Protection of Mice from Lethal Influenza Virus Infection with High Dose-Short Duration Ribavirin Aerosol

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An aerosol generated from a reservoir containing 60 mg of ribavirin per ml given for 2 h twice daily for 4 days afforded the same high level of protection against lethal influenza virus infection of mice as a longer, conventional treatment schedule (20 mg/ml given for 11 h daily for 4 days). Incremental decreases in ribavirin concentration made while maintaining the 2-h intermittent schedule provided progressively less protection of mice. Mice exposed to the 60-mg/ml doses had significantly increased pulmonary and serum drug levels when compared with mice given 20 mg of drug per ml, these increases were transient, and no evidence of pulmonary intolerance was detected. These studies suggest that protective effects of ribavirin against influenza virus infection can be achieved without untoward effects if higher doses and shorter periods of administration are used.

Ribavirin, 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide, is a broad-spectrum antiviral agent that has been used successfully in small-particle aerosol to treat respiratory syncytial (2, 6, 10), parainfluenza (1), and influenza (3, 5, 7, 11) virus infections of children and adults. In usual practice, ribavirin aerosols are administered for periods of 10 to 18 h/day. Such long treatment schedules discourage the use of ribavirin for the treatment of other than severe illness. If effective treatment could be achieved with shorter dosage periods, the use of small-particle aerosols of ribavirin could be extended to less severely ill patients.

In this study, we measured the effects of increasing the dose and shortening the duration of administration of ribavirin aerosol on influenza A/Hong Kong/68 virus infection of mice. Six- to eight-week-old random-bred CD-1 male mice obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were used in all experiments. The animals were housed in cages covered with barrier filters and fed mouse chow and water ad libitum.

The influenza A/Hong Kong/68 (H3N2) virus used in these studies was isolated in 1968 from a patient with influenza. The adaptation of this virus to mice and its characteristics were described in detail previously (14). In the present study, four median lethal doses of virulent, passage 10 virus were administered intranasally in 0.05-ml volumes to mice lightly anesthetized with ether.

Pulmonary virus titers were determined on day 4 after virus inoculation. Lungs were removed from mice killed by cervical dislocation. One lobe from each lung was placed in Formalin for histologic studies. The remaining lobes were homogenized with glass tissue homogenizers (catalog no. K886600; Kontes, Vineland, N.J.) and an overhead stirrer (model 903475; Wheaton Scientific, Millville, N.J.). The homogenates were clarified by centrifugation (100 × g) and tested for virus in 96-well tissue culture plates (catalog no. 76-013-05; Flow Laboratories, Inc., McLean, Va.) containing Madin-Darby canine kidney tissue cells (MDCK; Flow Laboratories), as described previously (9). Titers were expressed as the reciprocal of the last dilution of lung suspension that exhibited hemagglutination.

For histologic studies the lung tissues were embedded in low-melting-point paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin. Each section was coded by number and given to Donald Greenberg and Toshiaki Kawai, Department of Pathology, Baylor College of Medicine, for evaluation.

Mice were treated with ribavirin aerosols on days 1 through 4 after infection. The aerosol machines containing Collison nebulizers modeled after the design of K. R. May (4) used in these experiments and their use to deliver antiviral agents were described in detail previously (12, 13, 15). Ribavirin (ICN Pharmaceuticals, Inc., Costa Mesa, Calif.) suspensions in 0.9% saline (injection grade; Travenol Laboratories, Deerfield, Ill.) were prepared; they contained 20, 40, or 60 mg/ml. Zero-percent placebo controls were not run, because numerous previous experiments proved that aerosolized saline has no effect on pulmonary virus titers or disease. Each preparation was added to the appropriate aerosol generator reservoir and delivered at 12.5 liters/min to animals kept in plastic cages covered with plastic tops.

In both experiment 1 and experiment 2 (Table 1), all groups of mice exposed to small-particle aerosols of ribavirin had fewer deaths than the groups containing virus-inoculated untreated control mice. In experiment 1, highly significant protection was observed in the group treated with 20 mg of ribavirin per ml for 11 h/day for 4 days (group 2), in the group of mice treated with 40 mg/ml twice daily for 4 days (group 4), and in the group given 60 mg/ml twice daily for 4 days (group 5). In experiment 1, significant protection from death was observed in the group of mice given 20 mg of ribavirin per ml twice daily for 2 h (group 3). Similar results were obtained in experiment 2, with the exception that mice receiving 20 mg of drug per ml twice daily were not significantly protected (P > 0.05) from lethal disease. In both experiments, complete protection was observed only in groups 2 and 5.

All mice exposed to small-particle aerosols of ribavirin in experiment 2 had reduced virus titers in their lungs as compared with virus-inoculated untreated control mice (Table 1). Maximal reductions in virus titer were observed in groups 2 (1.6 log10) and 5 (1.4 log10), the groups which received 20 mg of ribavirin per ml for 11 h/day and 60 mg of ribavirin per ml twice daily, respectively. Mice receiving 40

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mg of drug per ml twice daily also had significantly reduced pulmonary virus titers, in contrast to those receiving 20 mg/ml two times a day (Table 1).

Sections of lung from untreated influenza virus-infected control mice collected on day 4 after inoculation of virus exhibited evidence of bronchiolitis, focal pneumonia, and necrosis of the bronchiolar epithelium (Table 2). No necrosis was observed in sections of lung from any ribavirin-treated group, and minimal histopathologic alterations were seen in sections of lung from infected mice treated with 20 mg of drug per ml for 11 h/day and 60 mg/ml twice daily. Intermediate evidence of disease was observed in lungs of mice given 20 or 40 mg of ribavirin per ml intermittently.

Lungs and sera were collected for measurement of ribavirin levels at various times during or after aerosol therapy. Three to five mice per group were tested at each interval. Measurements were made with a high-performance liquid chromatography (HPLC) system (Waters 840; Waters Associates, Inc., Danvers, Mass.). All measurements were made at ambient temperature on a Microsorb C18 stainless steel HPLC column (particle size, 5 μm; length, 25 cm; inner diameter, 4.6 mm; Rainin Instrument Co., Emeryville, Calif.) protected by a Guard-Pak precolumn with a C18 insert (Waters Associates). Before being added to the column, each sample was deproteinized by ultrafiltration through CF25 cones (Amicon Corp., Danvers, Mass.). The ultrafiltrate was adjusted to pH 8.8 and applied to a phenylboronate column. Bound ribavirin was then eluted, collected, and lyophilized to dryness. After lyophilization, each sample was suspended in 0.02 M ammonium phosphate containing 1% methanol (pH 5). Analysis of ribavirin was performed at 207 nm with 0.02 M ammonium phosphate buffer (pH 5.1) containing 1% methanol as the mobile phase. Strongly absorbed substances were eluted from the column after each analysis with a gradient of 0 to 80% acetonitrile in water. Flow rates were maintained at 1.0 ml/min throughout each separation.

Measurement of lung homogenates for ribavirin levels by HPLC (Fig. 1, top panel) indicated significantly increased peak levels of drug in the lungs of mice administered 60 mg of ribavirin per ml (116 ± 22 nmole per lung) as compared with peak pulmonary drug levels in mice administered the 20-mg/ml drug concentration (23 ± 5 nmole per lung; \( P = 0.004 \)). However, after termination of drug delivery, pulmonary drug levels declined rapidly, and by 4 h they were no higher than levels seen in mice given the lowest ribavirin dosage. Serum drug levels in mice given 60 mg/ml (bottom panel) also reached maximal and significantly higher levels (23 ± 3 versus 9 ± 1 μm/ml for mice exposed to 20 mg of drug per ml; \( P = 0.04 \)) at 2 h but declined more slowly than pulmonary levels.

It is important that the higher treatment dosages were not associated with detectable pulmonary toxicity; no untoward effects were noted, although ribavirin levels were significantly higher in lungs and sera of mice given the 60-mg/ml dose than in mice given the 20-mg/ml dose. The higher drug

| Group no. | Administration schedulea | Reservoir conc (mg/ml) | HPLC-determined pulmonary drug levelsb | No. of deaths/totals (%)c | Virus titer per lung (GMT ± SD [log_{10}]d in exp 2 |
|-----------|--------------------------|------------------------|----------------------------------------|--------------------------|---------------------------------------------------|
| 1         | 1 x 11                   | 20                     | 29.5 ± 7.4                             | Expt 1: 13/19 (68)       | 16/21 (66)                                        |
| 2         | 2 x 2                    | 20                     | 20.7 ± 4.1                             | Expt 1: 2/20 (0)        | 20/20 (0)                                         |
| 3         | 2 x 2                    | 40                     | 16.6 ± 2.2                             | Expt 1: 3/20 (15)       | 2/20 (10)                                         |
| 4         | 2 x 2                    | 60                     | 46.0 ± 8.8                             | Expt 1: 0/20 (0)        | 0/20 (0)                                          |

- a Number of exposures per day to small-particle aerosols × duration (hours) of each exposure. Treatments were begun on day 1 after virus infection and given again on days 2, 3, and 4.
- b Mean nanomoles ± standard deviation per lung determined by HPLC analysis of homogenates prepared from lungs of mice killed on day 4 of treatment immediately at the end of 2-h treatment period 1 (for group 2, this was a 4 h after continuous aerosol). Uninfected mice given the same treatment as the infected mice in group 5 had a retained dose of 37.6 ± 9.5 nanomol per lung.
- c Cumulative number of deaths per group on day 21 after virus inoculation.
- d The geometric mean titer (GMT) for each experimental group was compared with the GMT of virus in the lungs of untreated controls by using Student's t test.
- e Statistically significant (P < 0.01) compared with the untreated control group using chi-square contingency tables (8) and 1 df.
- f 0.01 < P < 0.05.

### TABLE 2. Comparison of the histologic findings in lungs of mice infected with influenza A/Hong Kong/68 virus and treated with different doses of ribavirin

| Group | Administration schedulea | Ribavirin reservoir conc (mg/ml) | Virus inoculated | Bronchiolitis | Focal pneumonia | Necrosisc |
|-------|--------------------------|---------------------------------|-----------------|--------------|----------------|-----------|
| 1     | 1 x 11                   | 20                              | +               | +            | +              | +         |
| 2     | 2 x 2                    | 20                              | +               | +            | +              | +         |
| 3     | 2 x 2                    | 40                              | +               | +            | +              | +         |
| 4     | 2 x 2                    | 60                              | +               | +            | +              | +         |
| 5     | 2 x 2                    | 60                              | +               | +            | +              | +         |

- a Sections were prepared from lungs collected on day 4 after virus inoculation. +, Evident; + +, evident in all fields. There were no cases in which numerous foci were evident in all fields. Each group comprised four animals.
- b Number of exposures to small-particle aerosol × duration (hours) of each exposure. Treatments were begun on day 1 after virus infection and given again on days 2 and 3.
- c Necrosis of bronchiolar epithelium.
levels were transient and rapidly declined when the delivery of aerosol was terminated.

The results of this study show that a ribavirin aerosol produced from a generator reservoir containing 60 mg of ribavirin per ml given 2 h twice daily for 4 days affords the same high level of protection against lethal influenza virus infection as 20 mg/ml given for 11 h daily for 4 days. The latter reservoir concentration and duration of treatment are representative of those routinely used to treat selected respiratory infections of humans (3, 5, 6, 10, 11). A 40-mg/ml dose given 2 h twice daily also provided significant protection, although the degree of protection was less than that with the higher dosage. The 20-mg/ml dose given 4 h intermittently gave minimal protection. These results suggest that the protective effect of ribavirin can be achieved with a significantly shorter duration of treatment if higher doses of drug are used; moreover, because the total dosages of drug delivered to the mice in groups 1 and 5 were similar (Table 1), total dosage and not duration of treatment may be the primary factor determining efficacy.

The importance of these findings is that if successful treatment of respiratory virus disease with small-particle aerosols of ribavirin can be achieved with shortened dosage periods and without loss of efficacy, this approach could be used to treat moderate respiratory syncytial virus disease.

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