EFFICACY OF POST-EXPOSURE TREATMENT OF YELLOW FEVER WITH RIBAVIRIN IN A HAMSTER MODEL OF THE DISEASE

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Abstract. Ribavirin was evaluated as a potential treatment of yellow fever (YF) in a hamster model of the disease. Ribavirin treatment during the first five days after YF virus infection improved survival, reduced tissue damage in target organs (liver and spleen), prevented hepatocellular steatosis, and normalized alanine aminotransferase levels. The results of this study suggest that ribavirin may be effective in the early treatment of YF, and that its mechanism of action in reducing liver pathology in YF virus infection may be similar to that observed with ribavirin in the treatment of chronic hepatitis C virus infection.

INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) is approved in the United States for use in respiratory syncytial virus aerosol therapy, hepatitis C virus (HCV) combination therapy, and under an investigational new drug protocol as a monotherapy for several viral hemorrhagic fevers (VHFs). In the case of VHFs, ribavirin has been shown to be effective in treatment of Lassa and other arenaviral hemorrhagic fevers (VHF). Hemorrhagic fever with renal syndrome, and Crimean-Congo hemorrhagic fever.

In previous studies, ribavirin treatment of rhesus monkeys infected with yellow fever (YF) virus did not improve survival; consequently, the compound was considered ineffective against this VHF. At the time, the rhesus monkey was considered the animal model of choice for YF, despite the fulminant nature of the infection in this primate species. Because more recent studies have shown in vitro activity of ribavirin against YF virus, we decided to re-examine the efficacy of the drug in a hamster model of the disease.

The present report shows the results of these studies that indicate that early treatment of YF virus infection with ribavirin reduces liver damage and increases survival in a hamster model of the disease.

MATERIALS AND METHODS

Virus. The Jimenez strain of YF virus, which had been serially passaged 11 times in hamster liver, was used in all experiments. A standard dose of 10⁶ tissue culture infectious dose₅₀ (TCID₅₀) units of the virus was administered by intraperitoneal inoculation to each animal with the group designation YF (Table 1).

Animals. The animals used in the study were adult (6–8 weeks old) female Syrian golden hamsters (Mesocricetus auratus) obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with infected animals and YF virus was carried out in biosafety level-3 facilities.

Drug treatment trials. A total of 86 hamsters were used in the study; the animals were divided into seven groups, as outlined in Table 1. Group YF-NT (n = 16) received 10⁶ TCID₅₀ of YF virus intraperitoneally, but no ribavirin. Group UC-24 (n = 10) received ribavirin, but no virus. The other five groups (YF-24, YF-48, YF-72, YF-96, and YF-120) were infected with YF virus, and ribavirin treatment was begun at preset times (24, 48, 72, 96, and 120 hours, respectively) after virus inoculation. The animals received an initial loading dose (LD) of 80 mg/kg of ribavirin (Virazole®; ICN Pharmaceuticals, Inc., Costa Mesa, CA) on the first day of treatment, and a daily dose (DD) of 40 mg/kg each subsequent day until the seventh day after virus inoculation, when therapy was discontinued. The animals were examined daily for signs of illness, and blood samples were collected for 11 consecutive days from the retroorbital sinus for virus titration and for liver function studies.

On the sixth day after inoculation of YF virus, two hamsters from each group were killed, and samples of liver, mediastinal lymph nodes, spleen, kidney, adrenal gland, lung, heart, and pancreas were taken for histopathologic examination.

Histologic examination. Tissues taken at necropsy on the sixth day were placed immediately in 10% buffered formalin solution for fixation. After 24 hours, the samples were transferred to 70% ethanol for storage. Tissue samples were subsequently processed and embedded in paraffin for histologic sectioning, staining with hematoxylin and eosin, and microscopic examination, as described previously.

Histopathology scoring system. Hematoxylin and eosin–stained tissue sections were evaluated blindly for the following pathologic changes: liver (steatosis, necrapoptosis, portal inflammation, lobular inflammation), spleen (necrosis, macrophage proliferation, lymphoid depletion), pancreas (pancreatic necrosis), lymph nodes and thymus (lymphoid depletion), kidney (tubular degeneration), lung (reduction of airspace, hemorrhage), and heart (myocardial inflammation). Pathologic changes were scored on a scale from 0 to 4, as previously described, where 0 was assigned to normal tissue, and 4 to severe pathology.

Immunohistochemistry. Unstained paraffin sections were deparaffinized with xylene and processed for viral antigen detection, as previously described.

Virus titrations. Titration of daily blood samples from the infected hamsters was done in 24-well tissue culture plates seeded with C6/36 cells, as previously reported.
ver virus titers in the blood were calculated as the TCID_{50}/mL, using the method of Reed and Muench. 15

Liver function studies. Determinations of serum alanine aminotransferase (ALT) levels were done on fresh serum from clotted blood, using a commercial kit (Infinity ALT; Sigma Diagnostics, St. Louis, MO) according to the manufacturer’s instructions.

Antibody detection. Sera from the surviving hamsters, obtained 21 days after virus inoculation, were examined for antibodies to YF virus by the hemagglutination-inhibition (HI) test.16 Antigen for the HI test was prepared from brains of newborn mice inoculated intracerebrally with YF virus; the infected brain was treated by the sucrose-acetone extraction method.16 Hamster sera were tested by HI at serial two-fold dilutions from 1:20 to 1:2560 at pH 6.6, as previously described.13

### RESULTS

**Effect of ribavirin treatment on survival.** Table 1 shows the mortality rate among the seven groups of hamsters included in this study. Forty-three percent (6 of 14) of the non-treated, YF virus-infected hamsters (YF-NT) died within 5–8 days after YF virus infection, and all of the uninfected control animals (UC-24) that received ribavirin alone survived. The YF virus-infected animals that were given ribavirin treatment at 24, 48, and 72 hours after infection (groups YF-24, YF-48, YF-72, respectively) also all survived. The mortality rate in the two YF virus-infected groups given ribavirin therapy at 96 and 120 hours after infection (groups YF-96 and YF-120) was 13.7%. The eight surviving hamsters in the untreated infected control group (YF-NT), and all survivors in the five ribavirin-treated groups had HI antibodies to YF viral antigen when examined 21 days after virus inoculation. Thus, the administration of ribavirin, following YF virus infection, improved survival in each of the five treatment groups tested (YF-24, YF-48, YF-72, YF-96, and YF-120).

**Effect of ribavirin treatment on viremia.** In contrast to the survival pattern, the level of viremia in the untreated infected controls and the ribavirin-treated animals (YF-NT, YF-24, YF-48, YF-72, YF-96, and YF-120) was similar. Figure 1 shows the mean level of viremia among hamsters in the untreated infected control group (YF-NT) and in the YF virus-infected groups that were given ribavirin therapy at 24, 48, and 72 hours after virus inoculation (YF-24, YF-48, and YF-72, respectively). The mean viremia levels in the other two ribavirin-treated groups (groups YF-96 and YF-120) are not shown in the figure, but were not significantly different from those of the others. Thus, treatment with ribavirin did not seem to affect the level or duration of viremia in the animals after YF virus infection.

### Table 1

<table>
<thead>
<tr>
<th>Group designation</th>
<th>Number of animals†</th>
<th>YF virus infection</th>
<th>Ribavirin treatment (hours)‡</th>
<th>Percent mortality (no. dead/total)†</th>
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<tr>
<td>YF-NT</td>
<td>16</td>
<td>Yes</td>
<td>None</td>
<td>43 (6/14)</td>
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<td>UC-24</td>
<td>10</td>
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<tr>
<td>YF-120</td>
<td>10</td>
<td>Yes</td>
<td>120</td>
<td>13 (1/8)</td>
</tr>
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</table>

* YF = yellow fever; NT = no ribavirin treatment; UC = uninfected control.
† Two animals in each group were killed on day 6 post-infection to obtain tissues for histopathologic examination. These animals were not included in calculating percent mortality.
‡ The hours indicate the time interval between inoculation with YF virus and start of ribavirin treatment.
Effect of ribavirin treatment on liver function. The degree of hepatocellular injury following YF virus infection was measured by daily ALT determinations on sera of the surviving hamsters. Figure 2 shows the daily mean ALT levels in uninfected hamsters treated with ribavirin (UC-24), in YF virus-infected non-treated animals (YF-NT), and in hamsters treated with ribavirin following YF virus infection (YF-24, YF-48, YF-72, YF-96, and YF-120). The latter five treatment groups were combined in Figure 2 for simplicity, since their mean values were similar. Based on measurement of the ALT levels, treatment with ribavirin appeared to markedly reduce liver injury in the YF virus-infected animals.

Effect of ribavirin treatment on organ histopathology. When examined at necropsy six days after YF virus infection, the livers of the ribavirin-treated hamsters were grossly normal in appearance and consistency. In contrast, livers of the infected control animals (YF-NT) were pale in color and were soft and fragile in consistency. Microscopic examination of liver sections of two untreated YF virus-infected hamsters (YF-NT) revealed massive necroptosis, microvesicular steatosis, and inflammation (Figure 3). In contrast, liver sections of the YF-infected, ribavirin-treated hamsters had less necroptosis and did not show microvesicular steatosis. The ribavirin-treated hamsters in groups YF-24, YF-48, YF-72, YF-96, and YF-120 also had a reduced number of Councilman bodies and inflammatory cells per high power field, compared with the YF control (YF-NT) animals.

The depletion of lymphoid areas in the spleen and reduction of lymphocytes in lymph nodes were also reduced by ribavirin treatment. The splenic white pulp in the ribavirin-treated animals was well preserved. Although the proliferation of resident macrophages was similar in the untreated and treated groups, tangible body macrophages were rarely observed in the treated groups (Figure 4).

A reduction of tissue damage in the ribavirin-treated animals was observed in other organs and tissue types as well. For example, the extensive necrosis, observed in adrenal glands of the untreated infected control (YF-NT) animals, were absent in the ribavirin-treated animals, as was pancreatic necrosis (Figure 4). The reduced tissue damage observed in ribavirin-treated animals correlated with their overall better clinical appearance and survival. In general, the behavior of the ribavirin-treated hamsters remained relatively normal; in contrast, animals in the untreated infected control group became lethargic, anorectic, and somnolent, regardless of whether or not they eventually survived.

In an attempt to understand how ribavirin might exert its antiviral effect in YF virus infection, immunohistochemical (IHC) staining of tissue sections was done to study the localization of viral antigen. In general, staining for YF viral antigen was much more intense and diffuse in the untreated infected control group (YF-NT) than in the ribavirin-treated groups. The most striking finding was in the liver, where there was only scattered and low-intensity YF antigen staining in hepatocytes of the ribavirin-treated animals, while hepatocytes of the YF control group (YF-NT) were intensely stained (Figure 3). There was an absence of YF antigen staining in lymph nodes, splenic white pulp, pancreas, and kidney of the ribavirin-treated animals, but those same tissues in the YF control animals showed multiple zones of YF viral antigen staining. Based on the IHC and histopathologic findings, there was a good correlation between the degree of tissue damage and the presence and intensity of YF viral antigen.
DISCUSSION

Despite the availability of an effective vaccine, YF remains enzootic in tropical forested areas of sub-Saharan Africa and South America. The World Health Organization estimates that approximately 200,000 cases of YF occur annually, most of them in Africa. The case-fatality rate of YF in Africa is estimated to be approximately 24%. Because of the frequency and speed of air travel from these YF-endemic regions, there is now concern that the virus could be transported to other geographic areas where there are large concentrations of susceptible people and the urban mosquito vector (*Aedes aegypti*), and that urban epidemics of YF could reappear. At present, there is no specific therapy for YF.

In the present study, ribavirin proved to be effective in the early treatment of YF virus infection in the hamster model when used at an optimal loading dose of 80 mg/kg and a daily dose of 40 mg/kg. Survival was 100% when treatment was begun on days 1, 2, or 3 post-infection, and was 87% when therapy was started on days 4 or 5 after infection. In contrast, survival was only 57% in the infected (untreated) controls.

In addition to better survival, the ribavirin-treated animals also had reduced histopathology. The reduction in tissue damage was observed in a variety of organs, but was most striking in the liver, spleen, and pancreas, the organs that are usually most severely affected in YF. Regardless of when ribavirin treatment was started, the livers of treated animals had less necroptosis, inflammatory cells, and no microvesicular steatosis, compared with livers of the untreated infected control animals. A similar histologic pattern has been observed in non-fatal YF virus infections from previous studies. As confirmation of the reduced liver pathology in the treated animals, their ALT levels also remained at normal values, and the intensity of YF viral antigen staining in their hepatocytes was markedly diminished. Interestingly, the level and duration of viremia in the YF virus-infected animals was unaffected by treatment with ribavirin. A similar pattern has also been observed in chronic HCV infection following treatment with ribavirin. Ribavirin treatment of HCV patients generally produces a reduction (normalization) of serum ALT levels and liver histopathology, although the HCV viral load in plasma is unchanged.

A second significant difference noted between the ribavirin-treated hamsters and the untreated infected controls was the reduced depletion of lymphoid cells in spleen and lymph nodes of the treated animals. The histopathologic changes
observed in the spleen, and to a lesser extent in the lymph nodes, of the untreated YF virus-infected hamsters consisted of a marked depletion of lymphoid tissues accompanied by lymphocytic necrosis, proliferation of resident macrophages, and an increase in foamy, debris-loaded macrophages (tangible-body macrophages).

The retention of many lymphoid cells in the ribavirin-treated animals may indicate less damage to the immune system, thus allowing these animals to more effectively control the infection.

Although ribavirin has activity against a variety of different...
types of viruses, its precise mechanism of action is unknown. At present, there are four proposed mechanisms of action, which can be divided into two types. \(^\text{22,23}\) The first group consists of two postulated indirect mechanisms: 1) enhancement of host T cell-mediated immunity against viral infection through switching the T cell phenotype from type 2 to type 1, and 2) inhibition of the host enzyme inosinate monophosphate dehydrogenase. \(^\text{22}\) The second mechanistic group consists of two other hypotheses that ribavirin directly affects viral replication by 1) direct inhibition of RNA polymerase, or 2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold of “error catastrophe.” \(^\text{22,23}\) The results of our study would seem to suggest an indirect effect by one or both of the mechanisms in the first group, since the level and duration of YF viremia were unaffected by ribavirin treatment (Figure 1).

It is also important to note that no toxicity or histopathology were observed in the uninfected ribavirin-treated animals (group UC-24). The behavior, ALT values, and microscopic appearance of tissues in animals of this group were normal, despite the relatively high loading and treatment doses of ribavirin (80 mg/kg and 40 mg/kg, respectively) that were given. The absence of untoward effects could be partially accounted for by a higher ribavirin clearance in rodents compared with primates. Also, the drug was only given to the animals for a maximum of seven days. The adverse effects of ribavirin therapy in humans (anemia, depression, pruritus, nausea, cough, chest pain, etc.) are usually associated with treatment for longer periods, such as in HCV. \(^\text{21,24}\)

In previous treatment trials of YF in rhesus monkeys, \(^\text{8}\) lower doses of ribavirin were used (30–50 mg/kg LD and 15–30 mg/kg DD). In those studies, \(^\text{8}\) no significant differences were noted between the treated and the control groups. Initially, we tried a lower ribavirin dose (40 mg/kg LD and 20 mg/kg DD) in YF virus-infected hamsters and a beneficial effect was noted. However, ultimately we decided to use a higher dose for the experiments described here. Thus, the lower ribavirin dosage regimens used in rhesus monkey studies may not fully explain the different outcomes observed in hamsters and macaques. Infection with YF virus in the rhesus monkey generally produces a fulminant rapidly fatal disease, \(^\text{9}\) whereas YF virus infection in the adult hamster model \(^\text{13,14}\) is a less severe illness with mortality rates in the range of 20–60%. This is closer to the mortality rate in humans, which Monath and Tsai \(^\text{25}\) estimate to be approximately 20% in clinically apparent (jaundiced) cases of YF. The clinical course (evolution) of YF in humans is slightly longer than in hamsters, with death occurring about the ninth or tenth day. \(^\text{25}\) In our hamster model of the disease, maximum liver pathology and death usually occur about the six or seventh day after infection. \(^\text{13,14}\) If the analogy with the hamster model is correct, then treatment of human cases of YF with ribavirin in the early intoxication phase of the disease \(^\text{25}\) (fifth to seventh day of illness, when jaundice first becomes apparent) might be beneficial.

In summary, results of our preliminary studies indicate that ribavirin is effective in the treatment of YF virus infection in a hamster model of the disease. Treatment with ribavirin during the first five days after YF virus infection resulted in enhanced survival, and reduced tissue damage in target organs (i.e., liver and lymphoid tissues). Further experimental studies and perhaps limited clinical trials would seem warranted.

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