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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 Catrall, Reginald Gorczynski, Edward Cole, Laisum Fung, Jin Wen Ding, Ming Feng Liu, Ori Rotstein, M. James Phillips, and Gary Levy

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 from 941 ± 80 to 34 ± 11 μM/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. In 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. The Journal of Immunology, 1998, 160: 3487–3493.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic guanosine analogue that possesses a broad spectrum of activity against DNA and RNA viruses (1). Earlier studies have shown that ribavirin has immunosuppressive activity, reducing lymphocyte proliferation to mitogens in vitro and prolonging skin allograft survival in rats (2, 3). Previous reports have shown that ribavirin attenuates the course of an experimental model of fulminant hepatitis in mice produced by murine hepatitis virus strain 3 (MHV-3) (4). Studies in our laboratory as well as others have demonstrated a causal relationship between macrophage activation, production of proinflammatory mediators such as TNF and IL-1 as well as the unique procoagulant activity fgl2 prothrombinase, and the subsequent development and pathogenesis of MHV-3-induced liver disease (5, 6).

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×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 5% thiglycollate (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.

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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-susceptible A/J and MHV-3-resistant A/J mice, respectively. The procedures were previously described (8). The cell lines were recovered from draining lymph nodes of mice that had been injected in the footpad with MHV-3. The cell lines were subtyped by flow cytometry and cytokine production assayed by the ability of supernatants to support proliferation of CTL-2 and CT4.5 cells in the presence of anti-IL-2 and anti-IL-4 mAbs, respectively. IFN-γ activity was determined by inhibition of plaque formation induced by MHV-3 on L2 cells (8). Stable T cells were passaged with fresh medium and irradiated splenic mononuclear cells as described previously (8).

**Viral titer (plaque assay)**

The effect of ribavirin on viral titer were determined in a standard plaque assay (5). Briefly, monolayers of peritoneal macrophages from BALB/cJ mice were pretreated with either ribavirin (0–500 μg/ml) or medium (RPMI 1640), and 2 × 10^5 PFU of MHV-3 was then added. Following a 10-h incubation at 37°C with 5% CO₂, cells were harvested, subjected to one cycle of freeze-thawing, and assayed for viral titers on monolayers of L2 cells.

**PCA**

MHV-3-infected macrophages, at a multiplicity of infection (MOI) of 2.5, were incubated with ribavirin for 8 h in supplemented RPMI 1640. Uninfected macrophages and MHV-3-infected macrophages without drug treatment were set up as negative and positive controls, respectively. Macrophages were evaluated for functional PCA in a one-stage clotting assay as previously described (5). Following incubation, samples to be assayed for PCA were washed three times with unsupplemented RPMI 1640 and resuspended at a concentration of 10^6/ml. Samples were assayed for the ability of supernatants to support the spontaneous clotting of normal circulating human platelet-poor plasma. Milliliters of PCA were assigned by reference to a standard curve generated with serial log dilutions of a standard rabbit brain thromboplastin (Dade Division, American Hospital Supply, Miami, FL). Media and reagents were assayed showing no PCA activity.

**Cytokine assay**

**TNF.** MHV-3 (at a MOI of 2.5) or LPS (10 μg/ml, Sigma)-stimulated BALB/cJ macrophages were incubated with ribavirin (0–500 μg/ml) for 8 or 4 h, respectively. Supernatants were collected, and TNF-α concentrations were assayed by ELISA. Monoclonal hamster anti-murine TNF-α Ab (Genzyme, Boston, MA) was coated to ELISA plates overnight at 4°C. After being washed with Tris buffer (pH 8.0), plates were blocked with 100 μl of 5% BSA in each well for 1 h at room temperature. Following washing, 100 μl of standards or samples were added and incubated at room temperature for 3 h. Subsequently, 100 μl of polyclonal rabbit anti-mouse TNF-α Ab (Genzyme, IP-400) were added to each well, and plates were incubated at 4°C overnight. Goat anti-rabbit IgG alkaline phosphatase conjugate (100 μl) (Jackson ImmunoResearch Laboratories, West Grove, PA) was added, and plates were incubated at room temperature for 1 h. Following washing, 100 μl of FSAP (dilutase phosphate, 1:10 in substrate buffer) were added, and plates were incubated for an additional 10 min at room temperature with shaking. Plates were read by Microplate Fluor (Beckman Instruments, Fullerton, CA). Units were assayed by comparison to a mouse IFN-γ standard (Genzyme).

**IL-1.** MHV-3 (at a MOI of 2.5) or LPS (10 μg/ml)-stimulated macrophages were incubated with ribavirin (0–500 μg/ml) for 8 or 4 h, respectively. Production of IL-1β was measured by ELISA (R&D Systems, Minneapolis, MN). An affinity-purified polyclonal Ab specific for mouse IL-1β was precoated onto the microtiter plate. Standards, controls, and samples were added to the wells for 2 h at room temperature. After washing, an enzyme-linked polyclonal Ab specific for mouse IL-1β was added to the wells, and plates were incubated at room temperature for 2 h. Following washing, a substrate solution was added to the wells. After 30 min of incubation at room temperature, the stop solution was added, and plates were read by Titertek Multiskan MCC/340. Ribavirin was added to the supernatants from MHV-3- or LPS-infected macrophages at a final concentration of 1–500 μg/ml. Effects were read by inhibition of plaque assay for TNF-α and IL-1β.

**IL-4 and IFN-γ.** Production of IL-4 and IFN-γ in the T cell lines (9) was measured by bioassay. For the Th1/Th2 cell line, 2 × 10^4 cells were first stimulated with peritoneal cells from B10BR (C3H) and cultured with ribavirin (0–500 μg/ml) for 24 h in a final volume of 200 μl in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U of penicillin, and 100 μg of streptomycin (Flow Laboratories) per ml in 96-well flat-bottom microtest plates (Falcon Laboratories, Grand Island, NY). For the MHV-3-specific Th1 (3E9.1) and Th2 (3B6.8) cell lines, the lines were cocultured in growth factor-free medium with MHV-3-infected, irradiated splenic mononuclear cells as APCs, and the supernatants were collected 18 h later. Supernatants were collected for measurement of IFN-γ and IL-4 production by the ability of supernatants to inhibit or support the proliferation of WEHI 297 and CT4.S cells, respectively. Cultures were incubated for 40 h in a humidified CO₂ atmosphere at 37°C, pulsed with 1 μCi of [3H]Tdr (sp. act. 2 Ci/mmol; Amersham, Arlington Heights, IL), and harvested 26 h later onto glass fiber filters. Total cell-associated radioactivity was measured in a Beckman scintillation counter, and bioassay data were expressed as nanograms per milliliter for IFN-γ and units per ml for IL-4, derived from a standard curve with mouse recombinant IFN-γ and IL-4 (Genzyme). Ribavirin was added to WEHI 297 and CT4.S cells at different concentrations (1–500 μg/ml), showing no effect on the assay system (data not shown).

**Northern (RNA) blot analysis**

The effect of ribavirin on mRNA levels of the MHV-3-induced PCA, fgl2 prothrombinase, TNF, and IL-1 were assayed by Northern blot analysis. Northern (RNA) blots were hybridized with 6 S acid-guanidium hydrochloride extraction in a modified procedure described by Armstein and Cox (10). RNA was resolved on a 1% agarose gel containing formaldehyde and transferred onto a nitrocellulose membrane (Bio-Rad, Oakville, Ontario, Canada). A 1.3-kb fgl2 prothrombinase cDNA (11), a 1.4-kb TNF cDNA (12), and a 2-kb IL-1 cDNA (13) were excised from plasmids, purified by agarose gel electrophoresis, and labeled by using a random-priming DNA labeling system (Pharmacia, Montreal, Quebec, Canada) with α[32P]dCTP (sp. act. 3000 Ci/mmol; Amersham). Membranes were prehybridized for 4 h at 42°C in a mixture containing formamide (50%), 5 × Denhardt’s solution, 0.2% SDS, 100 μg of denatured salmon sperm DNA per ml, and 5 × SSPE. Hybridization was conducted overnight at 42°C in the same fresh mixture. The membranes were then washed under medium to high stringency conditions, and the membranes were exposed to Kodak XAR-5 film with intensifying screens for 16 to 24 h at ~70°C. To confirm that equivalent amounts of RNA were loaded in all lanes, the membranes were probed with α-tubulin cDNA.

**Statistical analysis**

Statistical analysis was conducted by Student t test, and a p value of 0.05 or less was considered to be statistically significant. Results were reported as the mean ± SD for at least three separate experiments, each performed in triplicate.

**Results**

**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 to 500 μg/ml. However, in macrophages, the maximal inhibitory effect of ribavirin was only 1 log, from 2.40 ± 0.02 × 10^7 PFU/ml to 1.55 ± 0.07 × 10^6 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/10^6 cells) in comparison with basal values of 63 ± 15 mU/10^6 cells. With ribavirin treatment over the range 100 to 500 μg/ml, macrophages PCA decreased significantly from 941 ± 80 mU/10^6 cells to 34 ± 11 mU/10^6 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

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**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 × 10^6 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 for 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 3E9.1, 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.
compared with no ribavirin treatment. IL-4 were measured in the supernatants as described in Materials and Methods.

m
g
(2 h before infection with 100 PFU of MHV-3. Groups of mice either ribavirin at a dose of 75 mg/kg/day i.p. or saline beginning (C3H/HeJ), and resistant (A/J) to MHV-3 were treated daily with increase in IL-4, but no increase in IFN-γ whereas sera from MHV-3-infected BALB/cJ mice had an increase in levels of IL-4 consistent with a Th1 response. Sera from MHV-3-infected semisusceptible C3H mice had a cytokine profile similar to that of susceptible BALB/cJ mice.

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

<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>0</td>
<td>85 ± 5</td>
<td>114 ± 12</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>500</td>
<td>78 ± 8</td>
<td>114 ± 12</td>
</tr>
</tbody>
</table>

a 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 × 10⁴) were cultured with various concentrations of ribavirin for 24 h. IFN-γ and IL-4 were measured in the supernatants 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).

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 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

FIGURE 7. Effect of ribavirin on mRNA levels of TNF-α and IL-1β in BALB/cJ macrophages stimulated with LPS by Northern blot analysis. Thirty micrograms of total RNA extracted from macrophages that had been stimulated with LPS at 10 µg/ml added to each lane and hybridized with TNF-α cDNA (A) or IL-1 cDNA (B) as described in Materials and Methods. Lane 1, macrophages alone; Lane 2, macrophages + ribavirin (300 µg/ml); Lane 3, macrophages + LPS; Lane 4, macrophages + LPS + ribavirin (500 µg/ml); Lane 5, macrophages + LPS + ribavirin (100 µg/ml); Lane 6, macrophages + LPS + ribavirin (1 µg/ml). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to ensure equal amounts of RNA in all.

In vivo. To determine the effect of ribavirin on levels of IL-4 and IFN-γ in vivo, mice fully susceptible (BALB/cJ), semisusceptible (C3H/HeJ), and resistant (A/J) to MHV-3 were treated daily with either ribavirin at a dose of 75 mg/kg/day i.p. or saline beginning 2 h before infection with 100 PFU of MHV-3. Groups of mice (n = 4) were sacrificed daily, and sera were collected and assayed for IL-4 and IFN-γ. Similar to previous results (21), sera from MHV-3-infected A/J mice had an increase in levels of IFN-γ, but no increase in levels of IL-4 consistent with a Th1 response whereas sera from MHV-3-infected BALB/cJ mice had an increase in IL-4, but no increase in IFN-γ consistent with a Th2 response. Sera from MHV-3-infected semisusceptible C3H mice had a cytokine profile similar to that of susceptible BALB/cJ mice.

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

<table>
<thead>
<tr>
<th>Treatment Protocol</th>
<th>IL-4 (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BALB/cJ</td>
<td>A/J</td>
</tr>
<tr>
<td>Saline</td>
<td>28.4 ± 4.1</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>6.2 ± 1.1*</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>5.5 ± 2.0</td>
<td>156 ± 8</td>
</tr>
</tbody>
</table>

a 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 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).

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 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

* 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 × 10⁴) were cultured with various concentrations of ribavirin for 24 h. IFN-γ and IL-4 were measured in the supernatants 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)
inhibitory 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. Ribav-
irin 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, CCL4, galactosamine, and ace-

detaminophen, 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 IFN-alpha, while no effect was seen on IFN-
while preserving Th1 cytokine production. These data provide a
rational potential clinical utility of ribavirin either alone or in com-
bination with IFN-alpha in patients with hepatitis or as a substitute in
IFN-alpha-responsive patients.

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