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Short Communication

Experimental infection of mice with Borna disease virus (BDV): Replication and distribution of the virus after intracerebral infection

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To develop an animal model resembling natural asymptomatic Borna disease virus (BDV) infections, BDV He/80 rat brain homogenate was passaged four times in adult SJL/J mice. Within 12 months of observation, mice did not develop overt signs of disease. Nucleotide sequencing of the rat isolate and the mouse isolates at the fourth passage revealed no difference in the deduced amino acids. Viral RNA was found in brain, heart, kidney, lung, liver, and urinary bladder. Infectious virus was isolated from brain, but also from heart and lung tissue. Immunohistochemically, BDV was demonstrated in nerves in the abdominal cavity, ganglion coeliacum, and adrenal glands, but not in organ parenchyma. Occasionally, viral RNA was detected in mononuclear blood cells. *Journal of NeuroVirology* (2001) **7,** 272–277.

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Borna disease virus (BDV) is the etiological agent of a nonpurulent encephalomyelitis in a wide range of animal species including horses, sheep, cattle, cats, and ostriches (Rott and Becht, 1995; Gonzalez-Dua *et al*, 1997; Hatalski *et al*, 1997). BDV infections have been reported worldwide (Kao *et al*, 1993; Malkinson *et al*, 1995; Bahmani *et al*, 1996; Nakamura *et al*, 1996). Severe clinical cases of Borna disease (BD) in horses, sheep, and cats are recognized in certain areas of Germany, Switzerland, Austria, United Kingdom, Sweden, and Japan (Metzler *et al*, 1976; Lange *et al*, 1987; Lundgren *et al*, 1993; Herzog *et al*, 1994; Nowotny and Weissenböck, 1995; Nakamura *et al*, 1996; Reeves *et al*, 1998). Animals show various clinical symptoms ranging from impaired coordination to paralysis and death.

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Possible virus reservoirs as well as the natural route of BDV transmission are still unknown. In this regard it is important to note that many seropositive animals remain asymptomatic (Kao *et al*, 1993; Herzog *et al*, 1994). In order to investigate whether mice might serve as reservoirs for persistent infections, an animal model closely resembling the natural asymptomatic BDV infection was developed. The mouse strain SJL/J (Charles River, Germany) has been reported to be relatively resistant to clinical signs of BD (Rubin *et al*, 1993). Seven 4-week-old SJL/J mice were infected intracerebrally (i.c.) with a BDV He/80 rat brain homogenate (provided by S. Herzog, University of Giessen, Giessen, Germany). This was followed by three consecutive passages of brain homogenates in 8, 15, and 22 animals, respectively. Mock infections were performed with three animals each. Initial infection and all passages were performed by injecting 40 μl of the BDV brain homogenate corresponding to 4×10^4 rabbit embryonic brain (REB) cells/$TCID_{50}$ bilaterally in each brain hemisphere using a Hamilton syringe. Virus stocks were prepared from brains after they had been removed aseptically and homogenized on ice by douncing in Glasgow modified Eagle's medium containing 2% fetal calf serum as 10% (wt/vol) suspensions. After three brief (15 s) ultrasonic pulses, the material was clarified

Table 1 Development of BDV-specific antibody titers in experimentally infected SJL/J mice*

	Days p.i.		
Passage	*21*	*49*	*72*
1	—	7.8 ± 0.32	9.2 ± 0.96
3	3.4 ± 1.1	11.2 ± 0.32	12.8 ± 0.32
4	6.4 ± 1.2	12.4 ± 0.48	13.4 ± 0.48

Sera of the infected animals were titrated on acetone-fixed persistently BDV-infected MDCK cells. Anti-BDV antibodies were visualized using a fluorescence-labeled goat anti-mouse IgG. Five animals per group, selected randomly, and investigated at different time points are shown.
*Antibody titers are expressed as 10 $\log_2$.

Table 2 Detection of viral RNA in organs of eight BDV-infected SJL/J mice 10 weeks p.i.

	*RT-PCR**	
Organs	*Positive/total*	*Percent*
Brain	8/8	100.0
Heart	5/8	62.5
Urinary bladder	4/8	50.0
Lung	3/8	37.5
Kidney	2/8	25.0
Liver	2/8	25.0

*Performed according to Vahlenkamp *et al*, 2000.

by centrifugation at 4°C for 10 min at 1000 × g. Supernatants were subsequently stored at −70°C.

Within the observation period of up to 12 months postinfection (p.i.), none of the animals developed any clinical signs of BD. After each passage, infection was confirmed by serology, virus titration, RT-PCR, and immunohistological analysis.

Using the indirect immunofluorescence assay (IFA; Herzog and Rott, 1980), BDV-specific antibodies were detected 7 weeks p.i. in mice infected with first passage virus stock (BDV He/80 rat brain homogenate), but as early as 3 weeks p.i. in mice infected with third and fourth passage virus stock. In the latter case, the animals also had developed higher antibody titers; in each group mean, antibody titers differed by $\leq \log_2$ dilution steps (Table 1).

The presence of BDV was investigated by inoculating REB cells with brain homogenates and subsequent demonstration of BDV p40 in IFA using the monoclonal antibody Bo 18 (Haas *et al*, 1986; Morales *et al*, 1988). In all mice with BDV-specific antibodies, virus titers ranged between $10^{5.25}$ and $10^{5.75}$ $TCID_{50}$/g (data not shown).

To determine the minimal mouse infectious dose (MID), 10-fold dilutions of the brain homogenate from the fourth passage containing $10^{5.75}$ REB-TCID50/ml were applied i.c. to three animals per dilution and screened for the development of BDV-specific antibodies 10 weeks p.i. The presence of BDV-specific antibodies was detected in the animals inoculated with a 10^{-5} dilution of the virus stock, whereas animals inoculated with the 10^{-6} dilution remained seronegative. The minimal MID was calculated to be 0.22 REB/$TCID_{50}$, according to 50% endpoints (Reed and Münch, 1938).

RT-PCR was applied (Vahlenkamp *et al*, 2000) to investigate the distribution of BDV in brain, heart, lung, liver, kidney, and urinary bladder. These organs were taken from eight animals of the fourth passage euthanized 10 weeks p.i., and RNA was extracted according to Chomczynski and Sacci (1987). BDV p40 RNA was detected in eight out of eight brains (100%), five out of eight hearts (62.5%), four out of eight urinary bladders (50%), three out of eight lungs (37.5%), two out of eight kidneys (25%), and two out of eight livers (25%; Table 2). The presence of infectious virus in peripheral organs was investigated by inoculating REB cells with homogenates of heart, urinary bladder, lung, kidney, and liver derived from two animals of the fourth passage. BDV antigens were detected in

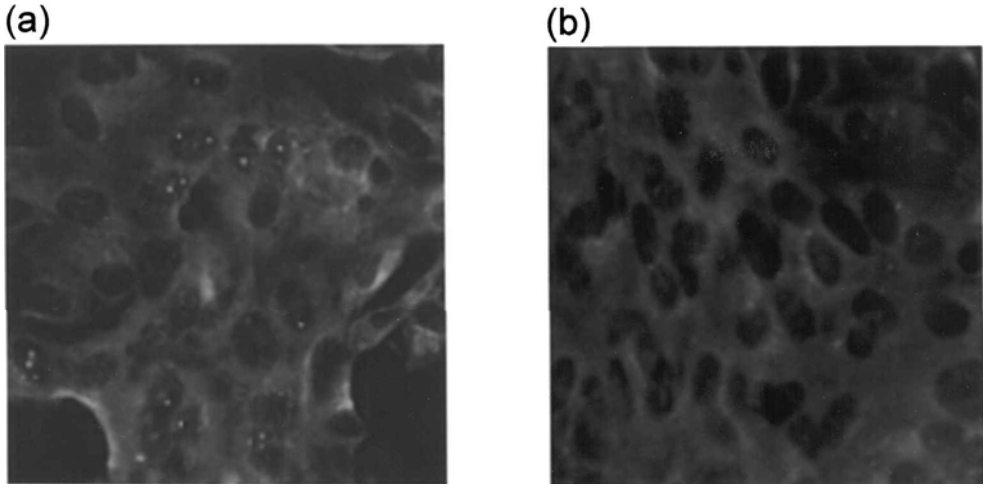

Figure 1 Indirect IFA on acetone-fixed REB cells. (**a**) Detection of BDV-specific intranuclear fluorescence in REB cells inoculated with heart tissue of two experimentally BDV-infected mice. (**b**) REB cells inoculated with heart tissue of a mock-infected animal.

REB cells inoculated with homogenates from heart and lung (Figure 1).

At days 25, 40, and 87 after the fourth passage, one animal each was euthanized, and the brain was examined immunohistochemically. The peroxidase–antiperoxidase method was applied to formalin-fixed (10%, nonbuffered formalin) and paraffin-embedded tissue sections, using the monoclonal antibody Bo18. Staining for BDV p40 was restricted at day 25 p.i. to few ganglion cells in the hippocampus; at day 40 p.i., viral antigen was also demonstrated in some ganglion cells in the frontal cortex (Figure 2a); at day 87 p.i., p40 antigen-positive ganglion cells in the cortex were numerous (Figure 2b). Generally, staining was bilateral-symmetrical.

Brain and various tissue specimens as well as specimens from ganglion coeliacum and intra-abdominal nerves obtained from mice euthanized 6 months ($n = 1$), 8 months ($n = 6$), 11 months ($n = 5$), and 12 months ($n = 5$) after the fourth passage were also examined. In these long-term infected animals, p40 was expressed by ganglion cells throughout the entire brain, but was not detected in the parenchyma of heart, lung, liver, spleen, kidney, and urinary bladder. However, few cells in the ganglion coeliacum (Figure 2c), single to numerous axons of peripheral nerves in the abdominal cavity (Figure 2d) and few cells in the adrenal gland stained positive for BDV p40. In control animals, staining for BDV p40 never was observed (Figure 2e).

Recently, we detected viral RNA had been detected in cells of the peripheral blood in naturally BDV-infected animals (Vahlenkamp *et al*, 2000). Therefore, cells of the peripheral blood of the experimentally infected mice were also investigated. Using a nested set of p24-specific primers, weak RT-PCR products were detected in three out of three animals of the third passage 8 months p.i. (data not shown). With a nested set of p40-specific primers, BDV RNA was detected clearly in cells of the peripheral blood of one out of three additional mice. The peripheral blood cells of the uninfected animals remained negative.

To determine genome differences between the rat brain homogenate used for the initial infection and the mouse brain homogenates, the p40, p24, and gp18 coding regions of the first passage virus stock (BDV He/80 rat brain homogenate) and the mouse brain homogenates of the first and the fourth passage were sequenced. The sequences of the three coding regions analyzed were identical, except for one mutation (C to T at residue 342) in the mouse-derived BDV gp18 coding region. This nucleotide change did not result in a change in the deduced amino acid sequence. Except for this nucleotide mutation, all sequences were identical to the reference BDV strain He/80. Compared with BDV strain V the nucleotide sequence homology to BDV p40, p24, and gp18 was 95.5%, 97.4%, and 95.9%, respectively.

Most studies on the pathogenesis of BD were performed in Lewis rats, highly susceptible to the deleterious effects of BDV (Narayan *et al*, 1983; Richt *et al*, 1990; Stitz *et al*, 1995). The clinical course and histopathology of BD vary with the age of the animal at the time of infection. In newborn or immunosuppressed rats, BDV replicates to high titers in the brain after intracerebral infection. Viral spread via neuronal pathways is accompanied by extraneuronal replication in various organs, and BDV is shed in urine and saliva (Herzog *et al*, 1984; Morales *et al*, 1988). In these rats, BDV was isolated from cells of the peripheral blood and bone marrow (Sierra-Honigmann *et al*, 1993; Rubin *et al*, 1995). In contrast, BDV infections of immunocompetent rats revealed that viral replication remains restricted to the central and the peripheral nervous system (Carbone *et al*, 1987). Neutralizing antibodies seem to play a role in preventing generalized BDV infections since passive immunization of immunoincompetent rats with neutralizing antiserum results in a restriction of BDV to neuronal tissue (Stitz *et al*, 1998).

A major difference between the well-established rat model of experimental BD and experimental BDV infections of mice is that infection of adults induces overt neurological disease in rats, but not in mice. Kao *et al* (1984) reported the infection of newborn Balb/c, DBA/2J, C3H, STU, and C57BR/Cd mice. Four months p.i., 95% of the animals, of all strains, had developed antibodies with maximum titers of 40 000. None of the animals had clinical signs of BD. Rubin *et al* (1993) used adult animals for infection. In SJL/J mice, BDV was only detected when inoculated with the second mouse passage brain homogenate; no clinical signs were observed. In MRL/+ mice, clinical signs such as hyperactivity and aggression were reported, however only when animals were inoculated with fourth mouse passage homogenates. In newborn MRL/+ mice, Hallensleben *et al* (1998) reported marked clinical disease with ataxia, movement disorders, paraparesis, apathy, and death; a lower incidence or a lesser severity of the disease was also observed in newborn C3H, CBA, BALB/c, and C57BL mice.

The molecular basis of these differences is not well understood at present. The expression of distinct alleles of the MHC class I antigen has been suggested to affect the severity of the disease (Hausmann *et al*, 1999). CBA, C3H, and MRL mice which harbor the haplotype H-2^k develop the most prominent disease symptoms; mice with other haplotypes, e.g., BALB/c (H-2^d) or C57BL/6 (H-2^b), have a more moderate course. The frequency at which BDV induces neurological disease, however, seems to be controlled by other genetic traits (Hausmann *et al*, 1999). The haplotype of SJL/J mice (H-2^s) does not seem to support the development of clinical symptoms after BDV infection because in the experiments described here none of the animals developed neurological or behavioral disease symptoms for up to 12 months p.i.

It has been speculated that BDV replication in adult mice is limited more strictly to the central nervous

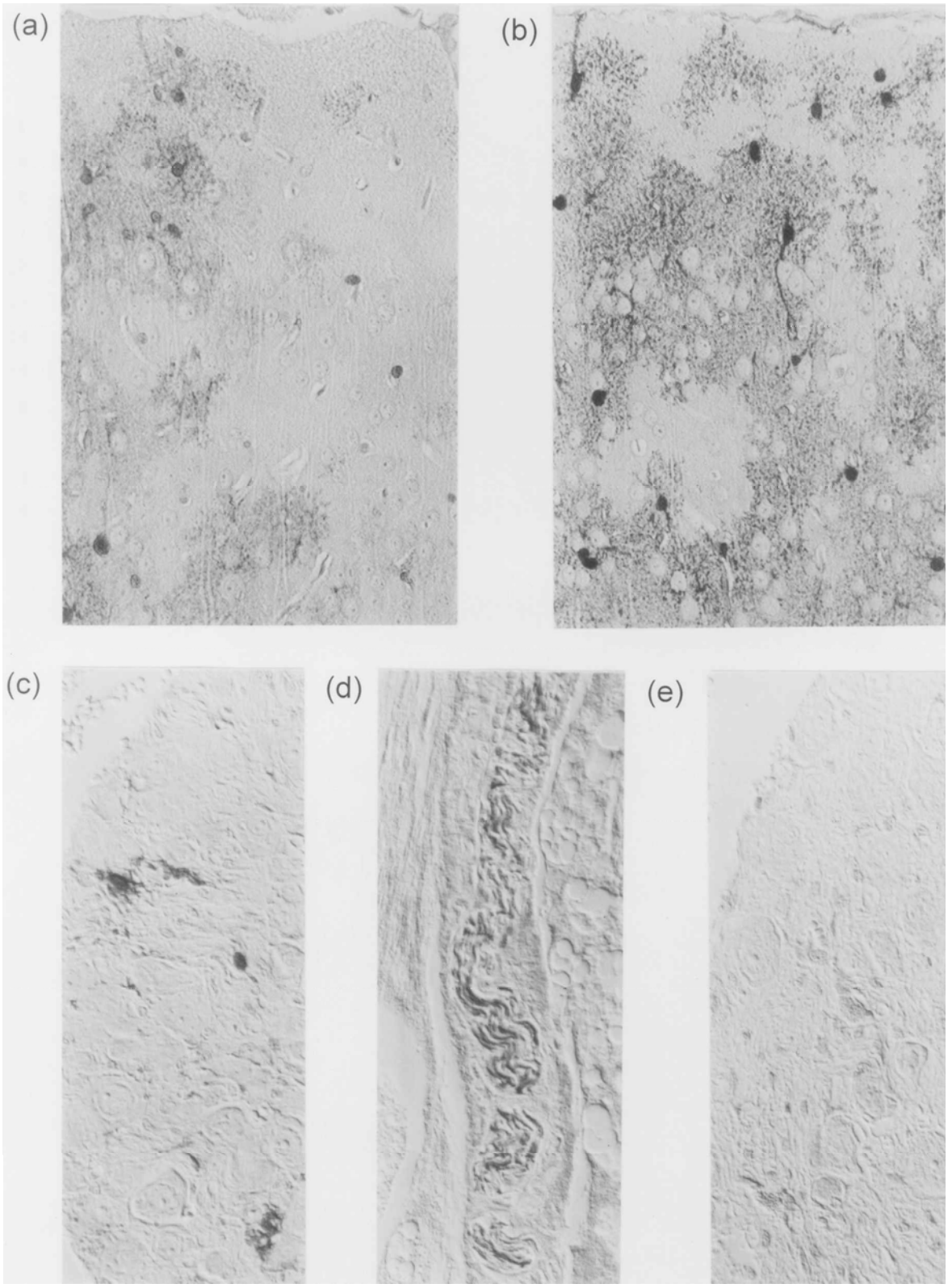

Figure 2 Immunohistochemical demonstration of BDV p40 antigen. (**a**) Few positive ganglion cells are found in the frontal cortex at day 40 p.i. (**b**) At day 87 p.i., numerous positive ganglion cells are found in the frontal cortex. (**c**) Few positive cells are observed in the ganglion coeliacum (11 months p.i.). (**d**) A peripheral nerve in the abdominal cavity shows BDV p40-positive axons (11 months p.i.). (**e**) In a control animal, the ganglion coeliacum is negative for BDV p40 antigen. Peroxidase–antiperoxidase method, Papanicoulaou's counterstain, ×500.

system (CNS) than in newborns; viral antigens might therefore not be present in sufficient quantities at peripheral sites for an efficient priming of T cells (Hallensleben *et al*, 1998). Superinfection of persistently infected B10.BR (H-2^k) mice with recombinant BDV p40 resulted in severe neurological disease and encephalitis (Hausmann *et al*, 1999). None of the previous studies examined the replication and distribution of the virus outside the CNS.

In our experiments, SJL/J mice proved to be very susceptible to experimental BDV infection, but did not develop overt signs of BD. The minimal MID was 0.22 REB/$TCID_{50}$ and infectious virus was isolated after the first passage. Nucleotide sequence analysis of BDV in the rat brain used for the initial infection and in mouse brain homogenates revealed a very high degree of conservation. Outside the CNS, viral RNA was detected most frequently in the heart, urinary bladder, and lung; a lower frequency was detected in other organs such as liver and kidney. This might be explained by different amounts of nervous tissue present in these organs. Indeed, immunohistochemical analysis confirmed that BDV-specific antigens were only present within their nervous tissues. The presence of viral RNA in cells of the peripheral blood, however, reveals that BDV also spreads to extraneuronal cells, as observed in experimentally infected rats (Sierra-Honigmann *et al*, 1993; Rubin *et al*, 1995).

We also isolated BDV from peripheral tissues of heart and lung. This shows that infectious virus is also present in peripheral tissues of these mice, despite the fact that none of the animals showed any overt signs of clinical disease. Due to the small volumes of body secretions and excretions of these animals and the known difficulties to isolate BDV in cell culture, we did not perform infection experiments with these materials.

In an attempt to test whether immunocompetent, persistently infected mice shed infectious virus to the environment in their secretions and excretions, we house infected and uninfected immunocompetent animals together in one cage for up to 1 year. None of the naive animals, however, got infected. Further experiments have to be performed to elucidate whether (i) the immune response in these animals is involved in the distribution of the virus, and (ii) persistently infected newborn or immunosuppressed adult mice shed virus to neighboring animals or to their offspring. Experimental BDV infection of mice also enables to exploit the potential of this genetically well-defined model to get insights in the mechanisms involved to sustain the persistent infection without developing clinical signs of BD.

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