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Ribavirin Inhibits Viral-Induced Macrophage Production of TNF, IL-1, the Procoagulant fgl2 Prothrombinase and Preserves Th1 Cytokine Production But Inhibits Th2 Cytokine Response

Qin Ning,* Deron Brown,* Jean Parodo,† Mark Cattral,§ Reginald Gorczynski,† Edward Cole,* Laisum Fung,* Jin Wen Ding,* Ming Feng Liu,* Ori Rotstein,† M. James Phillips,‡ and Gary Levy*2*

Ribavirin, a synthetic guanosine analogue, possesses a broad spectrum of activity against DNA and RNA viruses. It has been previously shown to attenuate the course of fulminant hepatitis in mice produced by murine hepatitis virus strain 3. We therefore studied the effects of ribavirin on murine hepatitis virus strain 3 replication, macrophage production of proinflammatory mediators including TNF, IL-1, and the procoagulant activity (PCA), fgl2 prothrombinase; and Th1/Th2 cytokine production. Although ribavirin had inhibitory effects on viral replication (<1 log), even at high concentrations complete eradication of the virus was not seen. In contrast, at physiologic concentrations (up to 500 μg/ml), ribavirin markedly reduced viral-induced parameters of macrophage activation. With ribavirin treatment, the concentrations of PCA, TNF-α and IL-1β all decreased to basal concentrations: PCA from 941 ± 80 to 34 ± 11 mU/10⁶ cells; TNF-α from 10.73 ± 2.15 to 2.74 ± 0.93 ng/ml; and IL-1β from 155.91 ± 22.62 to 5.74 ± 0.70 pg/ml. The inhibitory effects of ribavirin were at the level of gene transcription as evidenced by Northern analysis. Both in vitro and in vivo, ribavirin inhibited the production of IL-4 by Th2 cells, whereas it did not diminish the production of IFN-γ in Th1 cells. In contrast, ribavirin had no inhibitory effect on TNF-α and IL-1β production in LPS-stimulated macrophages. These results suggest that the beneficial effects of ribavirin are mediated by inhibition of induction of macrophage proinflammatory cytokines and Th2 cytokines while preserving Th1 cytokines.


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Abbreviations used in this paper: MHV-3, mouse hepatitis virus strain 3; PCA, procoagulant activity; musfiblp, mouse fibrinogen-like protein; HCV, hepatitis C virus; PFU, plaque-forming units; MOI, multiplicity of infection.

To examine the mechanisms of the beneficial effect of ribavirin in this experimental model of viral hepatitis, we studied its effects on MHV-3 replication; its effects on macrophage production of TNF, IL-1, and PCA and Th1/Th2 cytokine production in vitro.

Materials and Methods

Mice

Female BALB/cJ, A/J, and C3H/HeJ mice, 6 to 8 wk of age, were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). The animals were kept in microisolator cages and housed in the animal facilities at the Toronto Hospital, and were fed with standard laboratory chow diet and water ad libitum.

Virus

MHV-3 was obtained from American Type Culture Collection (Rockville, MD) and was plaque purified on monolayers of DBT cells and grown to titers of 2 × 10⁶ plaque-forming units (PFU)/ml in 17 CL1 cells. Virus was harvested by centrifugation at 4500 x g for 1 h at 4°C and was assayed on monolayers of L2 cells in a standard plaque assay (5).

Cells

Peritoneal macrophages were harvested from BALB/cJ mice 4 days after i.p. administration of 1.5 ml of 3% thiolgylcolate (Difco Laboratories, Detroit, MI) as previously described (6). Macrophages were resuspended in RPMI 1640 (ICN Biomedicals, Costa Mesa, CA) supplemented with 2 mM glutamine (Sigma Chemical, St. Louis, MO) and 2% heat-inactivated FCS (Flow Laboratories, Mississauga, Ontario, Canada). Macrophages were >95% pure as determined by morphology and nonspecific esterase stain (7). Viability exceeded 95% by trypan blue exclusion.

A Th1/Th2 cell line was derived from C3H/HeJ mice that had been immunized with 1 × 10⁶ irradiated (2000 rad) B10BR spleen cells and poly(IC) i.p. T cells were recovered from spleen, diluted, and incubated with 2 × 10⁵ irradiated B10BR cells and IL-2 in 96-well U-bottom plates.
Cells were fed weekly by adding fresh medium and rechallenged every 10 days with freshly harvested and irradiated B10BR spleen cells. Wells showing proliferation were transferred to T-25 tissue culture flasks. Stable T cells were then passaged and redistributed at limiting dilution under the same conditions (with fresh medium and irradiated B10BR spleen cells). CD4+ Th1 (3E9.1) and Th2 (3B6.8) cell lines were derived from MHV-3-resistant A/J and MHV-3-susceptible BALB/cJ mice, respectively. Addition of ribavirin to peritoneal macrophages from BALB/cJ mice had no toxic effects as determined by trypan blue exclusion (>95% viable).

**Effect of ribavirin on macrophages and Th2 cytokines**

**Cytotoxicity of ribavirin**

Addition of ribavirin up to 500 μg/ml to peritoneal macrophages freshly isolated from BALB/cJ mice had no toxic effects as demonstrated by trypan blue exclusion (>95% viable).

**Effect of ribavirin on the growth of MHV-3 in macrophages**

The addition of ribavirin to macrophages from BALB/cJ mice inhibited MHV-3 replication as demonstrated in Figure 1. The inhibitory effects showed a dose-dependent pattern over the range 10^4 to 500 μg/ml. However, in macrophages, the maximal inhibitory effect of ribavirin was only 1 log, from 2.40 ± 0.07 to 1.30 ± 0.05 PFU/ml at a concentration of 100 μg/ml with no further increase at a concentration as high as 500 μg/ml compared with no ribavirin treatment. The inhibition was not due to toxic effects of ribavirin on cells as demonstrated by trypan blue exclusion.

To determine whether ribavirin directly affected the ability of virus to infect cells, macrophages were pretreated with ribavirin for 2 h before addition of MHV-3, following which cells were cultured for an additional 10 h, harvested, and assayed for viral titers. There was no significant difference on inhibition of viral replication in the presence or absence of preincubation suggesting...
that the inhibitory effect was not due to inhibiting entry of MHV-3 into the cell (Fig. 1).

Effects of ribavirin on functional PCA and transcription of mRNA for fgl-2 in MHV-3-infected macrophages

Macrophages infected with MHV-3 for 8 h demonstrated a significant rise in PCA (941 ± 80 mU/10⁶ cells) in comparison with basal values of 63 ± 15 mU/10⁶ cells. With ribavirin treatment over the range 100 to 500 μg/ml, macrophages PCA decreased significantly from 941 ± 80 mU/10⁶ cells to 34 ± 11 mU/10⁶ cells (maximum 94% inhibition) (p < 0.001). The inhibitory effect of ribavirin showed a concentration-dependent manner (Fig. 2). To determine whether ribavirin inhibited the one-stage clotting assay, ribavirin (500 μg/ml) was added to MHV-3-infected macrophages just before the PCA assay. Ribavirin had no inhibitory effect on the ability to determine PCA (data not shown).

Northern blot analysis showed MHV-3-induced mRNA of fgl-2 was inhibited by ribavirin. This effect was dose dependent with inhibition seen at 100 μg/ml and complete inhibition observed at 500 μg/ml of ribavirin, as seen in Figure 3.

Effect of ribavirin on production and mRNA transcription of TNF in macrophages

Macrophages produced significantly higher levels of TNF-α in response to MHV-3 infection (10.73 ± 2.15 ng/ml) in comparison with the basal values (0.03 ± 0.03 ng/ml). Ribavirin significantly

FIGURE 1. Inhibitory effect of ribavirin on MHV-3 replication in BALB/cJ macrophages and L2 cells. Confluent monolayers of macrophages in six-well plates were infected with 2 × 10⁶ MHV-3 in the presence or absence of indicated concentration of ribavirin for 10 h. For the pretreatment group, cells were incubated with ribavirin for 2 h before MHV-3 infection. Viral titers were measured on monolayers of L2 cells in a standard plaque assay. The bars represent the mean ± SD of four independent experiments compared with control (ribavirin, 0 μg/ml) by an unpaired t test (* two-tailed p < 0.01).

FIGURE 2. Effect of ribavirin on BALB/cJ macrophage PCA induced by MHV-3. One million macrophages were stimulated with MHV-3 at a MOI of 2.5 and harvested after 8 h for measurement of PCA activity. Values represent the mean ± SD of three separate experiments done in triplicate. *represents p < 0.01 when compared with MHV-3-stimulated macrophages.

FIGURE 3. Effect of ribavirin on transcription of mRNA of fgl2 in MHV-3-infected BALB/cJ macrophages. Thirty micrograms of total RNA extracted from macrophages that had been stimulated with MHV-3 at a MOI of 2.5 were added to each lane and hybridized with musfiblp cDNA (E1B) as described in Materials and Methods. Lane 1, macrophages alone; Lane 2, macrophages + ribavirin (500 μg/ml); Lane 3, macrophages + MHV-3; Lane 4, macrophages + MHV-3 + ribavirin (500 μg/ml); Lane 5, macrophages + MHV-3 + ribavirin (100 μg/L); Lane 6, macrophages + MHV-3 + ribavirin (1 μg/ml). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to ensure equal amounts of RNA in all lanes.
inhibited the production of TNF-α in MHV-3-infected macrophages over the range 100 to 500 μg/ml. The inhibitory effect showed a dose-dependent pattern with a maximum 75% inhibition in TNF-α (10.73 ± 2.15 to 2.74 ± 0.93 ng/ml) (p < 0.001) (Fig. 4). By Northern blot analysis, addition of ribavirin prevented increased levels of TNF mRNA transcription seen in response to MHV. The reduced effect was first detected at a concentration of 100 μg/ml with complete inhibition at 500 μg/ml (Fig. 5A).

**Effect of ribavirin on production and mRNA transcription of IL-1 in macrophages**

Supernatants from macrophages were analyzed for production of IL-1. The dose-dependent inhibitory effect of ribavirin on the IL-1β production of MHV-3-stimulated macrophages is depicted in Figure 6. Ribavirin significantly inhibited IL-1β production from 155.91 ± 22.62 to 5.74 ± 0.70 pg/ml over the range 10 to 500 μg/ml (maximum 96% inhibition) (p < 0.001). This inhibitory effect was at the level of gene transcription as evidenced by Northern blot analysis (Fig. 5B). The mRNA concentration for IL-1 was inhibited by ribavirin (100 μg/ml) and was undetectable when cells were treated with 500 μg/ml ribavirin.

To determine whether the inhibitory effect of ribavirin on transcription of mRNA for IL-1 and TNF was specific or a general phenomenon, the ability of ribavirin to inhibit transcription of mRNA for these cytokines following stimulation with endotoxin (LPS) was determined. Ribavirin had no effect on mRNA levels of TNF-α and IL-1 in LPS-stimulated macrophages even when added at concentrations up to 500 μg/ml (Fig. 7).

**Effect of ribavirin on Th1/Th2 cytokine profiles**

**In vitro.** To determine whether ribavirin differentially affected production of Th1/Th2 cytokines, ribavirin (1–500 μg/ml) was added to the T cell lines. Supernatants were collected and assayed for the production of IFN-γ and IL-4 as described above. Ribavirin, when added at concentrations from 1 to 500 μg/ml, had no inhibitory effect on the production of IFN-γ (84.5 ± 3.7 ng/ml) by either the Th1/Th2 line or the Th1 cell line 3F9.1, as shown in Table I. Addition of ribavirin at a concentration higher than 100 μg/ml inhibited the production of IL-4 by both the Th1/Th2 line and the Th2 line 4B6.8 (Table I). A maximum 97% inhibition (p < 0.01) was seen at concentrations of ribavirin ≥100 μg/ml and was not due to toxic effect of ribavirin on Th2 cells as shown by trypan blue exclusion.

**FIGURE 4.** Effect of ribavirin on BALB/cJ macrophage TNF-α production induced by MHV-3. One million macrophages from BALB/cJ mice were stimulated with MHV-3 at a MOI of 2.5. TNF levels in the supernatants were measured by ELISA after incubation for 8 h. Data are presented as mean ± SD for three separate experiments done in duplicate. * represents p < 0.01 compared with macrophages + MHV-3.

**FIGURE 5.** Effect of ribavirin on mRNA level of TNF-α and IL-1β in BALB/cJ macrophages stimulated with MHV-3 by Northern blot analysis. Thirty micrograms of total RNA extracted from macrophages that had been stimulated with MHV-3 at a MOI of 2.5 were added to each lane and hybridized with TNF-α cDNA (A) or IL-1β cDNA (B) as described in Materials and Methods. Lane 1, macrophages alone; Lane 2, macrophages + ribavirin (500 μg/ml); Lane 3, macrophages + MHV-3; Lane 4, macrophages + MHV-3 + ribavirin (500 μg/ml); Lane 5, macrophages + MHV-3 + ribavirin (100 μg/L); Lane 6, macrophages + MHV-3 + ribavirin (1 μg/ml). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to ensure equal amount of RNA in all lanes.

**FIGURE 6.** Effect of ribavirin on BALB/cJ macrophage IL-1β production induced by MHV-3. One million macrophages from BALB/cJ mice were stimulated with MHV-3 at a MOI of 2.5. IL-1β levels in the supernatants were measured by ELISA after incubation for 8 h. Data are presented as mean ± SD for three separate experiments done in duplicate. * represents p < 0.01 compared with + MHV-3.
compared with no ribavirin treatment. IL-4 were measured in the supernatants as described in Materials and Methods.

Ribavirin (500 mg/kg) was administered daily i.p. to BALB/cJ mice as previously described (8). Th1/Th2, Th1 (3E9.1), and Th2 (4B6.8) cells (2 x 10^5) were cultured with various concentrations of ribavirin for 24 h. IL-4 and IFN-γ were measured on day 3 post-MHV-3 infection in sera and are mean ± SD from four mice per group. IL-4 and IFN-γ were measured as described in Materials and Methods. All mice infected with 100 PFU of MHV-3.

Table II. Effect of ribavirin on cytokine release following MHV-3 infection in vivo

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Treatment Protocol</th>
<th>IL-4 (pg/ml)</th>
<th>IFN-γ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cJ</td>
<td>Saline</td>
<td>28.4 ± 4.1</td>
<td>2.8 ± 11</td>
</tr>
<tr>
<td></td>
<td>Ribavirin</td>
<td>62.6 ± 1.1*</td>
<td>5.5 ± 2.0</td>
</tr>
<tr>
<td>A/J</td>
<td>Saline</td>
<td>2.3 ± 1.1</td>
<td>171.1 ± 11</td>
</tr>
<tr>
<td></td>
<td>Ribavirin</td>
<td>2.1 ± 1.2</td>
<td>156 ± 8</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>Saline</td>
<td>22.4 ± 8.2</td>
<td>15.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Ribavirin</td>
<td>37.2 ± 2.1*</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

*Concentrations of IL-4 and IFN-γ were measured on day 3 post-MHV-3 infection in sera and are mean ± SD from four mice per group. IL-4 and IFN-γ were measured as described in Materials and Methods. All mice infected with 100 PFU of MHV-3. * P < 0.01 compared with saline-treated mice.

Treatment of mice with ribavirin resulted in a marked inhibition in levels of IL-4 in semisusceptible and susceptible mice, whereas it had little effect on production of IFN-γ in resistant mice similar to the observed effects in vitro in the Th1, Th2, and Th1/Th2 lines (Table II).

**Discussion**

Viral hepatitis is a major health problem accounting for significant morbidity and mortality. An incomplete understanding of the pathogenesis of viral hepatitis has limited the development of successful medical approaches to its treatment (14). Recent studies have suggested that the antiviral agent ribavirin alone or in combination with IFN may be of benefit in the treatment of patients with hepatitis B (15) and C (1, 16–18). However, the mechanism for the beneficial effect of ribavirin remains unknown given that ribavirin appears not to eradicate viral replication. Our laboratory has been interested in determining the mechanism for susceptibility to viral hepatitis and has extensively examined the immunopathogenesis of viral hepatitis using an experimental animal model of acute and chronic liver disease induced by mouse hepatitis virus strain 3 (MHV-3) (8, 19–22). Studies to date have suggested that resistance to MHV-3 is associated with a predominant Th1 response, the production of IFN, neutralizing Abs, and cytotoxic T cells (8, 22). In contrast, in susceptible mice, viral infection of macrophages leads to a marked inflammatory response including sustained production of TNF, IL-1, a unique procoagulant fgl2 prothrombinase encoded by the gene fgl2 located on mouse chromosome 5, and is associated with a Th2 cellular immune response and production of nonneutralizing Abs (23). Previous studies in our laboratory have demonstrated that susceptibility to MHV-3 correlates with macrophage activation and not viral replication (20). The importance of the fgl2 prothrombinase, which can directly cleave prothrombin to thrombin, has been demonstrated by the fact that treatment of mice with a high titered neutralizing Ab

Table I. Effect of ribavirin on cytokine production by T cell lines

<table>
<thead>
<tr>
<th>Ribavirin (μg/ml)</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-4 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Th1/Th2</td>
<td>3E9.1</td>
</tr>
<tr>
<td></td>
<td>16 ± 1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>16 ± 1</td>
<td>0.3 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>11 ± 4</td>
<td>0.2 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>1 ± 1*</td>
<td>0.1 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.1*</td>
<td>0.2 ± 0.10</td>
</tr>
</tbody>
</table>

* The Th1/Th2 cell line was derived from C3H/HeJ mice. 3E9.1 is a Th1 cell line derived from A/J mice as previously described (8). 4B6.8 is a Th2 cell line derived from BALB/cJ mice as previously described (8). Th1/Th2, Th1 (3E9.1), and Th2 (4B6.8) cells (2 x 10^6) were cultured with various concentrations of ribavirin for 24 h. IFN-γ and IL-4 were measured as described in Materials and Methods. Results represent the mean ± SD of three experiments done in triplicate. (* indicates P < 0.001 compared with no ribavirin treatment.)
to fgl2 prothrombinase fully protected these mice from the lethal-
ity of MHV-3 (6). In contrast, inactivated virus (MHV-3) did not
induce the production of these inflammatory mediators and did not
cause disease (24). Previous studies have shown that ribavirin pro-
longed the course of MHV-3 infection and increased the survival of
animals (1, 4). The present studies were undertaken to deter-
mine the mechanism of the beneficial effects of ribavirin in the
experimental animal model of MHV-3.

Our data confirm that although ribavirin has minimal (<1 log)
hibitory effects on replication of MHV-3 in vitro, even at
very high concentrations complete eradication of the virus was
not seen, as has been previously reported (1, 25, 26). However,
ribavirin at concentrations that are achieved in vivo (4) almost
totally inhibited the production of proinflammatory mediators
TNF, IL-1, and PCA in BALB/cJ macrophages in vitro. Riba-
vin was unable to cause similar inhibition of LPS-induced
inflammatory cytokines. This may be due to the fact that in-
duction of TNF, IL-1, and PCA is due to LPS induction of IFN
which is not inhibited by ribavirin (27, 28).

In several other animal models of liver injury, including those
due to virus infection, endotoxin, CCL, galactosamine, and ace-
ptomogen, the hepatic injury is associated with fibrin deposition,
sinusoidal thrombosis, and accumulation of the inflammatory cells
(29–37). In the hepatocellular necrosis associated with these
pathologic processes, resident macrophages within the liver
(Kupffer cells) exhibit morphologic features of activation and re-
lease a number of inflammatory mediators, including TNF, IL-1,
proteolytic enzymes, and eicosanoids, as well as superoxide anions
and nitric oxide (36). Furthermore, Chisari (38), using a hepatitis
B surface Ag transgenic mouse model, has shown that although
CD8+ CTL initiates hepatocyte injury, macrophages and their in-
flammatory mediators, in particular IL-1 and TNF, are responsible
for massive hepatic necrosis. Inactivation of these macrophages
prevents hepatic necrosis.

Macrophages generate a wide range of mediator molecules
which may contribute either directly or indirectly to the develop-
ment of fulminant hepatitis by inducing PCA (37). TNF and IL-1
production by macrophages can stimulate endothelial cell produc-
tion of immune coagulants and increase neutrophil-endothelial in-
teraction, thereby potentially promoting microvascular thrombo-
sis (39, 40).

The immune system plays an essential role in the outcome of
viral infection. One mechanism of the immune response in vivo
involves CD4+ Th cells, which, through the production of cyto-
kines, control the development of immune effector mechanisms
such as Ab production, generation of cytotoxic T cells, and mac-
rophage activation. Following Ag exposure, Ag-specific Th cells
differentiate along two pathways (41). Th1 cells, through the pro-
duction of IL-2, IFN-γ, and lymphotixin, mediate cellular immu-
nity, which is essential for clearance of viral and other pathogens
(42–51). Th2 cells, conversely, produce IL-4, IL-10, and IL-13 and
are most effective in providing help for B cell differentiation to
plasma cells. IFN-γ production by Th1 cells inhibits the prolifer-
ation of Th2 cells (43), whereas treatment with neutralizing Abs to
IFN-γ promotes the development of a Th2 response (44, 45).

Our data also showed that ribavirin diminished IL-4 production
both by the Th1/Th2 line and the MHV-3-specific Th2 cell line
4B8.6, while no effect was seen on IFN-γ production by Th1 cells,
thus preventing the shift to a Th2 response.

In vivo, treatment of MHV-3-infected semisusceptible C3H/HeJ
and susceptible BALB/cJ mice with ribavirin resulted in inhibition
of IL-4 production, but similar doses of ribavirin did not inhibit
production of IFN-γ production in resistant A/J mice. This may
explain the benefit of ribavirin on amelioration of MHV-3 infec-
tion and the increased survival in vivo previously reported (4).

A Th1 response has been associated with host resistance, and a
Th2 response has been associated with susceptibility in murine
models of leishmaniasis, candidiasis, listeriosis, and MHV-3 (46,
47). Recently, in acute and chronic hepatitis B virus infection in
humans, a Th1 response has also been associated with resistance
and a Th2 response with susceptibility (38, 48). Furthermore, im-
munomodulatory treatments that have converted a Th1 to a Th2
response have resulted in the loss of resistance (38), and, con-
versely, induction of a Th1 response has led to resolution of the
infection (48). In hepatitis C, studies on cytokine profiles have
been reported only in patients with chronic disease in contrast to
the model of acute fulminant hepatitis induced by MHV-3. The
reports in patients with HCV are somewhat conflicting but suggest
a poor or absent Th1 response in these patients (49–51). Recent
studies have shown that ribavirin has beneficial effects on serum
aminotransferase concentrations and necroinflammatory activity
of liver biopsies in patients with chronic HCV infection. However,
ribavirin has no effect on serum HCV RNA titers, and biochemical
relapse is universal after cessation of therapy (15–17). The discord-
dance between the virologic and biochemical responses in patients
receiving ribavirin suggests that the beneficial effect of ribavirin
may not be mediated by inhibition of viral replication. Another
possibility is that ribavirin modulates the immune response to
HCV.

In conclusion, our study suggests that ribavirin is a very potent
inhibitor of viral-induced proinflammatory mediators. The benefi-
cial effects of ribavirin in both experimental animal models and
clinical treatment may be related to its ability to markedly reduce
macrophage activation and diminish Th2 cytokine production
while preserving Th1 cytokine production. These data provide a
rational potential clinical utility of ribavirin either alone or in com-
promise with IFN-α in patients with hepatitis or as a substitute in
IFN-α-nonresponsive patients.

Acknowledgments
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