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Short communication

Ribavirin inhibits Borna disease virus proliferation and fatal neurological diseases in neonatally infected gerbils

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ABSTRACT

By using neonatal gerbils, we assessed the effect of ribavirin on the proliferation of Borna disease virus (BDV) in the brain. The intracranial inoculation of ribavirin reduced viral propagation in the acutely infected brain, resulting in protection from fatal neurological disorders. We found that the treatment with ribavirin markedly reduces the numbers of OX-42-positive microglial cells, but does not activate expression of Th1 cytokines, in BDV-infected gerbil brains. Our results suggested that ribavirin directly inhibits BDV replication and might be a potential tool for the treatment of BDV infection.

Borna disease virus (BDV) induces Borna disease (BD) in naturally infected horses and sheep, which is characterized by severe non-purulent meningoencephalitis with massive perivascular and parenchymal infiltration (Ikuta et al., 2002a,b; Rott and Becht, 1995). Numerous reports have demonstrated that asymptomatic natural infections of BDV occur worldwide in a variety of vertebrate species, suggesting that the host range of this virus includes all warm-blooded animals (Ludwig and Bode, 2000; Tomonaga et al., 2002). In addition, mounting evidence suggests that humans could be a target for BDV infection (Billich et al., 2002; Carbone, 2001; Ikuta et al., 2002a,b), indicating that BDV presents a possible risk as a zoonotic pathogen. At present, several drugs have been reported to have antiviral effects in BDV infection (Bajramovic et al., 2002, 2004; Bode et al., 1997; Volmer et al., 2005). Bajramovic et al. (2002, 2004) demonstrated that a nucleoside analog, 2′-fluoro-2′-deoxycytidine, as well as 1-β-D-arabinofuranosylcytosine, inhibited the replication and spread of BDV in cell culture and in vivo systems. Some reports have showed that amantadine is effective against a strain of BDV both in vitro and in vivo, although the effect of this drug on anti-BDV activity is still controversial (Bode et al., 1997; Hallensleben et al., 1997; Stitz et al., 1998).

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is a synthetic ribonucleoside analog that displays broad-spectrum antiviral activity and is currently used for the treatment of a wide range of DNA and RNA virus infections (Cameron and Castro, 2001; Parker, 2005). Previous studies revealed that ribavirin has an antiviral effect on BDV infection in neural and non-neural cell lines (Jordan et al., 1999; Mizutani et al., 1998). Treatment with ribavirin drastically reduced the levels of viral transcription and release in persistently infected cultured cells, suggesting this agent to be a good candidate for an anti-BDV drug. The effect of ribavirin against BDV-induced neurological disorders has also been examined in a rat model of persistent BDV infection (Solbrig et al., 2002). Adult Lewis rats were infected with BDV and received a daily intraventricular ribavirin injection from 21 days postinfection (p.i.), at which time the infected animals had developed BD-like symptoms. Interestingly, the intraventricular injection of ribavirin caused clinical improvement without changing the viral titer or RNA level in the rat brain. In addition, decreased numbers of microglia, as well as $CD4^+$ and $CD8^+$ T-cells, were observed in the brains of ribavirin-treated, persistently infected rats (Solbrig et al., 2002). From this observation, it has been concluded that ribavirin may reduce the morbidity of BD by impacting on microglial proliferation and its effects on the brain. However, the direct effects of ribavirin on the

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proliferation of BDV in the brain had not been made clear because the viral titer was unchanged in the rat brain by the ribavirin. In the present study, therefore, we assessed the effect of ribavirin on the proliferation of BDV in the brains by using acutely BDV-infected gerbil model.

Gerbils make an intriguing model for analyzing the neuropathogenesis of BDV (Lee et al., 2003; Watanabe et al., 2001, 2003). In previous studies, we demonstrated that despite the development of fatal neurological disorders and aggressive proliferation of BDV in newborn gerbil brains, no severe neuroanatomical alterations were observed (Watanabe et al., 2001, 2003). Furthermore, treatment with an immunosuppressant, cyclosporine A, did not inhibit the fatal disorders in BDV-infected neonatal gerbils (Watanabe et al., 2003). These results indicated that significant replication of BDV in specific areas of the central nervous system (CNS), but not the host immune response, contributes to the onset of the neurological diseases in newborn gerbils. From these observations, we concluded that the gerbil provides a unique model for understanding the direct damage to the CNS caused by the replication of BDV. Thus, the acutely infected neonatal gerbil is a good system with which to evaluate the effects of ribavirin on the proliferation of BDV in the brain.

To determine the dose of ribavirin to inject into neonatal gerbils (SLC, Shizuoka, Japan), we intracranially inoculated ribavirin in phosphate-buffered saline (PBS) into the subarachnoid cavity, which is the interval between the arachnoid membrane and pia mater, at the temporal position by using a microsyringe having two-step needle, which has a 2 mm × 0.4 mm diameter long piercing tip from postnatal day (PD) 10 at 3-day intervals. We used doses of 1.0, 5.0 or 10 mg/(kg shot) in a volume of 10 μl for 15 days. All animal experiments conformed to the guide for the care and use of laboratory animals of the Research Institute for Microbial Diseases, Osaka University. The newborn gerbils endured even the 10 mg/(kg shot) treatment without any weight loss or death (data not shown). Thus, the 10 mg/(kg shot) treatment was adopted in all following experiments. This dose is quite similar to the clinical dosages used in human cases (Carlsson et al., 2008; Engler et al., 2004; Tomoda et al., 2003). Since brain development and/or BDV expansion within the brain could affect the effectiveness of ribavirin, we next compared the starting day of ribavirin administration in acutely BDV-infected newborn gerbils. Newborn gerbils were intracerebrally inoculated at the left temporal position with 4 μl of 200 focus forming units (FFU) of BDV strain He/80 per animal within 24 h after birth (Watanabe et al., 2001) and then injected ribavirin (10 mg/(kg shot)) from either PD10, PD13, or PD16 at 3-day intervals as described above. The animals were monitored for changes in weight and clinical signs of neurological disorders and sacrificed at 25 days p.i., because previous studies revealed that a neonatal infection of 200 FFU BDV induces severe neurological disorders by PD25 (Watanabe et al., 2001, 2003). The gerbils injected with ribavirin from PD10 (P10-Rv) did not lose body weight following the infection, while the infected gerbils treated with ribavirin from PD13 (P13-Rv) and PD16 (P16-Rv) did lose weight, similar to the untreated, infected animals, BDV/Rv(−), from 16 days p.i. (Fig. 1A). A humoral immune response to BDV was detected in all the infected gerbils at PD25 (Table 1). In addition, only 1 of 8 animals (12.5%) had developed signs of neurological disease in P10-Rv gerbils by 25 days p.i., despite that all of the untreated animals developed fatal disorders (Table 1). On the other hand, 33.3 and 50% of the P13-Rv and P16-Rv gerbils, respectively, showed signs of neurological disease (Table 1).

We next investigated whether viral proliferation is inhibited by the administration of ribavirin in the brains of acutely BDV-infected gerbils. The P10-Rv gerbils were sacrificed at PD25 and the BDV RNA level in the cerebral cortex was determined by semi-quantitative a RT-PCR for the BDV nucleoprotein (N) region. Total RNA (2 μg) was reverse-transcribed, and the resultant cDNA was amplified with BDV N-specific primers. The analysis revealed that although the expressions of GAPDH are not affected by the treatment, 10 mg/(kg shot) of ribavirin, but not 1.0 mg/(kg shot), clearly reduces the level of BDV RNA expression in the brain (Fig. 1B), suggesting that the propagation of BDV within the brain could be suppressed by the ribavirin treatment.

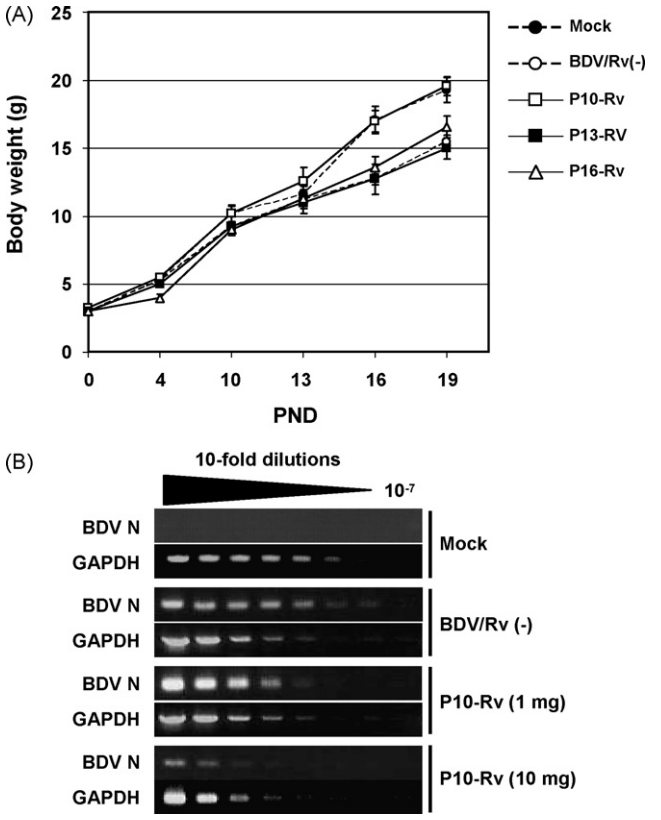

Fig. 1. Ribavirin administration in BDV-infected newborn gerbils. (A) Body weight changes of BDV-infected neonatal gerbils receiving ribavirin at 10 mg/(kg shot) from PD10 (P10-Rv, $n = 8$), PD13 (P13-Rv, $n = 6$) or PD16 (P16-Rv, $n = 4$). Mock, mock-infected newborn gerbil; BDV/Rv(−), BDV-infected, ribavirin-untreated gerbil. Ribavirin was administrated 4, 3 and 2 times in P10-Rv, P13-Rv and P16-Rv gerbils, respectively, by 19 days postinfection. (B) Semiquantitative RT-PCR analysis of BDV N mRNA levels in P10-Rv gerbils. As a control for RNA input, the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was assayed (Lee et al., 2003).

Previous studies demonstrated that newborn gerbils infected with 200 FFU of BDV developed severe neurological disorders in

Table 1
Summary of ribavirin administration in BDV-infected neonatal gerbils

Ribavirin-treated group[a]	Number of heads	Neurological diseases (%)[b]	Anti-N antibody (%)[c]
NT	10	10(100)	10(100)
P10-Rv	8	1(12.5)	8(100)
P13-Rv	6	2(33.3)	6(100)
P16-Rv	4	2(50)	4(100)

[a] NT: no ribavirin treatment; P10-Rv: ribavirin (10 mg/(kg shot)) treatment from PD10; P13-Rv: ribavirin treatment from PD13; P16-Rv: ribavirin treatment from PD16.

[b] Number of animals that showed signs of neurological disease, including decrease of body weight, paralysis of hind legs, quadriparesis, hypopraxia, debility or blindness.

[c] Number of animals positive for BDV N antibody in serum on the day sacrificed determined by Western blot analysis.

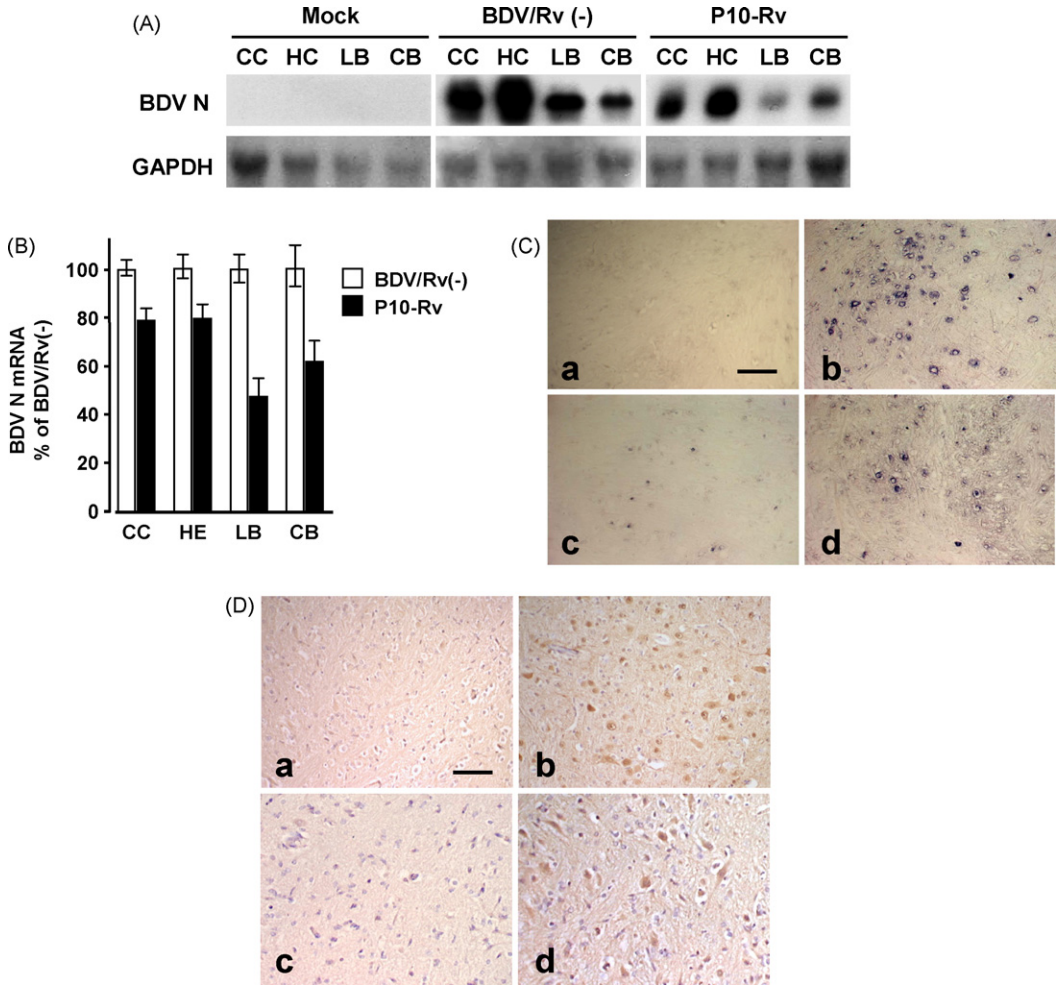

Fig. 2. Reduced proliferation of BDV in ribavirin-treated gerbil brains. (A) Northern blot analysis of P10-Rv gerbil brains. (B) Relative band intensities of N mRNAs in the brains of P10-Rv gerbils. The relative intensities were determined by optical densitometry from Northern blotting shown in (A) (±standard error). CC, cerebral cortex; HC, hippocampus; LB, lower brainstem; CB, cerebellum. (C and D) Expression of BDV replication products in the lower brainstem in neonatally infected gerbils at 25 days postinfection. In situ hybridization (C) and immunohistochemical (D) analyses were performed with an antisense riboprobe specific for the BDV N region and anti-N polyclonal antibody, respectively (Watanabe et al., 2001). Panel a, Mock-infected; b, BDV-infected, untreated; c, P10-Rv; d, P16-Rv. Scale bars; 40 μm.

correlation with significant viral proliferation in specific regions of the brains, including the lower brainstem, by 25 days p.i. (Watanabe et al., 2001). As shown in Fig. 2A and B, the level of viral N mRNA was reduced in all areas examined, especially in the lower brainstem, in P10-Rv gerbils. In situ hybridization using an antisense probe for the N region (Watanabe et al., 2001) revealed that P10-Rv drastically reduced the level of viral RNA in the lower brainstem at PD25 (Fig. 2C). We confirmed the expression level of BDV by an immunohistochemical analysis using rabbit anti-N polyclonal antibody (1:2000 dilution in PBS) generated in our laboratory by using recombinant N antigen (Watanabe et al., 2001). As shown in Fig. 2D, the level of BDV N was also found to be significantly reduced in the brain of P10-Rv gerbils at 25 days p.i. These observations demonstrated that the ribavirin treatment could efficiently repress the proliferation of BDV in the brains of acutely infected newborn gerbil.

A previous study revealed that microglial proliferation is impaired in the brains of BDV-infected, ribavirin-administered rats (Solbrig et al., 2002). To evaluate whether microglial activation is also reduced in the brains of acutely infected gerbils by ribavirin administration, we performed an immunohistochemical analysis using mouse OX-42 monoclonal antibody (1:100 dilution; Serotec; Oxford, UK), which is applicable to the gerbil brains (Hwang et al., 2004). As shown in Fig. 3A, numerous OX-42-positive rod cells were detected in the cortex and brainstem of BDV-infected, untreated gerbils at PD25. On the other hand, P10-Rv gerbils exhibited a marked reduction in the reactivity of OX-42 in the brain. This result suggested that, as reported in a rat model (Solbrig et al., 2002), ribavirin can affect microglial activity even in the acutely infected gerbil brain.

The antiviral activity of ribavirin is shown to be partially due to its ability to enhance Th1 immunity (Liu et al., 1998; Ning et al., 1998). Therefore, we investigated whether the expression of several cytokines, including Th1 cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor α (TNF-α), is enhanced in the ribavirin-treated, BDV-infected newborn gerbils. We performed semiquantitative RT-PCR with primers specific for gerbil cytokines (Lee et al., 2003; Watanabe et al., 2003). As shown in Fig. 3B, expression levels of these cytokines were not shown to increase in the cerebral cortex and lower brainstem by the ribavirin treatment.

In this study, we demonstrated that the ribavirin treatment significantly reduced BDV mRNA in the brains of acutely infected newborn gerbils. Interestingly, although the 10 mg/(kg shot) of ribavirin was fatal in rats (Solbrig et al., 2002), gerbils apparently well-tolerated at the same dose. This may lead to the different effects of ribavirin on BDV replication between the animals. On

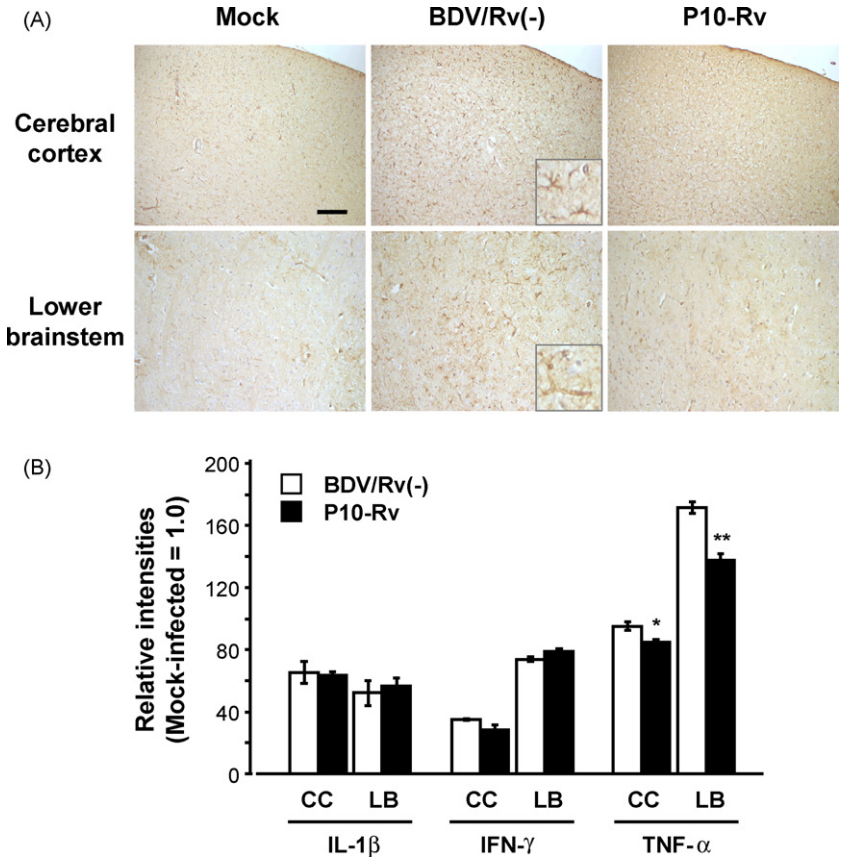

Fig. 3. Ribavirin reduces microglial proliferation, but not cytokine expression, in BDV-infected neonatal gerbils. (A) Reduction in numbers of microglia in the brains of BDV-infected gerbils. Ribavirin administered gerbils were immunostained with mouse OX-42 monoclonal antibody. Mock, mock-infected newborn gerbil; BDV/Rv(−), BDV-infected, ribavirin-untreated gerbil; P10-Rv, BDV-infected gerbil treated with ribavirin at 10 mg/(kg shot) from PD10. Scale bars; 100 μm. At least three sections from at least four animals in each group were investigated. Insets in BDV/Rv(−) show 3× magnificent view of rod-shaped positive cells. (B) Expression of cytokine mRNAs in BDV-infected, ribavirin-treated gerbils. The levels of IL-1β, IFN-γ and TNF-α mRNAs were analyzed with semiquantitative RT-PCR. As a control for RNA input, the level of GAPDH mRNA was assayed (Lee et al., 2003). The amplification products were resolved on 1.5% agarose gels and transferred onto nylon membranes. The membranes were then analyzed by Southern blot hybridization with digoxigenin-labeled specific probes for gerbil cytokines (Watanabe et al., 2003). The relative intensities to that of mock-infected gerbils were indicated. The intensity of each band on X-ray films was quantified using NIH image. Values were expressed as means ± standard error IL-1β: CC [BDV/Rv(−): 65.44 ± 7.08; P10-Rv: 63.38 ± 2.52], LB [BDV/Rv(−): 52.08 ± 7.99; P10-Rv: 56.46 ± 5.19], IFN-γ: CC [BDV/Rv(−): 35.05 ± 0.81; P10-Rv: 28.39 ± 2.87], LB [BDV/Rv(−): 73.78 ± 1.54; P10-Rv: 78.89 ± 1.62], TNF-α: CC [BDV/Rv(−): 95.25 ± 2.42; P10-Rv: 84.90 ± 1.64], LB [BDV/Rv(−): 171.56 ± 3.45; P10-Rv: 137.67 ± 4.07)]. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). CC, cerebral cortex; LB, lower brainstem.

the other hand, it is likely that the ribavirin enters into systemic circulation of the gerbils, because we administrated a 10 μl of ribavirin solution into the subarachnoid cavity of the animals. This might partially induce the difference in the susceptibility between the rats and gerbils. Our study revealed that the administration of ribavirin from PD10, when the BDV has not yet proliferated throughout the brain, could efficiently inhibit the viral expansion within the CNS. In previous studies (Watanabe et al., 2001, 2003), we showed that the expression areas of BDV mRNA and proteins in the brains are shifted in association with disease progression and that the disease onset may be correlated with BDV propagation within the brainstem region. Thus, the ribavirin treatment may prevent BDV to reach the brainstem of P10-Rv gerbils.

We previously concluded that both direct damage by BDV propagation and an indirect effect of the cytokines, such as IL-1β, are required for induction of fatal neurological disorders in gerbils (Watanabe et al., 2003). We found that the ribavirin treatment does not change the expression level of IL-1β, indicating that only an abnormal level of IL-1β is not enough to induce neurological disorders in the gerbils. On the other hand, the level of TNF-α was significantly decreased in both the cerebral cortex and lower brainstem regions of ribavirin-treated gerbils. It would be of interest to investigate the effect of TNF-α on the induction of the neurological symptoms in BDV-infected gerbils.

Ribavirin is known to exert antiviral effects through the following mechanisms; (i) interference with the capping of viral mRNAs (Bougie and Bisaillon, 2004), (ii) inhibition of the polymerase activity of viruses (Crotty et al., 2000) and (iii) induction of error catastrophe in viral genomes (Crotty et al., 2001; Severson et al., 2003). Previous reports have clearly showed that transcription of BDV is inhibited by ribavirin treatment in persistently BDV-infected cell lines, such as human oligodendrocytes and rat glioma cells (Jordan et al., 1999; Mizutani et al., 1998). These studies suggested that a reduction in the size of the intracellular GTP pool could be a mechanism for inhibition of the transcription and capping of BDV mRNA. The significant reduction of microgliosis suggested that the depletion of the GTP pools actually occur in the gerbil brain cells. On the other hand, despite the reduced activation of microglial cells by the ribavirin treatment, the expression levels of the cytokines in the treated gerbils appeared to be comparable to the untreated animals. Considering the ability of ribavirin to enhance Th1 immunity, it is conceivable that the ribavirin treatment enhances the expression of the cytokines from the other resident cells in the brains, such as astrocytes. Further studies will need to elucidate the sources of the cytokine expression in the ribavirin-treated animal brains.

In this study, we demonstrated that ribavirin could directly reduce viral propagation within the brains of acutely infected newborn gerbils. Our results indicate that ribavirin might be an effective tool for research into the replication and pathogenesis of BDV in vivo, as well as in the discovery of an antiviral strategy against infections of the CNS.

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