eight-week-old (25–28 g), random-bred, CD-1 mice obtained from Charles River Breeding Laboratories, Inc, Wilmington, MA, were used in all experiments. The animals were housed in cages covered with barrier filters and fed mouse chow and water ad libitum.

Three- to six-week old cotton rats (*Sigmoden hispidus*, 50–100 g) of mixed sexes were used in all experiments. These animals were obtained from a colony maintained by us. All animals were maintained in cages with barrier filters and fed water and food ad libitum.

Virus inoculation

Mice were inoculated with influenza A/HK by small particle aerosol (Wilson et al., 1980). Briefly, a 1:500 or a 1:750 dilution of a mouse lung pool of influenza A/HK/68 (H3N2) (1×10^6 $TCID_{50}$/ml) in 0.5% gelatin-MEM was administered by small particle aerosol for 20 min; the estimated exposure was approximately 2 $TCID_{50}$ of virus/animal. For the next 4 days (days +1 through +4), animals were treated with ribavirin or placebo aerosol. On day 4 (the day of peak virus titer) prior to drug administration, and on day 7, 5 mice from each group were killed and tested for virus as described below. The remaining animals were observed for mortality for 21 days.

Cotton rats (day 0) were weighed, anesthetized lightly with ether and inoculated intranasally (i.n.) with approximately 100 median cotton rat infectious doses ($CRID_{50}$) of RSV in 0.1 ml (Wyde et al., 1987; Wyde et al., 1990). On day +1 through day +3, animals were exposed to a small particle aerosol of ribavirin or placebo. On day 4, the day of maximum RSV pulmonary infection in untreated cotton rats, all animals were killed. The lungs of each animal were removed, weighed and assayed for virus levels as previously described (Wyde et al., 1990).

Virus quantification

Lung homogenates from animals inoculated with influenza A/HK/68 virus were serially diluted using minimal essential medium (MEM) and tested in Madin Darby canine kidney (MDCK) tissue cells using MEM containing trypsin (2 μg/ml) without fetal calf serum (FCS) as described previously (Wyde et al., 1987). 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.

In assays for RSV, serial half $\log_{10}$ dilutions of each virus sample were made in 2% FCS-MEM. Approximately 3×10^3 HEp2 cells were then added to each well. Plates were placed in a 35°C, 5% CO_2 incubator for 7 days. Wells were observed daily for formation of syncytia or other CPE. Mean virus titers ($\log_{10}$/g of lung tissue) were determined by calculating the means of the last dilutions in replicated rows that contained virus (Wyde et al., 1990).

Small particle aerosol treatment

Mice or cotton rats were placed in sealed plastic cages and exposed to aerosols as described previously (Gilbert and Wyde, 1988; Wyde et al., 1986).

The aerosol generator and particle characteristics were as described by Knight et al. (1986) and Wilson et al. (1980). The average concentration of ribavirin delivered in the 'high dose' or 'standard dose' aerosol was 600 or 200 μg of ribavirin/l of air for a reservoir containing 60 or 20 mg of ribavirin/ml, respectively. The median in vitro effective inhibitory concentration (ED_{50}) for this virus was 10 μg of ribavirin/ml.

Statistical analysis

Data analysis was performed using True Epistat™ statistical package from Epistat Services, Richardson, Texas. *P*-values are based on two-tailed analysis of these data by the Student's *t*-test, with ANOVA; Fisher's exact test; or by Life Table Analysis.

Results

Effect of duration of treatment on influenza infected mice

Influenza virus infected mice were treated with HDR for 15 min, 3 times a day or once daily for 45 min. Initially, the 3, 15 min treatments were given at 4 h intervals (08.00, 12.00, 16.00 h). As shown in Fig. 1A, when the virus challenge was low (i.e., mortality $<50\%$ in the control group), a daily aerosol treatment of 45 min completely protected mice from death ($P = 0.001$, Fisher's exact test). In contrast, when the virus challenge was high (i.e., mortality $>75\%$ in the control group; Fig. 1B, closed squares), the 3, 15 min treatment regimen prolonged survival (closed circles; $P = 0.006$, Gehan's Wilcoxon test), but did not significantly protect the mice from death ($P = 0.176$, Fisher's exact test). A single, 45 min treatment administered at 08.00 h each day failed to prolong survival or protect against death (closed triangles; $P = 0.206$, Gehan's Wilcoxon test, and 0.176, Fisher's exact test, respectively).

In the next experiment, ribavirin was administered 3 times daily at 8 h intervals (07.00, 15.00, 23.00 h) for 4 days beginning 24 h after virus exposure. When the virus challenge was low, this treatment regimen was effective in prolonging survival and protecting against death ($P = 0.014$, Gehan's Wilcoxon test, and 0.044, Fisher's exact test, respectively) (Fig. 2A). A single, 45 min treatment once daily for 4 days beginning 24 h after virus exposure was not effective against either prolonging survival or death. However, when the virus challenge was high, the 15 min, q.8 h treatment regimen was very effective in prolonging survival and protecting against death ($P < 0.001$, Gehan's Wilcoxon test, and $P < 0.001$, Fisher's exact test, respectively). Although the single, 45 min treatment repeated daily for 4 days did protect against death ($P = 0.043$, Fisher's exact test), it failed to significantly prolong survival and was not as effective in these parameters as the 3, 15 min treatments ($P = 0.012$, Gehan's Wilcoxon test, and 0.038, Fisher's exact test, respectively) (Fig. 2B).

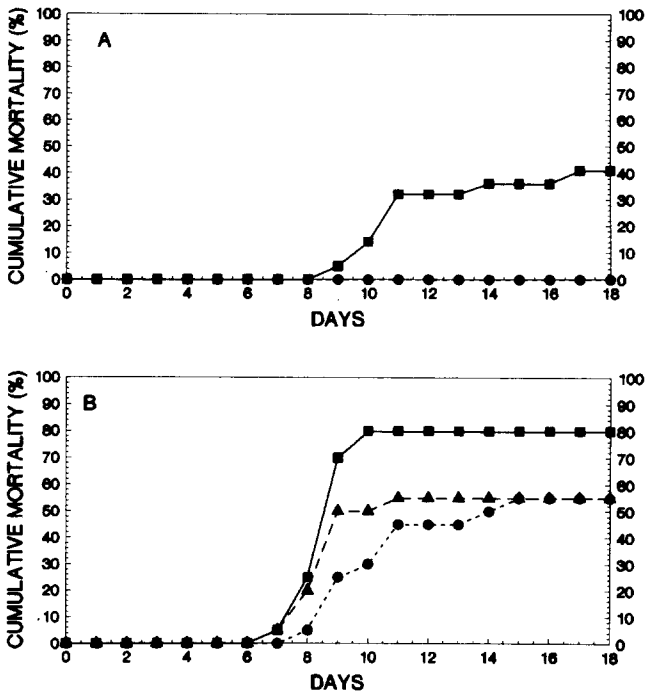

Fig. 1. Effect of high dose ribavirin aerosol treatment on the mortality of influenza virus infected mice. Mice (n = 20 in each group) were not treated (■) or treated with ribavirin aerosol for 15 min, 3 times during a single, 8 h period (●) on days +1 through +4. Experiments with low mortality (A) and high mortality (B) are shown. Animals treated once for 45 min (▲) on days +1 through +4 were included in B.

Table 1 displays pulmonary virus titers obtained on day 4 following virus inoculation (the day of peak virus titer). As indicated, significant reduction of pulmonary influenza virus titers occurred in mice given 3, 15 min treatments of ribavirin daily, but not in mice given a single, 45 min daily regimen (P = 0.006 and 1.0, respectively; Student's t test). Similar findings were seen on day 7 (3 days post-treatment) when there was little chance of ribavirin remaining in the lungs and affecting virus quantification (Gilbert and Wyde, 1988). On day +7, only animals given the 3, 15 min daily treatments had significantly lower pulmonary influenza virus titers compared to control animals (data not shown). The prolonged survival rate, protection from death and reduction in pulmonary virus titers in mice given the daily 3, 15 min treatments was similar to that reported for HDR given for 2 h, twice daily or the standard treatment regimen given over 11 h for the treatment of influenza in mice (Wyde et al., 1986; Wyde et al., 1987).

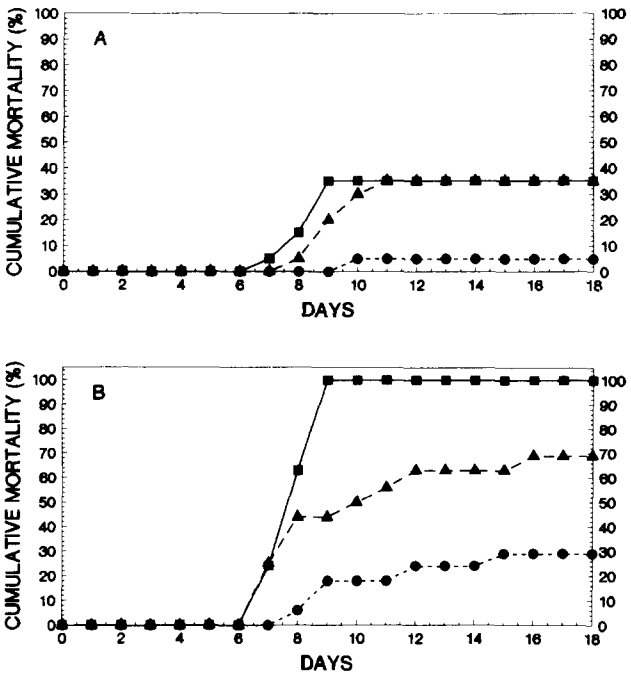

Fig. 2. Effect of high dose ribavirin aerosol treatment on the mortality of influenza virus infected mice. Mice (n = 20 in each group) were not treated (■), treated with ribavirin aerosol for 15 min every 8 h (●), or treated once for 45 min (▲) on days +1 through +4. Experiments with low mortality (A) and high mortality (B) are shown.

TABLE 1

Effect of the duration of ribavirin aerosol treatment on the pulmonary titer of influenza virus in mice and RSV in cotton rats

Treatment schedule[1]	Virus titer[2]		P value[3]
	Control	Treated	
Mice/influenza virus			
60 mg/ml; 3 × 15 min/day; q.8 h	5.7 ± 0.4	4.6 ± 0.5	0.006
60 mg/ml; 1 × 45 min/day	5.8 ± 0.4	5.7 ± 0.5	1.0
Cotton rats/RSV			
60 mg/ml; 3 × 15 min/day; q.8 h	4.46 ± 0.41	3.85 ± 0.65	>0.05
20 mg/ml; 1 × 18 h/day	4.46 ± 0.41	3.05 ± 0.22	0.0003
60 mg/ml; 3 × 30 min/day; q.8 h	4.47 ± 0.42	3.38 ± 0.51	0.017
20 mg/ml; 1 × 18 h/day	4.47 ± 0.42	3.38 ± 0.27	0.012

[1]Ribavirin concentration in the reservoir; duration of aerosol treatment for mice was 4 days and for cotton rats was 3 days starting 24 h after virus inoculation; [2]Mean $\log_{10}$ (± S.D.)/lung for mice (n = 5) or $\log_{10}$ (± S.D.)/g of lung for cotton rats (n = 4) determined on day +4 following virus inoculation; [3]Control vs. treated.

Effect of duration of treatment on RSV infected cotton rats

Because RSV infection of cotton rats does not cause death, only changes in pulmonary virus titers were used as an indication of drug effectiveness. RSV titers were determined on day +4, the day of peak pulmonary virus titer in untreated animals. When cotton rats were treated q.8 h for 3 days beginning 24 h after virus inoculation with HDR for only 15 min, there was no statistically significant reduction in pulmonary virus titer (Table 1), although the positive control (18 h of 20 mg of ribavirin/ml in the reservoir) was effective. However, when each treatment was extended to 30 min and repeated for 3 days, pulmonary virus titers were reduced significantly compared to titers in control animals ($P = 0.017$, Student's t test). This decrease in pulmonary virus titer in animals given the 3, 30 min treatments was observed in multiple experiments and was similar to that reported for HDR given 2 h, twice daily (Wyde et al., 1987). The results suggest that with RSV in the cotton rat, slightly longer periods of ribavirin aerosol treatment may be required than with influenza in mice.

Discussion

In early clinical trials and animal studies, influenza virus- or RSV-infected people or animals were treated with as much ribavirin aerosol as possible, without evidence of toxicity (reviewed in Knight and Gilbert, 1988). The standard treatment regimen was 20 mg of ribavirin/ml in the reservoir for 11–18 h. To make treatment less time consuming and more practical for administration to less severely ill individuals, and to reduce the time of possible exposure to aerosol by health care workers and family members, we have successively tested shorter delivery regiments and have determined the minimum time (and therefore, dose) for administration of HDR aerosol.

It has been determined that increasing the concentration of ribavirin in the reservoir from 20 to 60 mg/ml does not affect the aerodynamic characteristics of the aerosol (Englund et al., 1990; Byron et al., 1988) and that the output of ribavirin is proportional to the reservoir concentration (at ribavirin concentrations >60 mg/ml, the nebulizer may plug due to cooling and concentration of drug during the aerosolization process). Pharmacokinetic studies in mice have shown that ribavirin reaches higher concentrations in the lungs following HDR administration, but that it is cleared rapidly as with the standard dose (Gilbert and Wyde, 1988). In humans, HDR has been demonstrated to be safe and well tolerated in a group of elderly patients with chronic obstructive pulmonary disease, who were followed prospectively with pulmonary function tests (Liss and Bernstein, 1988). In a pediatric study, ribavirin concentrations in respiratory secretions were in the range predicted from the animal studies and were well tolerated (pulmonary function tests were

not done). Thus, HDR aerosol appears to be safe and may be as effective as the standard treatment.

Although the effectiveness of HDR aerosol administration used in the experiments performed in these studies with influenza virus appeared to vary according to the dose of challenge virus, 3 divided doses of ribavirin aerosol given at 4 or 8 h intervals daily were clearly more effective than an equivalent daily dose given only once. With high virus challenge, it was necessary to spread the treatment over 3 equal, 8 h periods (i.e., q.8 h) instead of 3 treatments within a single, 8 h period which was effective when low virus challenge (i.e., low mortality) occurred.

Calculations from pharmacokinetic data using a half-life of 2 h for ribavirin in the lungs (Gilbert and Wyde, 1988) indicated that the concentration of ribavirin in the lungs following HDR would decrease to below the in vitro 50% effective dose (ED_{50}; 10 μg/ml or 40 μM) after about 9 h post-treatment; it would be one-tenth this value by 16 h. Using multiple treatments of 15 min each at 8 h intervals, the concentration of ribavirin in the lungs remains above the in vitro ED_{50} value. These experiments suggest that the 15 min, q.8 h treatment regimen is an effective treatment for significantly reducing influenza virus in mice.

Based on aerosol dosage calculations from the above regimen (Knight et al., 1988), the daily dose of ribavirin necessary for the treatment of influenza virus-infected mice would be 8 mg/kg. For RSV-infected cotton rats, the calculated daily dosage would be approximately 15 mg/kg. In these experiments, the treatment period for RSV in cotton rats was 3 days (vs. 4 days for influenza virus in mice); a fourth day of treatment for cotton rats with RSV might have shown a greater antiviral effect. These dosage calculations have not taken into account that an additional amount of ribavirin will be swallowed from drug deposited on the pelt by mice and cotton rats. Thus, the actual doses of ribavirin given each animal may be larger than indicated from calculations based on deposition of inhaled aerosol. The amount of this increased exposure to oral ribavirin is uncertain; however, in the dosage comparisons made, such dosage would be about the same for all treated groups.

Thus, the effective daily aerosol dosage of HDR necessary for the treatment of influenza or RSV infections is estimated to be 8–15 mg/kg. If one extrapolates these calculations to human RSV disease by taking into account the concentration of ribavirin in the aerosol, the minute volume of the individual (or animal) and the duration of treatment, the time of treatment required for HDR aerosol to achieve a concentration of 10.8 mg/kg would be 2 and 4 h for infants and adults, respectively (Knight et al., 1988). This indicates that treatment of RSV infected infants with HDR for 2 h, 3 times daily (Englund et al., 1990) should be an effective and safe treatment regimen. Further clinical testing of the efficacy of this treatment regimen seems warranted.

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