Treatment of viral myocarditis with ribavirin in an animal preparation

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ABSTRACT The therapeutic effects of ribavirin, a broad-spectrum, antiviral agent, on experimental myocarditis caused by encephalomyocarditis (EMC) virus were investigated. Four-week-old DBA/2 mice were inoculated with 10 plaque-forming units (pfu) of EMC virus. Ribavirin in a dose of 100 (group 1, n = 20), 200 (group 2, n = 10), or 400 mg/kg/day (group 3, n = 10) was administered subcutaneously on days 0 to 12 after virus inoculation, and animals were observed for 12 days. Control animals were injected with saline (n = 20). Mice treated with ribavirin survived longer than controls (mean survival 6.7 days for group 1, 7.4 days for group 2, 7.7 days for group 3, and 5.2 days for control; p < .005). Myocardial virus titers on days 6 to 8 were significantly lower in group 2 (3.24 ± 0.49 log10 pfu/mg; p < .005) and in group 3 (1.70 ± 0.65 log10 pfu/mg; p < .001) compared with controls (4.09 ± 0.57 log10 pfu/mg). The incidence of gross myocardial lesions was significantly lower in group 1 (13/20, 65%), group 2 (2/10, 20%), and group 3 (0/20, 0%) compared with controls (20/20, 100%) (p < .05). Histologic examination showed extensive myocardial necrosis and cellular infiltration in untreated groups; there was less infiltration in groups 2 and 3 (p < .01) and less severe necrosis in group 3 (p < .01). Thus ribavirin effectively inhibited myocardial virus replication and reduced the inflammatory response and myocardial damage in an experimental preparation of viral myocarditis.


ALTHOUGH VIRUSES can seldom be demonstrated in the myocardium, and clinical evidence establishing viral infection is relatively infrequent, myocarditis is probably caused by viral infection more often than has been suspected previously.1 Our understanding of the factors that predispose to viral myocarditis in man is limited, and the underlying mechanisms remain to be established. Therefore therapy is directed primarily toward the control of symptoms (e.g., arrhythmias, heart failure), and no proven specific treatment is available.

Animal preparations of congestive heart failure associated with acute myocarditis after infection with encephalomyocarditis (EMC) virus2 and of subsequent congestive cardiomyopathy3 have been developed and described recently. These preparations are considered suitable for the evaluation of preventive and therapeutic interventions because the myocardial lesions are severe and occur frequently.

Ribavirin is a drug with broad antiviral activity against RNA and DNA viruses. Chemically, ribavirin is a synthetic nucleoside analogue, structurally related to inosine and guanosine. It has been shown that the prevention of influenza virus multiplication may depend on a selective inhibition of the RNA polymerase by ribavirin.4 Clinically, its efficacy has been demonstrated in measles,5 influenza,6 and respiratory syncytial virus infection.7 From the clinical trials reported, the principal side effects appear to be reversible anemia and transiently elevated bilirubin levels.8 Although studies in vitro showed that ribavirin is effective against picorna virus infection (e.g., coxsackievirus and poliovirus) in cultured cells,4 the effect of ribavirin on viral myocarditis has not been investigated.

In this study we investigated the effect of ribavirin on experimental myocarditis caused by EMC virus.
Materials and methods

**Viruses.** The M variant of EMC virus was used. Virus stock was prepared as described previously and was stored at -70°C until it was diluted for use.

Viral titers were determined by plaque formation on FL (human amnion) cell monolayers. Cells were suspended at a concentration of 1 x 10⁶/ml in Eagle’s minimal essential medium (EMEM) with 15% fetal calf serum (FCS) plus 100 µg/ml penicillin and streptomycin in six-well plastic plates and were allowed to grow for 4 days at 37°C in 5% CO₂.

Volumes (0.1 ml) of decimal dilutions of virus suspended in EMEM were adsorbed to FL cell monolayers for 60 min at 37°C in 5% CO₂. After adsorption, the cells were overlaid with 3 ml of medium containing 4% FCS and 1% methyl cellulose. After 2 days of incubation at 37°C in a humidified atmosphere containing 5% CO₂, cells were fixed with acetic acid and methanol (at a ratio of 1:3) and stained with crystal violet (1%), and plaques were counted with an inverted microscope.

**Ribavirin sensitivity assays in vitro.** The antiviral drug ribavirin (Virazole; 1-β-o-ribofuranosyl-1,2,4-triazole-3-carboxamide) was supplied by Viratek, Covina, CA. A stock solution of 100 µg/ml ribavirin in distilled water was prepared. Antiviral activity was assayed by a plaque-reduction method. Confluent monolayers of FL cells in six-well plastic plates were inoculated with 100 plaque-forming units (PFU) of EMC virus in 0.2 ml of medium containing various concentrations of ribavirin (0, 1, 10, 50, or 100 µg/ml).

After 2 days of incubation at 37°C, cells were fixed with acetic acid methanol and stained with crystal violet, and plaques were counted. Plaque formation was expressed as percent of plaques of control animals. The drug concentration required to reduce the number of plaques by 50% from the number in control wells (the median inhibitory dose or ID₅₀) was calculated from the graph relating plaque number and drug concentrations on a semilog plot (linear regression).³

**Virus inoculation and treatment of mice.** Four-week-old male DBA/2 mice (Jackson Laboratory, Bar Harbor, ME) were inoculated intraperitoneally with 0.1 ml of EMC virus diluted in EMEM to a concentration of 100 PFU/ml. Ribavirin was administered subcutaneously at a dose of 100, (group 1, n = 20), 200 (group 2, n = 10) or 400 mg/kg/day (group 3, n = 10), beginning immediately after virus inoculation until day 12. Control mice were injected with saline. Mice were observed daily, and, when found dead, complete necropsies were performed.

**Virus titers of tissues.** For infectivity assays, tissues were removed aseptically, weighed, and homogenized in 2 ml of EMEM. After centrifugation at 1500 g for 15 min at 4°C, supernatants were inoculated into FL cell monolayers and plaque assays were performed.

**Pathologic study.**

**Gross pathology.** Gross abnormalities on the surface of the heart were classified as follows: 0, no lesions; 1+, lesions involving less than 25% of the myocardium; 2+, lesions involving 25% to 50% of the myocardium; 3+, lesions involving 50% to 75% of the myocardium; and 4+, lesions involving 75% to 100% of the myocardium. After gross inspection the hearts were processed for histologic or virologic studies.

**Histopathology.** The hearts were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylin-eosin or von Kossa’s stain. Myocardial cell necrosis, cellular infiltration, and calcification were scored blindly on a scale of 1 to 4 in terms of severity. A 1+ score described a limited focal distribution of myocardial lesions. A 4+ score indicated the presence of multiple lesions over the entire heart, while scores of 2+ and 3+ were used to describe intermediate severity.

**Statistics.** Kaplan-Meier plots were made of the survival data and differences between the control and treatment survival curves evaluated by the Mantel-Cox log rank test. The Kruskal-Wallis test was used to evaluate the difference of gross and histologic gradings. Virus titers of the heart were examined by an analysis of variance followed by the Neuman-Keuls test for multiple sample comparison.

**Results.**

**Antiviral activity in vitro (figure 1).** The effect of ribavirin treatment of FL cells infected with 100 PFU of EMC virus is shown in figure 1. Treatment of cells with increasing concentrations of ribavirin resulted in progressive reduction of plaque formation. The mean percent plaque formation was 100.5% at a ribavirin concentration of 1 µg/ml, 94.9% at 10 µg/ml, 5.6% at 50 µg/ml, and 0.4% at 100 µg/ml (each n = 6). Thus a concentration of 50 µg/ml of the drug was effective in reducing the number of plaques produced by EMC virus, and plaque formation was completely inhibited at 100 µg/ml. Linear regression analysis showed good negative correlation of y = 116 - 55.5 log₁₀x (r = -.893, p < .001) between plaque-forming units and logarithm of drug concentration. ID₅₀ was 15 µg/ml.

**Ribavirin treatment of EMC virus-infected mice**

**Mortality (figure 2).** All 20 control mice died between days 4 and 7; 11 of these 20 mice died before day 6 (mean survival 5.2 days). In contrast to control animals, no mice in groups 2 and 3 died before day 6, and seven of 20 mice in group 1 died before day 6. All control animals died before day 8. However, eight of 20 mice in group 1, five of 10 mice in group 2, and seven of 10 mice in group 3 survived to day 7. Mean survival time was 6.6 in group 1, 7.4 in group 2, and 7.7 days in group 3. All treated groups survived significantly longer than controls (p < .005).

**Myocardial virus titer (table 1).** Myocardial virus titers were determined on the hearts of mice that had died on days 6 to 8. The mean myocardial virus titer of the controls was 4.09 ± 0.57 log₁₀ PFU/mg (n = 9). Those of ribavirin-treated groups were 4.17 ± 1.13 log₁₀ PFU/mg in group 1 (n = 10), 3.24 ± 0.49 log₁₀ PFU/mg in group 2 (n = 10, p < .005), and 1.70 ± 0.65 log₁₀ PFU/mg in group 3 (n = 9, p < .001, compared with controls). The degree of inhibition of viral replication in the heart was influenced by drug dosage, and 400 mg/kg ribavirin strongly inhibited viral replication.

**Gross pathologic findings.** All 20 control mice developed yellowish white patches on the surface of the heart, half of which were graded 3+ and 4+. On the other hand, seven of 20 mice (35%) in group 1, eight of 10 mice (80%) in group 2, and all of 10 mice (100%) in group 3 had no gross lesions. Gross lesions were sig-
significantly less severe in groups 1 through 3 compared with those in control animals (p < .05, .01, and .01, respectively).

**Histologic findings (table 2, figure 3).** Representative photographs of each grade of histologic changes are shown in figure 3. As indicated in table 2, cellular infiltration was found in all of the hearts of untreated mice and was grade 2+ to 4+ in 18 of 20 (90%). On the other hand, six of 20 hearts in group 1, all hearts of mice in group 2, and nine of 10 hearts in group 3 showed infiltration of grade 0 or 1+. Cellular infiltration was significantly less marked in groups 2 and 3 compared with that in controls (p < .01).

Myocardial necrosis was found in all of the hearts of untreated mice and was 3+ and 4+ in severity in half of the hearts. However, 16 of 20 mice in group 1, nine of 10 mice in group 2, and all of 10 mice in group 3 showed myocardial necrosis of grades 1+ and 2+. Myocardial necrosis was significantly less severe in group 3 compared with that in controls (p < .01).

Myocardial calcification was found in 13 of 20 untreated mice. Calcification was also found in the hearts of 19 of 20 mice in group 1 and in all mice in groups 2 and 3.

**Discussion**

Acute viral myocarditis may cause significant cardiac morbidity and mortality, but our understanding of the pathogenesis of myocarditis in man is limited. Consequently, therapy primarily directed toward the control of symptoms is disappointing.

The fact that exercise, hypoxia, and undernutrition aggravate experimental viral myocarditis points to the therapeutic importance of rest, adequate ventilation and oxygenation, and appropriate nutrition.

Steroids have been used in the therapy of viral myocarditis and have been considered by some to be of clinical value. However, there is evidence that in the acute stages of experimental viral infections, steroids render the myocardial lesions more severe and increase mortality.

During the stage of viral replication, a trial of anti-

**FIGURE 1.** Effect of ribavirin on EMC virus infection in FL cells. Treatment of cells with increasing concentrations of ribavirin resulted in progressive reduction of plaque formation.

**FIGURE 2.** Survival plots of untreated (solid line) and treated mice with 100 (left), 200 (middle), and 400 (right) mg/kg ribavirin (dotted line). All treated groups survived significantly longer than control animals.
viral agents would appear rational. Ribavirin is a synthetic nucleoside, structurally related to inosine and guanosine,\textsuperscript{19} which has been shown to have a remarkably broad spectrum of antiviral activity in laboratory systems.\textsuperscript{20}

Ribavirin has exhibited antiviral activity in vitro against many viruses tested, although the scope of this activity varies considerably with the virus, the cell line, and the parameter used for measuring that activity. The influenza viruses are among the most sensitive to ribavirin, and most investigators have reported ribavirin's minimum inhibitory concentrations against influenza to be in the range of 1 to 25 μg/ml.\textsuperscript{20} The antiviral experiments conducted with ribavirin in vivo generally confirmed the broad-spectrum antiviral activity of the compound seen in vitro, although the efficacy of the compound is dependent on the site of the infection, the manner of treatment, the age of the animals, and the virus dosage. Efficacy against experimental influenza has been seen after the administration of the drug by gavage, in the diet, intraperitoneally, subcutaneously, intravenously, intramuscularly, and by small-particle aerosol. The timing of ribavirin therapy against virus infections is important. The best effect was shown when therapy began before or at the same time as infection and continued for at least 4 days after infection.

Significant efficacy was seen, however, when therapy was begun as late as 24 hr after virus inoculation.\textsuperscript{21} Efficacy of ribavirin was also shown in animal experiments in vivo against parainfluenza, murine hepatitis, herpes, vaccinia,\textsuperscript{4} and Lassa fever viruses.\textsuperscript{22} More recently, clinical effectiveness of ribavirin has been reported in the treatment of measles,\textsuperscript{5} influenza,\textsuperscript{6} and respiratory syncytial virus infection.\textsuperscript{7}

In this study, ribavirin inhibited EMC virus replication in FL cell culture in vitro. Mice treated with ribavirin survived longer than control animals. Ribavirin inhibited EMC virus replication in the heart, and this inhibitory effect correlated well with the decrease of severity of myocardial damage and inflammatory cellular infiltration.

The cause of death of mice treated with a high dose of the drug may be related to encephalitis. Ribavirin has been reported to be inactive against many viral infections of the central nervous system because of failure of the drug to pass the blood-brain barrier.\textsuperscript{4} We found no significant decrease in virus titers of the brain in the mice treated with high doses of ribavirin compared with control animals. Microscopic examination of brain sections showed foci of spongiosis, neuron loss, and small round cell infiltrate. Occasional fragments of meninges diffusely infiltrated by chronic inflammatory cells were also seen. These changes were seen in mice not treated with ribavirin, as well as in mice treated with ribavirin (unpublished observations).

Ribavirin was found to be effective in reducing cellular infiltration and myocardial necrosis induced by EMC virus infection. Increased myocardial calcification in mice treated with ribavirin is considered to be related to the fact that treated mice survived longer (more than 6 days) than untreated mice; myocardial calcification became more prominent after day 7.\textsuperscript{3} In our preliminary experiments, we observed slight calcification in the hearts of three of 10 mice treated with

\begin{table}
\centering
\caption{Effect of ribavirin on EMC virus replication in the heart, days 6 to 8}  
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Group} & \textbf{Dose (mg/kg)} & \textbf{n} & \textbf{Mean ± SD} & \textbf{Range} & \textbf{p value} \\
\hline
Control & None & 9 & 4.09 ± 0.57 & 3.51-5.41 & NS \\
1 & 100 & 10 & 4.17 ± 1.13 & 2.51-5.49 & <.001 \\
2 & 200 & 10 & 3.24 ± 0.49 & 2.30-3.81 & .001 \\
3 & 400 & 9 & 1.70 ± 0.65 & 0.76-2.61 & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Effect of ribavirin on cardiac histopathology (No. of hearts)}  
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Infiltration} & \textbf{Control} & \textbf{1} & \textbf{2} & \textbf{3} \\
\textbf{(n = 20)} & \textbf{(n = 20)} & \textbf{(n = 10)} & \textbf{(n = 10)} \\
\hline
0 & 0 & 2 & 3 & 0 \\
1+ & 2 & 4 & 7 & 9 \\
2+ & 4 & 8 & 0 & 1 \\
3+ & 8 & 3 & 0 & 0 \\
4+ & 6 & 3 & 0 & 0 \\
\p value & NS & <.01 & <.01 \\
\hline
\textbf{Necrosis} & \textbf{Control} & \textbf{1} & \textbf{2} & \textbf{3} \\
\textbf{(n = 20)} & \textbf{(n = 20)} & \textbf{(n = 10)} & \textbf{(n = 10)} \\
\hline
0 & 0 & 2 & 0 & 0 \\
1+ & 3 & 7 & 3 & 9 \\
2+ & 7 & 7 & 6 & 1 \\
3+ & 5 & 1 & 1 & 0 \\
4+ & 5 & 3 & 0 & 0 \\
\p value & NS & NS & <.01 \\
\hline
\textbf{Calcification} & \textbf{Control} & \textbf{1} & \textbf{2} & \textbf{3} \\
\textbf{(n = 20)} & \textbf{(n = 20)} & \textbf{(n = 10)} & \textbf{(n = 10)} \\
\hline
0 & 13 & 1 & 0 & 0 \\
1+ & 4 & 5 & 3 & 0 \\
2+ & 1 & 2 & 7 & 5 \\
3+ & 1 & 6 & 0 & 5 \\
4+ & 1 & 6 & 0 & 0 \\
\p value & <.01 & NS & <.01 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Compared with controls. Ribavirin was given in the following doses (mg/kg/day): group 1, 100; group 2, 200; group 3, 400.
100 mg/kg/day ribavirin for 7 days without infection, but this calcification was not as severe as that seen in infected mice. Thus ribavirin may facilitate myocardial calcification, but the drug alone cannot account for calcification.

These results point to the therapeutic potential of ribavirin for viral myocarditis, if given early after infection. Exploration of the effects of this drug when given at a later stage, as well as in other viral infections known to cause myocarditis in man, appears warranted.

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FIGURE 3. Representative micrographs of the grade of histologic changes. A, 1+ infiltration, 1+ necrosis; B, 2+ infiltration, 2+ necrosis; C, 3+ infiltration, 3+ necrosis; D, 4+ infiltration, 4+ necrosis. (Hematoxylin-eosin; original magnification × 290.)
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