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Borna disease virus induces acute fatal neurological disorders in neonatal gerbils without virus- and immune-mediated cell destructions

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Abstract

Borna disease virus (BDV) is a noncytolytic, neurotropic RNA virus that is known to cause neurological disturbances in various animal species. Our previous experiment demonstrated that neonate gerbils develop an acute fatal neurological disease following infection with BDV (Watanabe et al., 2001, Virology 282, 65–76). The study suggested that BDV directly causes functional damage of neuronal cells resulting in the lethal disorder in neonatal gerbils. To extend this finding, we examined whether BDV can induce neurological diseases in the absence of virus- and immune-mediated cell destruction, by using cyclosporine A (CsA)-treated neonatal gerbils. Although CsA completely suppressed specific antibody production and brain inflammation in the infected gerbil brains, the fatal neurological disorder was not inhibited by the treatment. Furthermore, we demonstrated that CsA treatment significantly decreased brain levels of cytokines, except interleukin (IL)-1β, in the infected gerbils. These results suggested that BDV replication, as well as brain cytokines, at least IL-1β, rapidly induces fatal disturbances in gerbil brain. We demonstrate here that BDV exhibits a unique neuropathogenesis in neonatal gerbil that may be pathologically and immunologically different from those in two other established rodent models, rats and mice. With this novel rodent model of virus infection it should be possible not only to examine acute neurological disturbances without severe neuroanatomical and immunopathological alterations but also to analyze molecular and cellular damage by virus replication in the central nervous system.
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Introduction

Borna disease virus (BDV) is the prototype of a new family, *Bornaviridae,* within the nonsegmented negative-strand RNA viruses, *Mononegavirales* (de la Torre, 1994; Schneemann et al., 1995), and is characterized by low virus production, neurotropism, and nuclear localization of BDV transcription and replication (Cubitt and de la Torre, 1994). Although BDV was originally described as an agent of nonpurulent encephalomyelitis in horses in Germany (Rott and Becht, 1995), BDV infection has now been found in a wide range of vertebrates, including sheep, cattle, cats, and birds (Bode et al., 1994; Lundgren et al., 1995; Rott and Becht, 1995). Recent epidemiological studies suggested that

BDV infection also occurs in humans and that it may be related to certain human psychiatric diseases (Bode et al., 1995; Sauder et al., 1996; Salvatore et al., 1997; Waltrip et al., 1997; Nakamura et al., 2000).

At present, BDV neuropathogenesis has been extensively studied in rodent models, of which the rat is the best-characterized animal. In rats infected as adults with BDV, cellular immune response appears critical to the development of the neurological disorders characterized by locomotor abnormality (Narayan et al., 1983; Stitz et al., 1995; Lewis et al., 1999; Planz and Stitz, 1999). In contrast, neonatal rats do not show any immunopathological alterations in the brains following BDV infection (Hirano et al., 1983; Narayan et al., 1983). It has been demonstrated, however, that infected neonatal rats develop abnormalities in affective behavior and neuroanatomical disturbances characterized by degeneration of hippocampal neurons, cortical shrinkage, and cerebellar hypoplasia (Carbone et al., 1991; Bautista et al., 1995; Eisenman et al., 1999; Rubin et

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Table 1
Summary of CsA treatment on BDV-infected gerbils

Group	BDV infection	Dose of CsA (mg/kg)	Incidence of the disease[a] (%)	Antibody production[b]	Infiltration[c]
UI-0 ($n = 10$)	−	0	0	−	−
UI-20 ($n = 3$)	−	20	0	−	−
I-0 ($n = 9$)	+	0	100	+ +	+
I-5 ($n = 4$)	+	5	100	+ +	+
I-10 ($n = 4$)	+	10	100	+	±
I-20 ($n = 4$)	+	20	100	−	−

[a] Incidence of BDV-specific neurological disorders observed in gerbils at 25 dpi.
[b] Antibody to BDV P protein was measured by ELISA (Watanabe et al., 2000). Detailed titers of antibody are shown in Fig. 1.
[c] Induction of infiltrations was estimated by HE staining (Fig. 2) of the gerbil brains at 25 dpi −, none; ±, minimal; +, modulate.

al., 1999). Furthermore, chronic astrocytosis and microgliosis, as well as a progressive decrease in synaptic density and plasticity, were observed in the brains of neonatally infected rats (Gonzalez-Dunia et al., 2000). These observations have indicated that BDV infection can induce neuronal degenerations and dysfunctions without an immune-mediated mechanism. Therefore, neonatal rat infection is now considered to be a good model for understanding direct effects of BDV infection on the neuronal cells of brain. On the other hand, BDV may induce neuronal disturbances in neonatal rat brains by indirect routes, e.g., via altered expression of cytokines, tissue factors, nerve growth factors, and/or neurotransmitters caused by the infection (Hornig et al., 1999; Plata-Salaman et al., 1999; Sauder and de la Torre, 1999; Pletnikov et al., 2000; Zocher et al., 2000). In addition, mice are also frequently used for neuropathological analysis of BDV infection. Specific strains of mice exhibit severe neurological diseases following neonatal BDV infection. In the case of neonate mouse infection, it has been revealed that the neurological disorders are due to the indirect damage caused by host immune responses (Hallensleben et al., 1998; Hausmann et al., 1999).

Neonatal gerbils are emerging as an intriguing model for analyzing direct damage of neuronal cells by BDV infection. In a previous study, we demonstrated that despite development of acute fatal neurological disorders and aggressive proliferation of BDV in infected newborn gerbils, no severe neuroanatomical alterations, including astrocytosis and neuronal degeneration, were observed in the brains (Watanabe et al., 2001). From these results, we concluded that significant replication of BDV in specific areas of the central nervous system (CNS) contributes to the acute neurological disturbances in neonatal gerbils. In the report, however, we could not exclude the effects of infiltrations, antibody production, and cytokine expression in the infected neonates as secondary factors contributing to the lethal outcome. Although antibody and cytokine production seems not to directly induce neurological disorders in the BDV-infected rat model (Morimoto et al., 1996; Plata-Salaman et al., 1999; Sauder and de la Torre, 1999; Furrer et al., 2001), the roles of these brain factors in the induction of acute fatal disorders in virus-infected neonates are not fully understood.

In this study, to extend our knowledge regarding BDV-induced acute fatal neurological disorders, we further analyzed BDV-infected neonatal gerbils to which an immunosuppressive agent, cyclosporine A (CsA), was administered. Although CsA treatment could completely suppress antibody production and cell infiltration in the gerbil brains, fatal neurological disorders in neonates were not at all inhibited by the treatment. Moreover, the immunosuppression by CsA decreased the levels of brain cytokines, except interleukin (IL)-1β in the infected gerbils. These results suggested that, in addition to direct damage by BDV replication, expression of brain cytokines, at least IL-1β, may contribute to the induction of acute fatal disorders in the gerbils in the absence of neuronal cell destruction. Importantly, we show the possibility that neonatal gerbil infection is a unique and useful model for understanding acute neurological disturbances without neuroanatomical and immunopathological alterations.

Results

Neurological diseases in BDV-infected, immunosuppressed neonatal gerbils

To investigate the role of host immune responses and cytokine expression in BDV-induced neuropathogenesis, we intracranially inoculated BDV into a total of 34 neonatal gerbils with or without CsA treatment (Table 1). CsA was administered daily from 15 days postinfection (dpi), because newborn animals younger than neonatal day (ND) 15 were too immature to receive the treatment. Furthermore, because neonatally BDV-infected gerbils do not show specific antibodies to BDV, brain inflammation, and neurological disorders by the ND15 (Watanabe et al., 2001), it is not likely that some cellular immune responses had occurred by ND15 that triggered a cascade of events leading to the lethal outcome of the infected neonates. After ND15, neither relaxation nor exacerbation of symptoms was observed in the neonates treated with CsA. Almost all gerbils treated with any dose of CsA, as well as non-CsA-treated gerbils, developed acute and severe clinical disease around ND22 (Table 1). The neurological disorders are
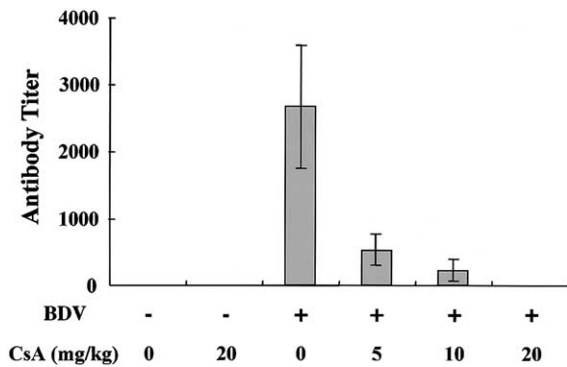
Fig. 1. Inhibition of antibody production in BDV-infected, CsA-treated neonatal gerbils. Sera from BDV-infected, CsA-treated neonatal gerbils were collected at ND25. Anti-BDV-P antibody production was estimated by antigen capture ELISA (Watanabe et al., 2000). Values were expressed as means ± SEM.

marked by paralysis of the hind legs with significant body weight loss until 25 dpi, as reported previously (Watanabe et al., 2001). In addition, quadriparesis, hypopraxia, debility and blindness were induced after the onset of the disease in some gerbils. During the observation period, none of the gerbils recovered from the disease. All of the diseased gerbils died by day 30 postinfection.

Suppression of BDV-specific antibody production in cyclosporine A-treated gerbils

To examine whether CsA treatment suppresses the humoral immune response against BDV infection, we determined the antibody titer to BDV 24-kDa phosphoprotein (P) in the infected gerbils by antigen capture enzyme-linked immunosorbent assay (ELISA) at 25 dpi. As shown in Fig. 1, non-CsA-treated neonatal gerbils produced virus-specific antibody at high levels. In CsA-treated, infected gerbils, the titer was drastically decreased in a CsA dose-dependent manner. At a dose of 20 mg/kg CsA, no antibody production could be observed in infected gerbils (Fig. 1, Table 1). This result reveals that CsA treatment successfully inhibits the humoral immune response in infected gerbils, indicating that antibodies are not involved in neurological diseases observed in infected neonates.

Effects of cyclosporine A treatment in BDV-induced pathological alterations and viral replication in gerbil brains

In a previous study, we demonstrated that no gross neuroanatomical disturbances were observed in the brains of neonatally infected gerbils. In this experiment, we also found minimal perivascular and meningeal infiltrations by hematoxylin–eosin (HE) staining in the cerebral cortex, diencephalon, and cerebellum in neonatally infected gerbils not treated with CsA (Fig. 2A, b and e). With CsA treatment, however, no inflammation was detected in the brains

at day 25 postinfection (Fig. 2A, c and f). Furthermore, we could not find any histological alternations in the BDV-infected, CsA-treated brains (Fig. 2B, b and d). As demonstrated in the previous study, no apoptotic signal was demonstrated in the brain sections from infected gerbils by terminal TUNEL assay (data not shown).

We investigated whether immunosuppression caused by CsA treatment could affect replication of BDV in gerbil brains. The virus-specific mRNAs were detected in gerbil brains by Northern blot analysis and in situ hybridization (ISH). As shown in Fig. 3, BDV P mRNA was comparatively detected in CsA-treated and nontreated, infected gerbil brains at 25 dpi (Fig. 3A). ISH using an antisense probe also revealed efficient viral gene production in the brains from both CsA-treated and nontreated, gerbils at the same level (Fig. 3B). The BDV sense probe for ISH also revealed no differences between the CsA-treated and nontreated gerbil brains (data not shown). Furthermore, as reported previously, viral mRNAs were found predominantly in the cerebellum and lower brainstem of diseased neonates at 25 dpi (Fig. 3B). These results demonstrated that CsA treatment has no effect on viral replication in the brains of infected newborn gerbils.

Cytokine expression in neonatally infected gerbil brains

Previous studies using a rat model demonstrated that brain cytokines may play a role in BDV-induced neuropathogenesis (Shankar et al., 1992; Hornig et al., 1999; Plata-Salaman et al., 1999; Sauder and de la Torre, 1999). It has been also suggested that cytokines may be directly activated in CNS resident cells by BDV infection, even in immunoincompetent rats (Morimoto et al., 1996). Therefore, to understand the role of brain cytokines in severe neurological disorders of BDV-infected neonatal gerbils, we investigated expression of cytokine mRNAs in BDV-infected gerbil brains. To detect brain cytokines of gerbils, we first cloned and sequenced several cytokine cDNAs, including IL-1α, IL-1β, IL-2, IL-4, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, specific for gerbil, and designed specific primers and probes for them. The gerbil-specific primers and probes used in this study are summarized under Materials and Methods. The alteration of cytokine expression was analyzed in the cerebral cortex and cerebellum at 25 dpi by semiquantitative reverse transcription (RT)-PCR followed by Southern blot hybridization. As shown in Fig. 4, IL-1α, IL-1β, and IFN-γ mRNAs were markedly increased in the brains of infected gerbils, whereas upregulation of IL-4 and TNF-α mRNAs was detected in only a few infected gerbils (Fig. 4). On the other hand, although expression of TGF-β mRNA slightly increased in the cerebellum of infected gerbils, both the cerebral cortex and cerebellum contained significant levels of the mRNA, even in uninfected gerbil brains (Fig. 4). These observations demonstrate that upregulations of IL-1α, IL-1β, and IFN-γ may be a characteristic of BDV-infected newborn brains.
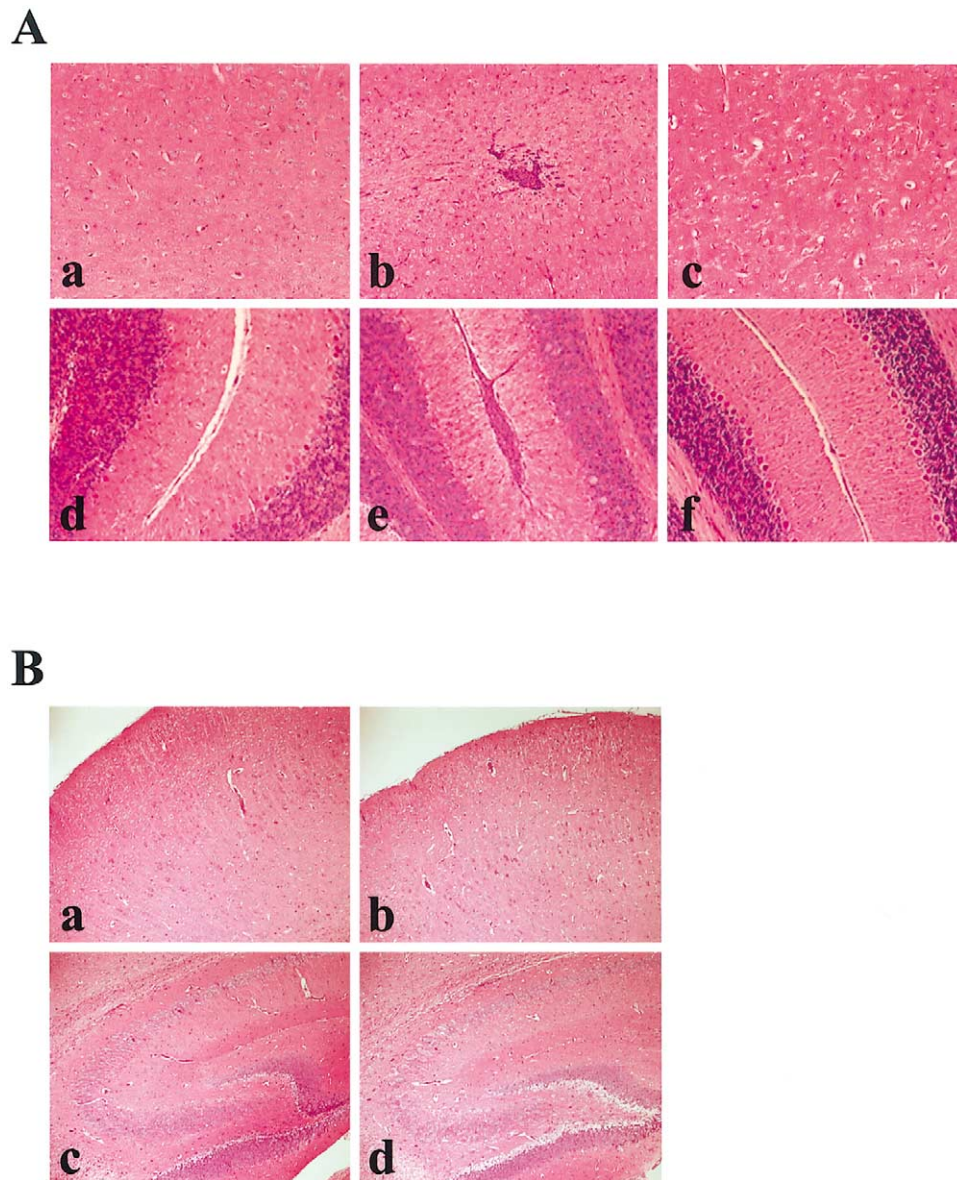
Fig. 2. Neuropathological analysis of BDV-infected, CsA-treated gerbil brains. (A) Brain sections from uninfected gerbil (a, d), BDV-infected gerbil (b, e), and BDV-infected, CsA-treated gerbils (20 mg/kg, I-20) (c, f) at 25 dpi were stained with HE. Cerebral cortex (a–c) and cerebellum (d–f) are shown. Infiltrated cells were observed in the cerebral cortex and meninges of BDV-infected, vehicle-treated gerbils but not CsA-treated animals. (B) HE staining of brain sections from uninfected gerbil (a, c) and BDV-infected, CsA-treated gerbils (20 mg/kg; I-20) (b, d) at 25 dpi. Cerebral cortex (a, b) and hippocampus (c, d) are shown.

CsA treatment altered cytokine levels in the CNS of BDV-infected neonatal gerbils (Fig. 4). The level of IL-1α mRNA was significantly decreased in the cerebellum of CsA-treated gerbils. Furthermore, we noted a marked reduction in IFN-γ mRNA in the treated brains. Interestingly, induction of IFN-γ mRNA in a gerbil brain was completely suppressed by treatment (Fig. 4). Although the downregulation of IFN-γ, as well as that of IL-4 and TGF-β, is not statistically significant, it was not necessarily linked to the induction of the neurological disorder in gerbils (Fig. 4). On the other hand, expression of IL-1β in infected brains was not affected by treatment with the immunosuppressive agent at 25 dpi (Fig. 4). We repeated the RT-PCR analysis at least

four times in each gerbil brain and obtained similar results in each experiment. Together with the deleterious effects of IL-1 on neuronal cell functions (Neveu and Liege, 2000; Konsman et al., 2002), these results suggest the possibility that expression of brain cytokines, at least IL-1β, contributes to the induction of fatal neurological disorders in newborn gerbils in the absence of neuronal cell destruction.

Discussion

In this study, we demonstrated that CsA treatment on a daily basis from ND15 could completely suppress antibody
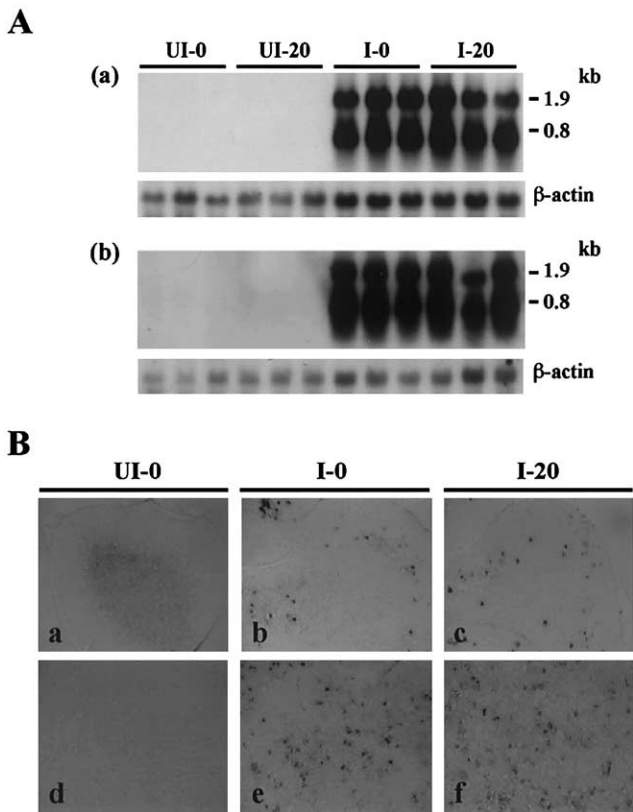
A

B

Fig. 3. Immunosuppression does not influence BDV replication in gerbil brains. (A) Northern blot hybridization. Aliquots of 5 μg of total RNA from cerebral cortex (a) and cerebellum (b) were separated by denaturing formaldehyde agarose gel electrophoresis, transferred to nylon membrane, and hybridized with a BDV P antisense probe. UI-0, uninfected vehicle-treated gerbils; UI-20, uninfected, CsA-treated gerbils (20 mg/kg); I-0, BDV-infected, vehicle-treated gerbils; I-20, BDV-infected, CsA-treated gerbils (20 mg/kg). (B) ISH. Sections of brains from uninfected gerbils (UI-0; a, d), BDV-infected gerbils (I-0; b, e), and BDV-infected, CsA-treated gerbils (20 mg/kg) (I-20; c, f) were hybridized with P antisense probe. (a–c) cerebellum; (d–f) lower brainstem.

production and cell infiltration in BDV-infected neonatal gerbils (Table 1, Figs. 1 and 2). However, the immunosuppression did not inhibit development of the fatal disorders in infected animals at all. These results indicate that antibody production and immune-mediated cell infiltration are not likely to be associated with the acute neurological diseases of BDV-infected neonatal gerbils.

In neonatal gerbils, both the direct and indirect effects of BDV infection could be involved in the acute fatal disorders. Disruption of the specialized cell functions by viral replication is most likely responsible for direct neurological disturbances in neonatal gerbil brains. Indeed, recent studies have demonstrated that BDV infection could affect several cellular factors and inhibit some cellular functions, such as cell process outgrowth and glutamate uptake, in cultured neural cells (Billaud et al., 2000; Kamitani et al., 2001; Hans et al., 2001; Planz et al., 2001). In addition to the previous study, we again found here that significant BDV replication in specific CNS areas, especially in the Purkinje

cells of the cerebellum and brainstem, is closely linked to the onset of the disease (Fig. 3). This observation provides us with the possibility that BDV replication has deleterious effects on neuronal functions in these regions, leading to fatal neurological disorders. The cerebellum is one of the brain regions that develop late and to a large extent post-natally. In the early postnatal weeks, Purkinje cells progressively lose the input from several climbing fibers and mossy fibers via granule cells. Furthermore, there is a reduction in Purkinje cell apical cone and dendrite growth, as well as in synaptogenic rate between mossy fibers and granule cells, in the maturation period. Interestingly, it has been known that Purkinje cells change their susceptibility to some viruses during this maturation period (Oliver et al., 1997). Considering that the Purkinje cells of the neonatal gerbils did not show BDV-positive signals until postnatal week 3 and were not destroyed by BDV infection, it is possible that BDV can infect only mature Purkinje cells of gerbils and disrupt some critical functions of the cells in the early maturation stage.

On the other hand, the role of cytokines is widely discussed in virus-infected CNSs as factors indirectly responsible for the induction of neurological dysfunction (Zhao and Schwartz, 1998; Neveu and Liege, 2000; Konsman et al., 2002). It is therefore likely that the altered levels of brain cytokines also play important roles in BDV-induced neurological abnormalities in infected gerbil brains. At present, however, it is difficult to assess the neurobiochemical status of infected gerbil brain, because only limited information and reagents, including gene sequences and antibodies to the brain factors, are available for gerbils. In this study, we selected several cytokines, that are relevant to an understanding of BDV pathogenesis and cloned cDNAs of them to use in further experiments. By using gerbil-specific primers, we detected marked alterations in expression levels of cytokine mRNAs in the brains of ND1-infected gerbils (Fig. 4). Expression of IL-1α, IL-β, IL-4, TNF-α, and IFN-γ mRNAs was upregulated in infected gerbil brains on examination at 25 dpi, as in cases of rat infection (Shankar et al., 1992; Morimoto et al., 1996; Hornig et al., 1999; Plata-Salaman et al., 1999; Sauder and de la Torre, 1999). Since CsA treatment suppressed invading immune-reactive cells and clearly depressed mRNA levels of some cytokines, including IL-4, IFN-γ, and TNF-α, it is likely that these cytokines are derived mainly from those invading cells. Incomplete depression of these cytokines might be due to a very low level of inflammatory cells, which were not detected after HE staining. On the other hand, expression of IL-1α and -1β mRNAs was not affected by CsA treatment in cerebral cortex and both cerebral cortex and cerebellum, respectively, indicating that these cytokines could be directly induced by BDV infection of CNS resident cells. Considering that CsA treatment could not inhibit the neurological diseases in the gerbils, the cytokines, of which expression was not altered by the treatment, may be involved in indirect effects on the neuronal abnormalities in the gerbil brains. In our experiment, IL-1,
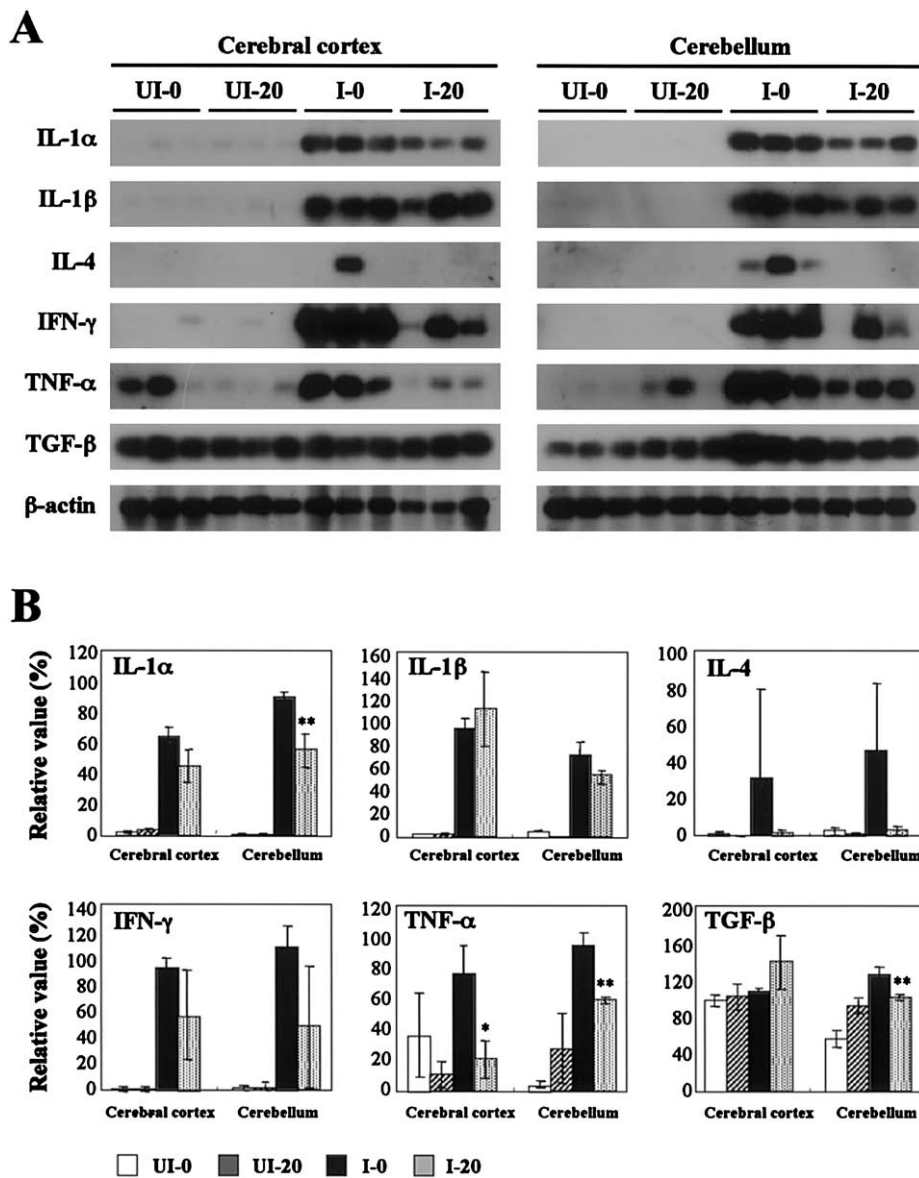
Fig. 4. Expression of cytokine mRNAs in BDV-infected, immunosuppressed gerbil brains. (A) Expressions of brain cytokines (IL-1α, IL-1β, IL-4, IFN-γ, TNF-α, TGF-β) in the cerebral cortex and cerebellum of BDV-infected newborn gerbils. The levels of cytokine mRNAs were analyzed with semiquantitative RT-PCR and DNA blot hybridization as described under Materials and Methods. For semiquantitative RT-PCR, PCR amplification was conducted in 30 cycles for IL-1α, IL-1β, IFN-γ, and TGF-β and 35 cycles for IL-4 and TNF-α. UI-0, uninfected vehicle-treated gerbils; UI-20, uninfected, CsA-treated gerbils (20 mg/kg); I-0, BDV-infected, vehicle-treated; I-20, BDV-infected, CsA-treated (20 mg/kg). Samples of three individuals from each group are shown. (B) The quantitative analysis of cytokine expressions. The band intensities from (A) were determined by NIH Image. Values were normalized to β-actin mRNA levels. Values were expressed as means ± SEM. Significant differences in BDV-infected, CsA-treated gerbils (I-20) compared with vehicle-treated gerbils (I-0): *$P < 0.02$, **$P < 0.01$ (Student's t test).

especially IL-1β, appeared to be a relevant candidate for the indirect effects. Although the downregulation of IL-4, IFN-γ, and TGF-β (cortex) mRNAs was also insignificant in the brains, these cytokines could not play critical roles in the induction of neurological diseases, because the expression of these cytokines was not necessarily correlated with the induction of neurological disorders in the gerbils. Note that there remains the possibility that a different class of brain molecules, expression of which was not examined in this study, may play more of a role in the fatal disorder of the gerbils.

Recently, several lines of evidence have suggested that IL-1β is involved not only in normal CNS development but also in induction of neurological diseases (Neveu and Liege, 2000; Konsman et al., 2002). It has been reported that expression of IL-1 is increased in several neurodegenerative disorders, such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Zhao and Schwartz, 1998; Rothwell and Luheshi, 2000). Although such an increase in IL-1 in brains might reflect a response to ongoing CNS damage and/or neuroprotective effects, there is growing evidence to suggest a close link between IL-1 expression and these

disease (Zhao and Schwartz, 1998; Rothwell and Luheshi, 2000). An abnormal level of IL-1 might cause fragility of neuronal cells in the CNS rather than neuronal protection. In fact, it has been reported that IL-1β-deficient mice are resistant to fatal neuroadapted Sindbis virus (NSV) inoculation into the cerebrum (Liang et al., 1999). This report demonstrated that IL-1β deficiency protects against lethal NSV encephalitis by a mechanism that does not involve the inhibition of virus-induced CNS apoptosis, which is suggestive of deleterious effects induced by IL-1β in acute CNS damage including virus infection. Thus, further studies to examine the role of IL-1 in BDV neuropathogenesis may be warranted. Furthermore, the fact that neonatal rats infected with BDV exhibit a sustained upregulation of IL-1β in the absence of a severe neurological disorder suggests that both direct damage by BDV replication and an indirect effect of cytokine upregulation in the brain may be required for induction of the acute fatal disorders.

The neuropathological and neurological outcomes in BDV-infected neonatal gerbils were totally different from those seen in rats or mice. In neonatal rat infection, BDV causes a life-long persistent infection with significant viral load in the brains (Hirano et al., 1983; Narayan et al., 1983). BDV-infected neonatal rats rarely show clinical manifestations and death. Their brain development, however, is markedly disrupted in specific brain regions (Carbone et al., 1991; Bautista et al., 1995; Eisenman et al., 1999; Rubin et al., 1999). These degenerations in the CNS must result in distinct deficiencies in emotional and cognitive functions observed in the infected rats. Very recently, Nishino et al. (2002) have reported that a mouse-passaged variant of BDV induces an acute fatal neurological disease in newborn Lewis rats. Although the neurological symptoms and replication area of BDV in the infected newborn rats were very similar to those in the neonatal gerbils observed in this study, the rats exhibited rapid degeneration of the hippocampal pyramidal neurons (Nishino et al., 2002). On the other hand, specific strains of neonate mice infected with BDV show severe, frequently fatal, neurological diseases (Hallensleben et al., 1998), which are very similar to disorders in neonatally infected gerbils. In neonatal mice, however, it has been revealed that the severe diseases are due to a cytotoxic T-cell-mediated immunopathological process (Hallensleben et al., 1998; Hausmann et al., 1999). Considering the differences in neonate models of BDV infection, the gerbil may provide a good model not only to gain an understanding of acute neurological abnormalities without cell destruction but also to analyze molecular damage to neuronal cells directly induced by virus replication in the CNS.

We demonstrate here that BDV has a unique neuropathogenesis in neonatal gerbils that may be pathologically and immunologically different from that in two other established rodent models, rats and mice. The effort to develop new animal models for complex brain disturbances will provide novel insights into the mechanisms by which neurotropic viruses and/or soluble brain factors impair cellular function without CNS destruction.

Materials and methods

Virus stock

Lewis rats were intracranially inoculated with BDV (He/80 strain) (Cubitt et al., 1994) and sacrificed 3 weeks after inoculation. Rat brains were homogenized in phosphate-buffered saline (PBS) and lysed by sonication. The lysate was clarified by centrifugation and frozen at $-80°C$ as a virus source. Viral titer was calculated by indirect immunofluorescence assay as previously described (Watanabe et al., 2001).

BDV infection and immunosuppression of gerbils

Neonatal gerbils were intracranially inoculated with 50 focus-forming units of BDV within 24 h of birth. CsA (Sandimmune, Novartis Phama AG, Switzerland; 5, 10, or 20 mg/kg per day) or the same volume of vehicle (corn oil, Wako, Japan) was orally administered on a daily basis from ND15. Almost all gerbils examined were sacrificed 25 days after inoculation.

ELISA

Microtiter plates were coated with 100 μl of PBS containing 5 μg of purified P recombinant protein for 14 h at 4°C. The plates were subsequently blocked with 150 μl of 0.5% skim milk in PBS for 3 h at 37°C. After three washes with 0.05% Tween-20 in PBS (PBST), the wells were incubated with 100 μl of serially diluted (1:100 to 1:12,800) gerbil serum for 1 h at 37°C. After five washes with PBST, the wells were incubated with 50 μl of 1:2000 diluted HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) for 1 h at 37°C. After washing, 100 μl of 0.4 mg/ml o-phenylendiamine with hydrogen peroxide was added. After color development for 1 min, 100 μl of 3 N H_2SO_4 was added. Antibody titer was defined as the final dilution at the point that the absorbance exceeded 0.3.

Histological analysis

Gerbil brains were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Thin sections (6 μm) were stained with hematoxylin and eosin for histological evaluation by light microscopy.

ISH

Thin sections (4 μm) of gerbil brains were deparaffinized and incubated in Tris–HCl before being treated with 0.1 mg/ml proteinase K in TE buffer for 20 min at 37°C and then fixed with 4% paraformaldehyde in Tris–HCl (pH 7.5). The sections were then incubated with hybridization solution (50% formamide, 3x SSC, 50 mM Hepes, pH 7.0, 5x Denhadt's solution, 250 μg/ml salmon sperm DNA) for 30 min at 37°C and were hybridized by incubation with a 10 μg/ml final

concentration of digoxigenin (DIG)-labeled riboprobes for 16 h at 50°C. After hybridization, sections were washed and incubated with alkaline phosphatase-labeled anti-DIG antibody. Specific signals were visualized with nitroblue tetrazolium and X-phosphate (Boehringer-Mannheim).

Cloning and sequencing of gerbil-specific cytokine cDNAs

Total RNA was isolated from gerbil brain homogenates using an RNA isolation kit (Nippon Gene Co., Tokyo, Japan). Aliquots of 0.8 μg of total RNA, 100 pmol of oligo(dT)$_{16}$ primer, and 50 units of MultiScribe reverse transcriptase (RT) (PE Applied Biosystems, Norwalk, CT, USA) were incubated at 25°C for 10 min, 48°C for 30 min for RT, and then 95°C for 5 min for RT inactivation. Two-microliter aliquots of the resulting cDNAs were used as templates for PCR amplification. The PCR was performed with 30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min with Taq DNA polymerase (Takara Ex Taq; Takara, Tokyo, Japan). PCR primers used for gerbil cytokines were as follows:

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IL-1α-sense       5′-CAG AAT TCC TTC TAT GAT GCA AG-3′
IL-1α-antisense   5′-TCG GAT CCC TGA GCA CTC ACA-3′
IL-1β-sense       5′-TTG AAT TCC TTC ATC TTT GAA GAA GAG CC-3′
IL-1β-antisense   5′-TGG GAT CCA CAC TCT CCA GCT GCA G-3′
IL-2 sense        5′-CAA GGA TCC GAA ACT CCC CA-3′
IL-2 antisense    5′-CTC AGG AAT TCC ACC ACA GT-3′
IL-4-sense        5′-CAC ATC CCT GAC GGT AGA ATT C-3′
IL-4-antisense    5′-CTG AGG ATC CCG GAG TTG TTC T-3′
TNF-α-sense       5′-ACT GGA ATT CGG GGT GTT TGG TC-3′
TNF-α-antisense   5′-GAG AAC CTG GGA ATT CAC GAG G-3′
IFN-γ-sense       5′-TGG AAT TCT ACT AGA GAA GAC ACA TCA GC-3′
IFN-γ-antisense   5′-ATG GAT CCG ACT CCT TTT CCG CTT C-3′
TGF-β-sense       5′-ACC TGG GCT GGA AGT GGA TC-3′
TGF-β-antisense   5′-ATG AAT TCT TGC GGC CCA CGT AGT AGA-3′
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PCR products were digested with *Eco*RI and *Bam*HI and cloned into pBluescript II SK(−) vector. Cloned cDNAs of gerbil cytokine fragments were sequenced with the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Semiquantitative RT-PCR analysis of cytokine expression

Expression of IL-1α, IL-1β, IL-2, IL-4, TNF-α, IFN-γ, and TGF-β mRNAs in BDV-infected gerbil brains was examined by semiquantitative RT-PCR followed by Southern blot hybridization. First-strand cDNAs were synthesized from aliquots of 1 μg of total RNA of gerbil brains. PCR amplification was performed in a total volume of 25 μl containing 2 μl of cDNA and 1.25 U of Taq polymerase (Amplitaq Gold; Perkin–Elmer). The serial dilutions of positive controls for each gene were amplified at 20, 25, 30, and 35 cycles to determine the optimal number of amplification cycles that produces a liner relationship between input RNA and PCR product. The PCR amplification was then conducted in 30 cycles (for IL-1α, IL-1β, IL-2, IFN-γ, and TGF-β) or 35 cycles (for IL-4 and TNF-α) under the conditions described above. As a control for RNA input, levels of β-actin (primers: 5′-ATG GTG GGA ATG GGT CAG AAG-3′ and 5′-ACG CAC GAT TTC CCT

CTC AGC T-3′) were assayed. 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 DIG-labeled specific probes for gerbil cytokines. The intensity of each band on X-ray films was quantified using NIH Image. The statistical significance of the expression level of each cytokines was determined with Student's *t* test using the Statcel software (OME Publishing Inc, Tokyo, Japan).

Northern blot analysis

We detected BDV RNAs in the gerbil brains by Northern (RNA) blot analysis using a BDV-P antisense probe as described previously (Watanabe et al., 2001). Briefly, the total RNAs were electrophoresed through 1% agarose gel, transferred onto nylon membrane, and hybridized with P antisense DIG-labeled probe. The signals were detected with anti-DIG antibody and chemiluminescence substrate and visualized by exposure to X-ray film.

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