Neuroprotection and Reduced Proliferation of Microglia in Ribavirin-Treated Bornavirus-Infected Rats

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In a rat model of Borna disease, intracerebral ribavirin caused clinical improvement without changes in virus titer or nucleic acid. Levels of microglia and infiltrating CD4 and CD8 cells were decreased, despite increases in mRNAs encoding interleukin-1β (IL-1β), IL-10, and gamma interferon in the brain. Intracerebral ribavirin may reduce morbidity through effects on microglia cell proliferation.

Bornavirus (BD) virus (BDV) is a nonsegmented negative-strand RNA virus that causes a multiphasic immune-mediated neurological syndrome (3, 8, 13, 20, 28–31, 38–40, 45). Two drugs have been reported to have antiviral activity: amantadine (2, 14, 17) and ribavirin (24, 32). The antiviral activity of amantadine is controversial (12, 21, 41), with discordance attributed to BDV strain differences. Ribavirin consistently inhibits in vitro replication and transcription of BDV (24, 32) but has not been tested in vivo.

Ribavirin (1-β-d-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic nucleotide analogue with broad antiviral activity. The drug is phosphorylated within mammalian cells (18). The monophosphate form inhibits GMP biosynthesis at the step of IMP conversion to xanthine 5’-phosphate (42). Ribavirin 5’-triphosphate can inhibit viral RNA polymerases (e.g., influenza virus [15, 47], vesicular stomatitis virus [44], and La Crosse virus [10]) and 5’-capping guanylation of viral mRNA (e.g., vesicular stomatitis virus [7, 18] and vaccinia virus [19]). Ribavirin is also mutagenic. Following integration into the genome by poliovirus RNA polymerase, it incorporates cytidine and uridine with the same efficiency (11). Ribavirin can enhance Th1 immunity (22, 27, 33, 43), a concern in BD, where vacccination with nucleoprotein in a Lewis rat model resulted in enhanced Th1 immunity and viral clearance but aggravated morbidity and mortality (26). Thus, we assessed the utility of ribavirin as an antiviral agent in Lewis rats.

The drug was administered through indwelling intraventricular catheters. Stainless steel guide cannulas were stereotactically implanted into the left lateral cerebral ventricles of uninfected adult male Lewis rats (200 to 225 g; Charles River Laboratories) in accordance with the atlas coordinates of Paxinos and Watson (from bregma, anterior-posterior, −0.8; medial-lateral, +1.5; dorsal-ventral, −4.8; IB bar set at −3.3) (35). Ribavirin (ICN Pharmaceuticals) in sterile phosphate-buffered saline (PBS) was delivered for 7 consecutive days at doses of 0, 1.25, 2.5, 5, and 10 mg/kg/day in volumes of 5 to 10 µl. The animal care and handling procedures used were in compliance with institutional and National Institutes of Health guidelines.

Ribavirin was tolerated at 1.25 and 2.5 mg/kg/day; however, weight loss and death occurred in the 5- and 10-mg/kg/day treatment groups. On day 8 of treatment, rats were sacrificed by decapitation. A 3-mm lateral, sagittal section of the cortex, contralateral to the side of drug administration, was removed, homogenized in water, and assayed for ribavirin concentration by radioimmunoassay (1). The detection limit of the system was 1 µg/ml. An increase in the ribavirin concentration with increasing doses was observed, with significant differences between groups by one-way analysis of variance (ANOVA) across doses $F(3,13) = 13.785, P = 0.0002$ (Table 1). Dosage regimens of 1.25 and 2.5 mg/kg/day were chosen for further testing.

Twenty-three adult male Lewis rats (200 to 225 g) were implanted with left lateral cerebral ventricular cannulas at Charles River Laboratories. Seven days later, the rats were infected intranasally with $1.6 \times 10^5$ focus-forming units of BDV strain He/80 (9). The rats were maintained for 21 days, at which time all had evidence of BD (hyperactivity, exaggerated startle response). Thereafter, rats received daily intraventricular ribavirin injections of 1.25 or 2.5 mg/kg/day in a total volume of 5 µl (eight rats per group) or 5 µl of PBS (seven rats) for 7 days. Animals were monitored for weight change, and clinical scores were based on appearance, activity, and pattern of respiration.

Weights of experimental animals were analyzed by a repeated-ANOVA design. Dose formed the independent factor, and time was the repeated measure. Weights were different in the three treatment groups. There was a significant group (dose) × time interaction, $F(2, 154) = 2.695, P = 0.0015$, with post hoc main effects showing significant differences at day 28, the last test day ($F = 7.647, P = 0.001$) (Fig. 1A).

Traits were scored as follows, with higher numbers corresponding to increased disability: disheveled appearance, 1 or 2; bloody nose and eyes, 1 to 4; responsivity with scores for ambulatory or supine, 1 to 4; normal or labored respiration, 1 to 4; weight loss, 1 to 4. Trait scores for individual animals were ranked as indicating mild, moderate, or severe disease and then analyzed by using the information statistic (25). A signif-

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significant overall difference was observed between the three experimental groups ($F = 14.57, df = 6, P < 0.05$) and the maximal effect between the 0- and 2.5-mg/kg/day groups ($F = 11.72, df = 3, P < 0.01$), with the 2.5-mg/kg/day group having the best clinical ratings (Fig. 1B).

After 7 days of treatment with ribavirin at either 1.25 or 2.5 mg/kg/day or with PBS, animals were decapitated. Brains of infected rats given PBS were hemorrhagic and friable. Brains of rats treated with ribavirin were neither hemorrhagic nor friable (data not shown).

The virus titer in the hippocampus was determined by an immunofocus assay (34) using C6 cells (rat astroglia) and antisera to the BDV N and P proteins. The values, in focus-

<table>
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<tr>
<th>Group (no. of rats)</th>
<th>Dose (mg/kg)</th>
<th>Ribavirin conc.a</th>
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<tbody>
<tr>
<td>1 (3)</td>
<td>0</td>
<td>Not detected</td>
</tr>
<tr>
<td>2 (5)</td>
<td>1.25</td>
<td>30.3 ± 6.96</td>
</tr>
<tr>
<td>3 (5)</td>
<td>2.50</td>
<td>38.5 ± 9.75</td>
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<tr>
<td>4 (4)</td>
<td>5.00</td>
<td>78.3 ± 7.19</td>
</tr>
<tr>
<td>5 (2)</td>
<td>10.00</td>
<td>228.5 ± 171.5</td>
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a Mean ribavirin concentrations are given in micrograms per gram (wet weight) of brain tissue ± the standard error of the mean.

FIG. 1. Clinical effects of ribavirin treatment of BDV-infected rats. (A) Body weights, plotted against experimental days, of infected rats receiving ribavirin at 1.25 or 2.5 mg/kg/day or a vehicle control (0-mg/kg/day dose). Body weight was significantly increased in the 2.5-mg/kg/day ribavirin treatment group in comparison with the 0-mg/kg/day dose group. Individual means comparisons revealed a significant increase on the seventh (last) day of treatment (*, $P < 0.05$). The values shown are means ± standard error of the mean. (B) Clinical scores of infected rats receiving ribavirin at 1.25 or 2.5 mg/kg/day or a vehicle control (0-mg/kg/day dose). Clinical scores were improved in the ribavirin-treated groups, with a maximal effect between the 2.5- and 0-mg/kg/day dose groups (**, $P < 0.01$).
forming units per gram of brain, were as follows: 0-mg/kg/day dose, 759,000; 1.25 mg/kg/day dose, 608,250; 2.5 mg/kg/day dose, 501,250. No significant group differences were observed \( F(2,20) = 0.6196, P = 0.54 \).

Viral N gene RNA in the prefrontal cortex (PFC) was quantitated by real-time PCR. Total RNA (0.5 g) from the PFC was reverse transcribed and subjected to real-time PCR analysis with an ABI Prism 7700 Sequence Detector (PE Biosystems) based on the following parameters: 45 cycles of 15 s of denaturation at 95°C and 1 min of annealing and elongation at 60°C. The primers were 5’-CAG TCA CGG CGC GAT ATG T, 5’-GCA CCC CTC CGT GAA CAA, and 5’-carboxyfluorescein -ATC CCA GGA CTG CAC GCT GCG-XT-carboxy-tetramethyl rhodamine (reporter probe). There were no significant differences between treatment groups: 0-mg/kg/day dose, 22,861; 1.25 mg/kg/day dose, 22,513; 2.5 mg/kg/day dose, 17,897 \( F(2,20) = 0.569, P = 0.575 \).

The distribution and quantity of viral mRNA were assayed in sagittal brain sections by in situ hybridization. Twenty-micrometer sections were hybridized with a 35S-labeled RNA probe complementary to the second transcription unit of BDV (encoding the P and X proteins) (37) and analyzed by autoradiography with MCID software (37). No significant differences in the quantity or distribution of viral RNA were observed between treatment groups.

Effects of ribavirin on immune responses to BDV in the brain were investigated immunohistochemically. In the cortex, CD4 cells, CD8 cells, and microglia were detected by using the W3/25 (CD4) (46), OX-8 (CD8) (23), and OX-42 (microglia) (36) monoclonal antibodies; a biotinylated goat anti-mouse immunoglobulin G (IgG) secondary antibody; and the chromogen 3,3’-diaminobenzidine (Vector Laboratories). Perivascular and parenchymal CD4 and CD8 cells were reduced in ribavirin-treated animals, with more robust differences observed between the 0-mg/kg/day and high-dose groups (Fig. 2A, B, and C). OX-42-positive microglia cells were reduced in the high-dose ribavirin group (Fig. 2E and F). Numerous microglia rod cells were present in the cortices of the 0-mg/kg/day dose group. Cell morphologies were similar across groups, with no group showing greater microglia cell activation. The numbers of microglia cells in the cortices of animals receiving ribavirin at 2.5 mg/kg/day were similar to those found in uninfected nontreated rats (data not shown). For detection of IgG in brain sections, biotinylated goat anti-rat IgG was used (Vector Laboratories). There were no differences in IgG staining across treatment groups.

Cytokine mRNA expression in the PFC was investigated by multiprobe RNase protection assay using 10 μg of total RNA per sample and probes corresponding to interleukin-1α (IL-1α), IL-1β, tumor necrosis factors alpha and beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, and IL-10. No differences were observed between treatment groups.
IL-4, IL-5, IL-6, IL-10, gamma interferon (IFNγ), IFNβ, granulocyte-macrophage colony-stimulating factor, transforming growth factor β1 (TGFβ1), TGFβ2, TGFβ3, lymphotoxin B, and migration inhibition factor (RiboQuant; PharMingen). Protected fragments were resolved on a 5% denaturing polyacrylamide gel and analyzed by phosphorimaging (Storm 840; Molecular Dynamics). There were significant increases in IL-1β, IL-10, and IFN-γ levels were observed in the 2.5-mg/kg/day ribavirin treatment group, compared to the 0-mg/kg/day dose group. The values shown are mean optical densities in arbitrary units ± the standard error of the mean. Groups were analyzed by ANOVA, followed by pairwise t-test comparisons. * P < 0.05; *** P < 0.0001 (relative to the 0-mg/kg/day dose group).

Antiviral activity by ribavirin in vivo has been attributed in part to its ability to enhance type I cytokine responses. In human hepatitis C virus infection and in murine models of hepatitis B virus infection, ribavirin can produce changes in Th1/Th2 balance and normalization of hepatic enzyme levels without reducing the viral load (4, 6, 22). In BD rats, ribavirin treatment results in increased levels of Th1 cytokines IL-1β and IFNγ but also increased levels of IL-10, a Th2 cytokine. The most dramatic histologic difference in ribavirin-treated BD rats was the reduction in the numbers of microglia cells. Reduced numbers of microglia cells would conceivably generate fewer neurotoxic products (e.g., proteases, reactive oxygen species, nitric oxide, excitotoxic amino acids, and inflammatory cytokines). Similarly, fewer CD4 and CD8 cells would generate fewer toxic cytokines.

The reduction in the number of lymphocytes in the high-dose ribavirin group may derive from relative preservation of the blood-brain barrier in the ribavirin-treated group. Alternatively, the reduction in both lymphocytes and microglia may signify a direct effect of the drug on dividing cells through depletion of cellular GTP pools (16).

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