

BORNA DISEASE VIRUS AND INFECTION IN HUMANS

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1. ABSTRACT

Borna disease virus (BDV) is a nonsegmented, negative-, single-stranded, highly neurotropic RNA virus with noncytolytic replication in the central nervous system. This virus causes neurological and behavioral disturbances primarily in horses and sheep, in addition to a variety of other vertebrate animal species and in laboratory animal models. BDV is now gaining much of the research attention, because the disturbances seen in animals resemble those of neuropsychiatric disorders in humans. These observations raise the possibility that BDV infection may be associated with certain human disorders.

Serological and molecular studies on many samples from human patients with a variety of psychiatric disorders have been performed. Some reported the presence and elevated levels of serum antibodies to BDV. Others reported the presence of BDV-RNAs or BDV-

antigens in the peripheral blood samples as well as in autopsied brains. Taken together these data support the possibility of human infection with BDV. On the contrary, others reported the complete absence of such BDV-markers from their samples, supporting the absence of a link between BDV infection and psychiatric disorders as well as excluding it as a human pathogen. Thus, BDV infection in humans is highly controversial. Further investigations are required to answer the question whether BDV is a human pathogen and moreover, to elucidate the possible role, if any, of BDV in the pathogenesises of these disorders.

2. INTRODUCTION

Borna disease virus (BDV) is the etiological agent of Borna disease that was first described more than 200 years ago in southern Germany as a fatal neurological

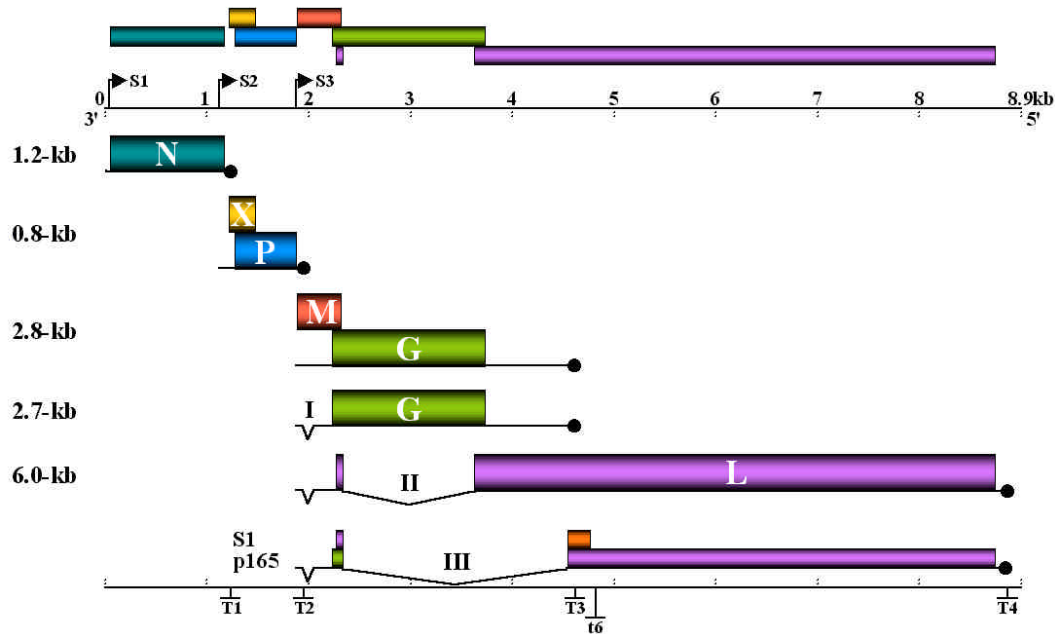


Figure 1. Genomic structure and transcription map of BDV. BDV ORF's are shown by boxes. The location of transcription initiation and transcription termination sites are marked as S and T, respectively. Positions of introns I to III are indicated.

disease of horses and sheep. The name refers to Borna, a city in Saxony, Germany, where a large number of horses died during an epidemic in 1894 and 1896. The experimental transmission of the disease to animals using brain homogenates from diseased horses together with filtration studies documented a viral etiology for the disease (1, 2).

BDV is a neurotropic enveloped virus. Molecular-biological studies on this virus revealed a nonsegmented, negative-, single-stranded (NNS) RNA genome that is similar to other viruses within the order *Mononegavirales*. Replication and transcription of the BDV genome take place in the nuclei of infected cells. Such unique features of this virus allowed it to be classified as a new family: *Bornaviridae*.

The main natural hosts of BDV are horses and sheep. However, naturally occurring Borna disease has been diagnosed in donkeys, goats, cattle, cats, rabbits and dogs. Further, a variety of warm-blooded animals including deer and zoo animals, such as vari monkeys, sloths, llamas, alpacas, and pigmy hippopotamus, have been documented to be naturally infected with BDV (3-5). Infection with BDV in humans, especially in patients with psychiatric disorders, was first reported by Rott *et al.* (6). These researchers used horse-derived BDV as antigen to analyze serum antibodies in these patients and in normal subjects. Additional serological and molecular epidemiological studies indicated that BDV can infect humans and the infection may have an association with certain neuropsychiatric disorders. However, these studies are very controversial, as we will discuss in detail. The purpose of this article is to review BDV infection in humans and discuss the possible association of BDV, if any, with certain human disorders.

3. VIROLOGY OF BDV

3.1. BDV genome structure and viral proteins

The BDV genomic RNA is approximately 8.9 kb in length (7, 8). As shown in figure 1, the BDV genome encodes six open reading frames (ORF's): p40 nucleoprotein ('N'), p24 phosphoprotein ('P'), gp18 or p16 matrix protein ('M'), gp94/gp84 membrane glycoprotein ('G'), p190 polymerase ('L'), and p10 protein with unknown function ('X'; refs. 9 & 10). These ORF's are transcribed in three units: the first transcription unit with a 1.2 kb mRNA for the 'N' protein; the second transcription unit with a 0.8 kb mRNA for the 'X' and 'P' in overlapping ORF's; and, the third transcription unit with several mRNA's generated by alternative termination of transcription and splicing of one to three introns (11-13) for the 'M', 'G', and 'L' proteins.

The 'N', 'P' and 'X' are the major proteins expressed in infected cells. The 'N' protein exists in two forms: 40- and 38-kDa. The latter lacks 13 N-terminal amino acids of the 40-kDa protein, because it is translated from the second AUG codon of the same mRNA. In cells independently transfected with plasmids encoding each of the two 'N' isoforms, p40 'N' and p38 'N' are accumulated in the nucleus and cytoplasm, respectively. This difference in the subcellular distribution of the two forms is derived from a nuclear localization signal (NLS) present in the N-terminal of p40 'N' (14, 15). Recently, p38 'N' was shown to have a role in the nucleocytoplasmic shuttling (16), as will be described later.

The 'X' protein does not have a NLS, but is localized in the nuclei of infected cells, which appears to be mediated by interaction with 'P' as well as with p40 'N' (17-19). The amino terminus of 'X' contains a leucine-rich

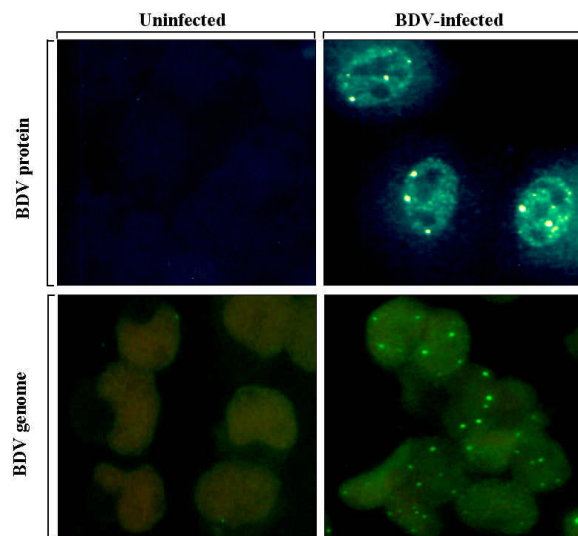


Figure 2. BDV expression in the nucleus of infected cells. Uninfected and BDV-infected cells were stained by IFA with anti-'N' antibodies (upper panels) and ISH with anti-BDV 'N' riboprobe (lower panels).

nuclear export signal (NES)-like motif, and has been shown to be critical for binding to 'P' (19, 20).

Although there is no direct evidence for the role of the 'P' protein in the life cycle of BDV, 'P' seems to have a function similar to that proposed for phosphoproteins of other known NNS RNA viruses, i.e., it serves as an essential cofactor in virus transcription and replication. The 'P' protein is phosphorylated predominantly by protein kinase epsilon and, to a lesser extent, by casein kinase II (21). It contains two strong NLSs (22, 23). The 'P' protein interacts with itself, with 'X' and 'N', and co-localizes with 'N' and 'X' in distinct areas within the nuclei of the infected cells (24). In addition to the p24 protein, the 'P' ORF also produces a 16-kDa protein by translation from the second in-frame AUG codon (25). This 16-kDa 'P' has been detected in BDV-infected cultured cells and in brains of experimentally infected animals.

Two glycoproteins, 'M' and 'G', have been identified. 'M' forms stable tetramers and contains hydrophobic sequences characteristics of membrane spanning proteins (26). Although the role of this protein in BDV life cycle remains to be elucidated, the 'M' protein was proposed to be present on the surface of viral particles and play a role in viral attachment to the cells (27, 28). However, 'M' was recently shown to be nonglycosylated (23a). In addition, 'G' protein, N-glycosylated with high-mannose and/or hybrid oligosaccharides, yields a full length type I membrane protein (29, 30) that is cleaved, and the C-terminal cleavage product is identified as gp43 (31). Both gp94/gp84 and gp43 are incorporated into the virion, while only gp43 is translocated to the cellular membrane (31). The gp94/gp84 was proposed to be involved in the attachment to the receptor, while gp43 is responsible for triggering the fusion events (32). A recent pseudotype approach based on a recombinant vesicular stomatitis virus

revealed that the N-terminal domain of the gp94/gp84 is sufficient for receptor recognition and virus entry (33).

The last BDV protein is 'L', predicted to encode the viral RNA-dependent RNA polymerase (7, 9). The 'L' protein is predominantly present in the nuclei of cells transfected with an 'L' expression plasmid in the absence of other viral proteins. Recently, over-expression of recombinant 'L' showed interaction with 'P' and further indicated that 'L' is phosphorylated by cellular kinases (34).

3.2. BDV transcription and replication in the nucleus

BDV belonging to the order *Mononegavirales* has similarity in its genome organization to other members of this order. However, BDV has several unique features. One of the most striking characteristics of BDV is its location for transcription (figure 2). BDV replicates and transcribes in the nucleus of infected cells (35). Strand-specific probes used for *in situ* hybridization (ISH) showed that there was differential localization of positive- and negative-strand RNA's within the nucleus and further, within the nuclei the sense-strand RNA's were preferentially localized within the nucleolus regions (36). The 'N' and 'P' proteins have nuclear localization activity, which is mediated by single and bipartite NLS's (15, 22, 23). These experiments suggested that the nuclear localization of the 'N' and 'P' proteins is critical for nuclear targeting of the BDV RNA-protein complexes, because these proteins interact with each other and are probably essential components of the viral RNA-protein complexes (17, 18, 35, 37). On the other hand, the NES-like sequence of BDV has been identified in the N terminus of the 'X' protein (18, 19). However, a recent study was not able to demonstrate nuclear export activity for the 'X' protein, despite the fact that the consensus leucine-rich sequence is found in the NES-like motif of the protein (20). More recently, the NES of BDV 'N' contains a canonical leucine-rich motif, and the nuclear export activity of the protein is mediated through the chromosome region maintenance protein pathway, indicating that BDV 'N' has two contrary activities: nuclear localization and export. These activities of 'N' may play a critical role in the nuclear-cytoplasm transport of BDV RNA-protein complexes in combination with other viral proteins (figure 3 and ref. 16). Interestingly, a region of the NES of p40 and p38 'N' proteins overlaps a binding site for the 'P' protein, suggesting that increased level of nuclear 'P' protein could negatively affect nuclear export activity mediated by the NES of 'N'.

The BDV particles (figure 4) produced by budding on the cell surface are spherical, enveloped, and approximately 130 nm in diameter, and had spikes 7 nm in length (38). A thin nucleocapsid, 4 nm in width, was present peripherally, which is in contrast to the thick nucleocapsid of hemagglutinating virus of Japan (38). However, BDV morphogenesis largely has not been clarified.

4. DIAGNOSIS OF BDV INFECTION

4.1. Serological diagnosis

Detection of antibodies to BDV in sera and cerebrospinal fluid (CSF) samples from infected animals

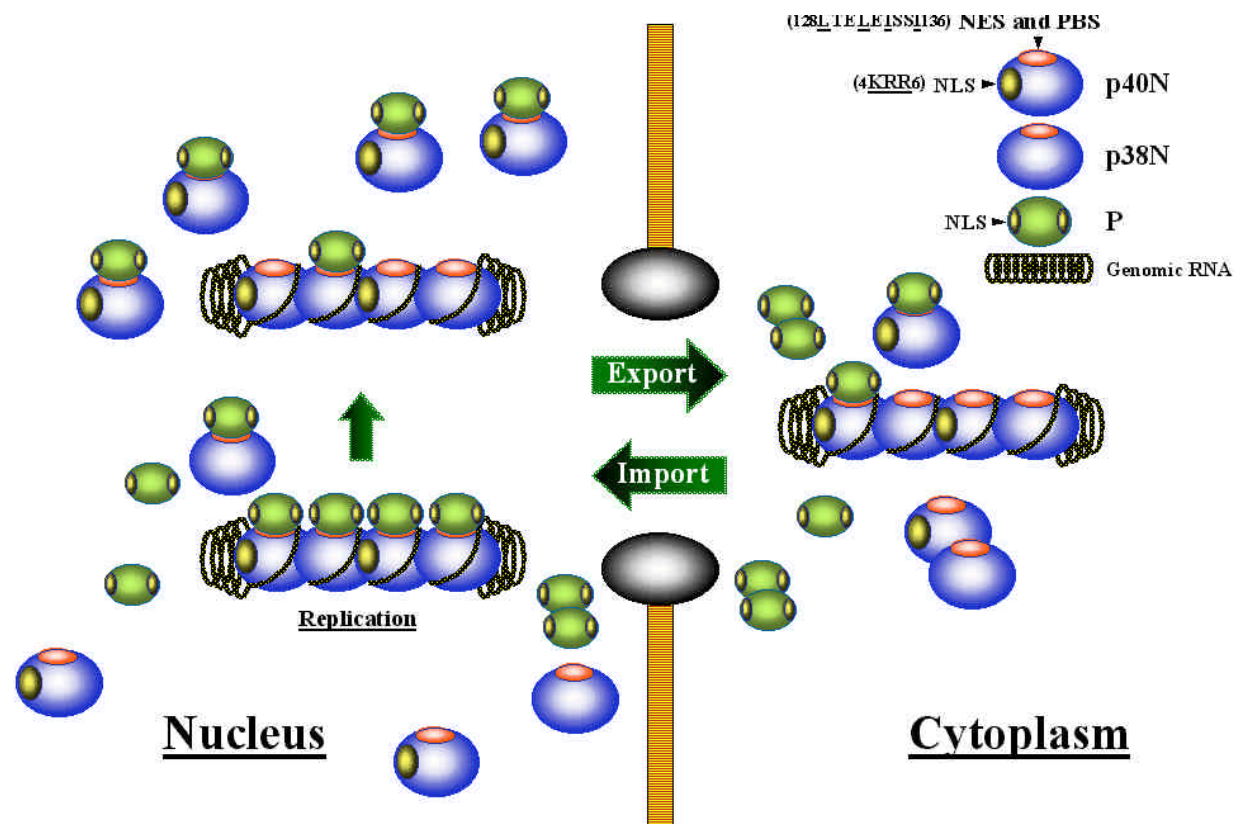


Figure 3. Model of nucleocytoplasmic transport of BDV RNA-protein complex. BDV genomic RNA is associated with multiple copies of BDV p40 and p38 'N'. BDV p40 'N' or 'P' is required for import of RNA-protein complex to nucleus by nuclear targeting. BDV 'N' also contains NES, which overlaps the P-binding site (PBS). It is postulated that the nuclear export activity of BDV 'N' is blocked by interaction with 'P' during its replication in the nucleus. A mechanism that triggers export of RNA-protein complex to the cytoplasm after replication may also exist. Concentrations of each viral protein in the nucleus seem to play an important role as a switch mechanism of RNA-protein complex. Modified from Fig. 7 in Kobayashi *et al.* (16).

and humans has mainly relied on the use of immunofluorescence assays (IFA's) (6). Many seroepidemiological studies have used a modified IFA that is based on double staining of BDV-infected cells using serum or CSF samples being tested and a monoclonal antibody (MAb) to the BDV 'N' or 'P' antigen (39). In this test, samples showing the same staining pattern as with the BDV-specific MAb were considered positive. However, several investigators have indicated that this IFA is highly unreliable and prone to produce false positives (40), especially when analyzing human sera which frequently exhibit only very modest titers of BDV antibodies. Increased sensitivity and specificity in BDV serology have been achieved with the introduction of Western blot (WB) assays using as antigens: BDV-infected cell extracts (40, 41), BDV proteins purified by affinity chromatography from infected rat brains (42) or from infected rabbit kidney (RK) cells (43), and/or recombinantly-expressed BDV proteins (44-46). The expression and purification of large amounts of recombinant BDV antigens has also facilitated the establishment of enzyme-linked immunosorbent assay (ELISA) for the detection of BDV antibodies in biological samples (45). The use of ELISA allowed a rapid and

quantitative analysis of BDV antibody titers in large number of samples, which should facilitate the epidemiological survey of BDV. Nevertheless, the reliability and reproducibility of these assays need to be validated before being considered as standard diagnostic tools for BDV infection (47). Recently, new serological techniques such as reverse-type (RT)-ELISA (47) and electrochemiluminescence immunoassay (ECLIA) (48) have been developed for the detection of BDV antibodies by the use of recombinant BDV proteins. For ECLIA, synthetic BDV peptides were also useful (49). These new techniques also remain for consideration as standard diagnostic tools for BDV infection.

A recent study reported that the interplay of BDV-specific circulating immune complexes (CIC's) with free antibodies and plasma antigens may give rise to detection "gaps"(50). Another point of contention is the specificity of the BDV antibodies in humans. Antibodies in human sera mainly recognize BDV 'P' protein, whereas animal sera preferentially recognize the 'N' protein. Sensitivity tests of immunoglobulins in up to 3 M urea suggest that antibodies in animals have high avidity to

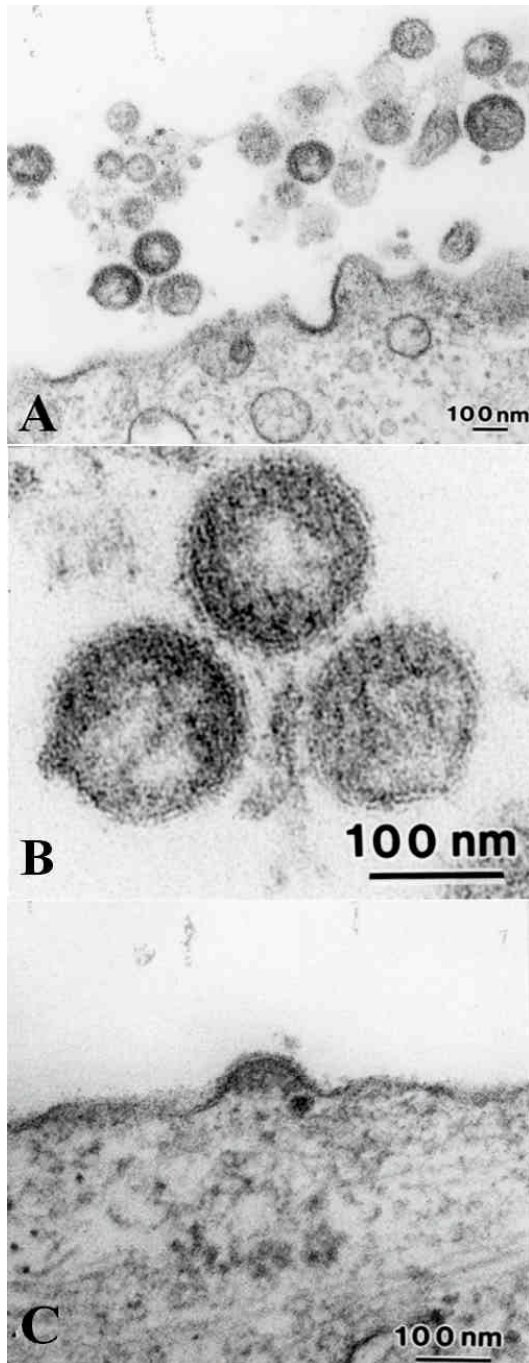


Figure 4. Morphology of BDV particles by electron microscopy. (A) Extracellular virus-like particles from persistently BDV-infected cells (A), and its high magnification (B) and budding profile (C). See also ref. 38. Figure courtesy of Toshiyuki Goto, Kyoto University, Kyoto, Japan.

BDV, whereas reactive antibodies in human sera have low avidity (51).

4.2. Molecular diagnosis

Molecular characterization of the BDV genome (7, 8) permits the development of specific and sensitive

reverse transcriptase-polymerase chain reaction (RT-PCR) procedures to detect BDV RNA in biological samples. The RT-PCR was first used to detect BDV RNA in the brains and organs of experimentally infected rats (52-54). Thereafter, RT-PCR has been instrumental in detecting BDV RNA in tissues such as brains from naturally infected animals including horses, donkeys, sheep, cattle, and cats (55-62).

Although BDV was initially believed to have exclusive tropism for brain cells, it also has been detected in non-neural tissues as well as body secretions from infected animals (55). Early RT-PCR studies failed to demonstrate BDV RNA in whole blood samples from adult rats infected with BDV (52). However, BDV RNA can be detected by RT-PCR in peripheral blood mononuclear cells (PBMC's), bone marrow (53) and thymic stromal cells (54) from neonatally infected rats. The RT-PCR procedures to examine the prevalence of BDV RNA in PBMC's from humans (44, 46, 63-83) and domestic animals (61, 83-89) have now been applied to investigate the molecular epidemiology of BDV infection. Most of these studies have focused on the detection of BDV 'N' (p40) and 'P' (p24) RNA sequences in tissues. The specificity of the RT-PCR-amplified products is then verified by Southern blot hybridization using BDV-specific probes. However, RT-PCR conditions, including the cell fraction from whole blood; the starting number of cells used to extract RNA; the amount of RNA used; the polymerases used; the number of PCR cycles; and, the methods used to estimate the sensitivity of the assay, greatly vary among studies preventing direct comparison of results obtained from different laboratories. The high sensitivity of RT-PCR also makes it prone to artifacts due to inadvertent contamination with laboratory sources of BDV. Moreover, the high degree of BDV sequence conservation makes it difficult to use sequence analysis to identify cases of contamination (74, 90). These concerns can be addressed by following the strict guidelines to prevent contamination during the RT-PCR assays. However, the extremely low level of BDV RNA in the PBMC - close to the sensitivity threshold of the RT-PCR - can affect the reproducibility of results between different laboratories.

5. NATURAL ANIMAL HOSTS OF BDV INFECTION

5.1. Natural Borna disease

A syndrome of progressive meningoencephalitis was recognized 100 years ago in horses and sheep, in an epidemic lasting from 1894 to 1896, near the town Borna, Germany. The incidence of fatal Borna disease in horses in the area was 100%, from a total of about 150,000 horses (91). However, severe cases of "classical" Borna disease still can be detected in endemic areas, although their incidence has remarkably decreased in the area (91, 92). The seasonal accumulation of cases is observed in the spring and early summer with a significant decrease in late autumn and winter (93-99).

The Borna disease in horses and sheep was mostly restricted to Germany, Switzerland, Austria and the Principality of Liechtenstein (97, 98, 100-111). However,

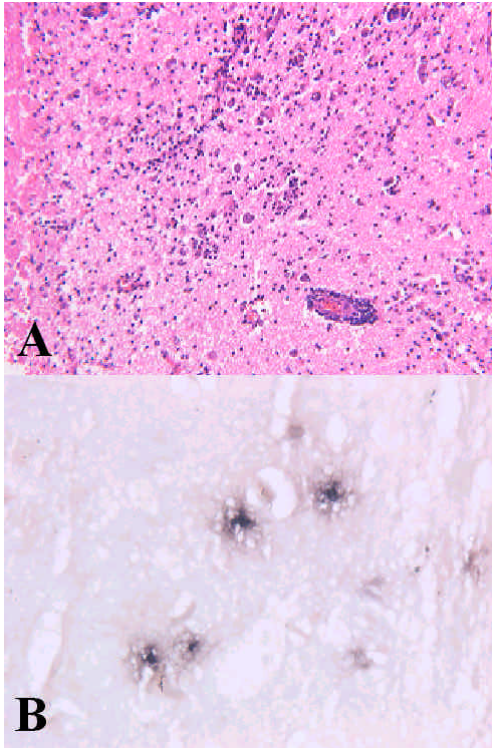


Figure 5. Non-suppurative encephalitis in the brain from a horse naturally infected with BDV. The neurological lesions are characterized by perivascular cuffing and mononuclear cell infiltration. A, the olfactory bulb region stained with hematoxylin and eosin. x20. B, the corpus striatum neurons stained by ISH using BDV-'N' (p40) antisense riboprobes. x60. Figure courtesy of Hiroyuki Taniyama, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan. See also (ref. 113).

Swedish horses with BDV infection also have been reported (112). In addition, two cases of classical Borna disease in horses were confirmed recently in Japan (113). Also, BDV has been demonstrated at a high rate in restricted regions of the brain from horses with locomotor disease with unknown etiology in Japan (62). Thus, these reports, together with that of subclinical cases of BDV infection in horses and sheep in many countries around the world, as described later, indicate that the geographical distribution of BDV appears not to have been fully estimated as previously thought.

The clinical manifestations and pathological findings in naturally BDV-infected horses (figure 5) and sheep are almost the same. However, the data reported are mostly obtained from horses. The pathological findings of BDV infection are usually restricted to the central nervous system (CNS; mainly in the gray matter), the spinal cord and the retina.

BDV infection in horses and sheep could be manifested as peracute, acute or subacute disease with meningoencephalitis. The typical clinical signs vary, and can include inappetence, simultaneous or consecutive alterations in behavior, circling, ataxia, blindness,

sometimes disturbances in fertility, rarely obesity, and in late stages paralysis followed by death (1, 4, 5, 94-96, 98, 114, 115). Occasional recovery can occur spontaneously despite a persistent infection in the CNS and sometimes a recurrent course of the disease may be noticed (94, 97, 116, 117).

5.2. Subclinical infection

Anti-viral antibodies in serum as well as viral nucleic acid have been detected in several tissue samples including PBMC's from apparently normal horses, indicating that asymptomatic infection is more frequent than it has been expected. BDV-specific antibodies or RNA were demonstrated in healthy horses from all over Germany, Holland, Poland, Sweden, France, Israel, North Africa, USA, Japan, Iran, Bangladesh, and China (42, 48, 55, 71, 83, 84, 87, 103, 112, 118-120). However, due to the significant differences in the sensitivities of the assays employed, the data obtained showing the prevalence of BDV varied considerably amongst the studies.

5.3. Natural Borna-like disease in other animal species

In cattle, a neurological disease resembling the classical clinical course and neuropathology seen in horses and sheep already had been documented long ago (2, 121). However, there was no etiological proof for BDV infection in these cases. Experimental infection of two calves with BDV had been successful (122). Thus, it was suggested that cattle are susceptible to BDV infection. However, it was not until 1994, when confirmed cases of naturally occurring Borna disease were reported in cattle, at approximately the same time in Germany, in endemic and unendemic areas, as well as in an endemic area in Switzerland (123, 124). Similarly, a cattle with Borna-like disease was recently found in Hokkaido, Japan (125) where two cases of horses with classical Borna disease had been found (113), as well as many subclinical cases in several animal species (84, 85, 88), including cattle (61).

A neurological disease in cats, named "staggering disease", was first described in Sweden by Kronevi *et al.* (126). Subsequent serological and molecular epidemiologic studies for BDV revealed high association of BDV infection in cats with staggering disease from Uppsala in Sweden, Berlin in Germany (127), Austria (128), the United Kingdom (89) and Japan (86).

BDV infection was demonstrated in paretic ostriches in Israel (129-131). The paresis syndrome was believed to be caused by an agent serologically related to BDV. Dogs have never been suspected as a possible host species for BDV infection, and neither natural nor experimental Borna disease had ever been described in dogs (114). However, in 1998, a dog with Borna-like disease was reported in Vorarlberg, Austria (132), and a second case was recently found in Japan (132a). Recently, BDV genome was detected in the brains of red foxes in France (120). In addition, mallards (*Anas platyrhynchos*) and jackdaws (*Corvus monedula*) in Sweden also were shown to be subclinically infected, and they may serve as a natural reservoir for BDV (133).

6. BDV INFECTION AND HUMANS

6.1. Epidemiological studies for BDV infection in humans

Epidemiological studies to assess the association of BDV infection with human diseases have been performed serologically and by use of molecular techniques, in like manner to studies of naturally occurring Borna disease in animals described in section 5. Initially, indirect IFA was used in these studies. Later, several serological methods were developed, including the use of RT-PCR in 1995 in molecular epidemiologic studies to detect BDV footprints in the PBMC's of patients. The results reported for patients with psychiatric disorders, chronic fatigue syndrome (CFS), and immunosuppressive state are summarized, according to the year of the report, in table 1 for anti-BDV antibodies, in table 2 for BDV antigens, and in table 3 for BDV RNA.

6.1.1. Psychiatric disorders

The wide host range of BDV, and the observations that animals experimentally infected with BDV exhibit behavioral disturbances reminiscent of some types of affective disorders in humans, prompted studies to investigate the association of BDV infection with human diseases. Serological analyses of BDV infection were carried out in patients with mental disorders, initially by use of horse-derived BDV as antigens in indirect IFA. Later, other laboratory techniques including ELISA, RT-ELISA, ECLIA, WB and T-cell proliferative response (TCPR) assay were also applied to study the epidemiology of BDV infection in humans.

Accumulated seroepidemiological data from different laboratories have shown an increased BDV seroprevalence in neuropsychiatric patients as compared to controls (6, 39, 40, 41, 43, 44, 46, 48, 63-65, 70, 74, 83, 134-142). However, considerable variations in the prevalence rates have been documented in various patient groups (table 1). These variations could be due to the different sensitivity of the methods used to detect anti-BDV antibodies. In contrast, several seroepidemiological studies found no evidence of association between BDV and neuropsychiatric disorders (47, 72, 81).

Anti-BDV seropositivity was associated with clinical diagnosis of deficit syndrome, a schizophrenia subgroup characterized by social withdrawal, neuroanatomic abnormalities and neurological disturbances (138). Magnetic resonance imaging (MRI) studies have also suggested a correlation between BDV seropositivity and cerebral atrophy in schizophrenic patients (143). Serologically positive reactions were shown to be associated with negative syndromes in schizophrenic patients (144), but not with age, age at onset, period of hospitalization, accompanying somatic diseases, a past history of tuberculosis, a history of transfusion, a familial history, or doses of psychotropic drugs (70).

These serological findings should be cautiously evaluated. In many cases, only limited information was provided about the composition of the subject group analyzed. Factors such as the geographic distribution, the heterogeneity of diagnoses and clinical status of the patients

may have significantly influenced these results. In addition, differences in experimental procedures to detect human serum antibodies to BDV could also have affected the results. Moreover, the IFA used in many of these studies is considered to be unreliable (40, 47, 145).

BDV antibody titers in human sera are usually very low (63), and sera from neuropsychiatric patients frequently contain auto-antibodies that react with nuclear structures (146, 147). Thus, the nuclear localization of BDV antigens detected by IFA in the infected cells makes it necessary to discriminate between BDV-specific staining and nuclear staining due to auto-antibodies by use of appropriate controls. The introduction of WB assays that use either BDV-infected cell extracts (40, 43) or recombinant BDV antigens expressed in *E. coli* (44) and in baculovirus (46) has provided increased specificity and sensitivity in BDV serology. Interestingly, results from studies conducted using this improved serologic test still show a significantly higher BDV seroprevalence in neuropsychiatric patients as compared to non-psychiatric control groups (40, 43, 46, 64, 140). Recently, TCPR assay showed significantly higher reactivity in mood disorders and schizophrenic patients than those by WB (142). In contrast, as described above, anti-BDV antibodies in humans was shown to have low avidity. It has been proposed that these antibodies are probably not induced by BDV, but rather by infection with an antigenically related microorganism of unknown identity, or by exposure to other related immunogens (51). Another interpretation for the low titers of anti-BDV antibodies in humans is the detection "gaps" mediated by BDV-specific CIC's (50). Serologic screening of 3000 sera from human patients and equines revealed that BDV CIC's may account for detection "gaps", and the infection rates may be 10 times higher than previously realized by standard serology testing (50).

The finding that virus-infected cells are present in the PBMC's of BDV-infected animals prompted studies to investigate whether the expression of viral antigen and RNA also could be detected in human PBMC's. Flow cytometric analysis was used to study viral antigen expression in PBMC's (63, 148). In addition, RT-PCR procedures were employed to detect BDV-specific nucleotide sequences, mostly at 'N' and/or 'P', in RNA samples extracted from PBMC's of patients as well as of healthy individuals (table 3). The number and nature of the infected cells in the PBMC's are still largely controversial. Highly sensitive RT-PCR procedures are needed to detect BDV RNA in PBMC's from BDV-infected animals because of the extremely low viral load in the PBMC's. In persistently infected rats, the prevalence of BDV-infected cells in PBMC's was estimated at one per 5×10^6 cells (54). Sauder and de la Torre (149) gave evidence suggesting an extremely low prevalence, in the range of one or two infected cells per 5×10^6 PBMC's from infected rats. Also, it was suggested that in PBMC's, BDV-infected cells have low levels of viral RNA, i.e., in the range of 100-500 copies of 'N'-RNA per cell (149). Although the number of PBMC's harboring BDV RNA in infected humans is unknown, some investigators reported that 10-17% of the monocytes from BDV-positive patients

Table 1. Detection of serum or CSF antibodies to BDV in subjects with various diseases

Reference	Disease	Assay	Comments
Rott <i>et al.</i> , 1985 (6)	Psychiatric	IFA	6% vs 0% ^b
	Affective disorders	IFA	4% vs 0%
Amsterdam <i>et al.</i> , 1985 (134)	Affective disorders	IFA	4.5% vs 0%
Bode <i>et al.</i> , 1988 (135)	Psychiatric	IFA	2% vs 2%
	HIV-positive	IFA	7.8% vs 2.0%
Rott <i>et al.</i> , 1991 (41)	Psychiatric/neurological diseases	IFA/WB	4%-7% vs 1%
Bode <i>et al.</i> , 1992 (39)	Chronic diseases ^a	IFA/IP	13%-14% vs 2%
Bode <i>et al.</i> , 1992 (156)	CFS	IFA	0%
Bechter <i>et al.</i> , 1992 (137)	Psychiatric	IFA	Significantly higher than controls (surgical patients)
Bode <i>et al.</i> , 1993 (136)	Psychiatric/Affective disorders	IFA	20% (increased from 2%-4% by follow-up studies)
Fu <i>et al.</i> , 1993 (43)	Affective disorders	WB	38% vs 16% in 'N'; 12% vs 4% in 'P'
Bode <i>et al.</i> , 1995 (63)	Psychiatric	IFA	50%
Kishi <i>et al.</i> , 1995 (64)	Psychiatric	WB	30%
Kishi <i>et al.</i> , 1995 (44)	Blood donors	WB	1%
Waltrip <i>et al.</i> , 1995 (40)	Schizophrenia	WB	8.9% vs 0% in 'N'; 27.8% vs 20% in 'P'
	Schizophrenia	IFA	16.7% vs 15%
Bechter <i>et al.</i> , 1995 (200)	Psychiatric		Increased CSF/serum index for anti-BDV antibodies in 10.5-29.0% (according to different methodological limits) of BDV-seropositive patients
Sauder <i>et al.</i> , 1996 (46)	Psychiatric	WB	14% vs 1.5%
	Affective disorders	WB	12% vs 1.5%
Igata-Yi <i>et al.</i> , 1996 (65)	Psychiatric	IFA	24% vs 11%
Nakaya <i>et al.</i> , 1996 (66)	CFS	WB	24%
Auwanit <i>et al.</i> , 1996 (165)	HIV-positive	ELISA	38%-48% in STD patients infected with subtype E 8.3% in prostitutes infected with subtype E 0% in pregnant women infected with subtype E 0% in intravenous drug users infected with subtype B 0% in HIV-1-negative individuals
Kitze <i>et al.</i> , 1996 (145)	Multiple sclerosis	IFA	0%
Waltrip <i>et al.</i> , 1997 (138)	Deficit schizophrenia	WB	33.3%
	Nondeficit schizophrenia	WB	8.2%
Richt <i>et al.</i> , 1997 (74)	Schizophrenia	WB	20%
Iwahashi <i>et al.</i> , 1997 (70)	Schizophrenia	WB	45%
Takahashi <i>et al.</i> , 1997 (71)	Blood donors	ELISA/WB	2.6%-14.8% in donors living near horse farms
Kubo <i>et al.</i> , 1997 (72)	Psychiatric	IFA/WB	0%
	Affective disorders	IFA/WB	0%-0.8%
	Schizophrenia	IFA/WB	0%-1%
	Controls	IFA/WB	0%
Horimoto <i>et al.</i> , 1997 (47)	Schizophrenia	RT-ELISA	0% vs 0%
Deuschule <i>et al.</i> , 1998 (139)	Major depression		6.3% in CSF
	Multiple sclerosis		0% in CSF
Yamaguchi <i>et al.</i> , 1999 (48)	Schizophrenia	ECLIA	3.08%
	Mood disorders	ECLIA	3.59%
	Other psychiatric	ECLIA	0%
	Neurological disorders	ECLIA	0%
	CFS	ECLIA	0%
	HIV-positive	ECLIA	1.18%
	Autoimmune	ECLIA	0%
	Blood donors	ECLIA	1.09%
	Multitransfusion	ECLIA	4.55%
Backmann <i>et al.</i> , 1999 (80)	HIV-positive	IFA	12.5% in patients with various neurological disorders; 8.0% in patients with AIDS dementia complex
Chen <i>et al.</i> , 1999 (140)	Schizophrenia	WB	12.1%
	Family members	WB	12.1%
	Mental health workers	WB	9.8%
	Controls	WB	2.9%
Nakaya <i>et al.</i> , 1999 (67)	CFS	WB	100% in 2 family clusters
Evengard <i>et al.</i> , 1999 (79)	CFS	ELISA/WB	0% vs 0%
Valenkamp <i>et al.</i> , 2000 (83)	Psychiatric	IFA	17.4% vs 0%
Tsuji <i>et al.</i> , 2000 (81)	Psychiatric/Schizophrenia	WB	0%
Rybakowski <i>et al.</i> , 2001 (141)	Psychiatric	ECLIA	10.2% (recent onset of disease)
		ECLIA	1.6% (>1 year illness)
Fukuda <i>et al.</i> , 2001 (142)	Mood disorders	TCPR	9%
	Schizophrenia	TCPR	4%
	Blood donors	TCPR	2%

Abbreviations used: HIV=human immunodeficiency virus; CFS=chronic fatigue syndrome; IFA=immunofluorescence assay; WB=Western blotting; IP=immunoprecipitation; ELISA=enzyme-linked immunosorbent assay; RT-ELISA=reverse-type ELISA; ECLIA=electrochemiluminescence immunoassay; TCPR=T-cell proliferative response; CSF=cerebrospinal fluid; STD=sexually transmitted disease. ^a Chronic progressive diseases of the brain and the immune system. ^b Controls.

Table 2. Detection of BDV antigens in subjects with various diseases

Reference	Disease	Tissue	Assay	Comments
Bode <i>et al.</i> , 1995 (63)	Psychiatric	PBMC	Flow cytometry	67%
de la Torre <i>et al.</i> , 1996 (171)	Hippocampal sclerosis	Brain	IHC	80%
Deuschle <i>et al.</i> , 1998 (139)	Major depression	CSF	ELISA	9.4%
	Multiple sclerosis	CSF	ELISA	10.5%
Ferszt <i>et al.</i> , 1999 (148)	Depression	PBMC	Flow cytometry	37.2% vs 1.01% ^a
Nakamura <i>et al.</i> , 2000 (175)	Schizophrenia	Brain	IHC	25% vs 0%
Bode <i>et al.</i> , 2001 (50)	Affective disorders	Plasma	ELISA	62% in Berlin and 52% in Hanover for CICs
	Healthy controls	Plasma	ELISA	24% for CICs

Abbreviations used: PBMC=peripheral blood mononuclear cell; CSF=cerebrospinal fluid; IHC=immunohistochemistry; ELISA=enzyme-linked immunosorbent assay; CIC=BDV-specific circulating immune complex.^a Controls.

express BDV antigens (63, 150). This would suggest that about 1% of the PBMC's are infected with BDV. If this is the case, detection of BDV RNA should not require the use of the highly sensitive nested RT-PCR procedure, even one assumes a very low viral load is present per cell. The reasons for this apparent discrepancy remain to be determined. Data from the rat model suggest that BDV is present in a very small fraction of circulating stromal cell precursors, rather than in the monocytes (54). In the human case, there was one report describing the possibility that the PBMC's are not the major targets, and granulocytes are the carriers for BDV (76). The PBMC's could have become infected during passage through the brain, or they may represent primary targets with the ability to spread the infection into the CNS, as proposed for other neurotropic viruses (151). However, intravenous injection of rats with cell-free BDV is not followed by the establishment of a persistent infection in the CNS (152). This argues for the hypothesis that cells within the PBMC's are not the primary targets for BDV.

Using RT-PCR procedures, BDV RNA has been detected in the PBMC's from four out of six hospitalized psychiatric patients (63). This initial study was followed by more comprehensive molecular epidemiological investigations by many groups, as shown in table 3. It has also shown that patients harboring detectable levels of viral RNA in their PBMC's are frequently seronegative for antibodies specific to BDV. Conversely, BDV-seropositive individuals frequently have non-detectable levels of viral RNA (46, 63, 64, 76). This situation is similar to that observed in domestic animals. In sharp contrast, there are also several studies reporting no detection of BDV RNA in the PBMC's from humans (72, 74, 78, 153, 154), or when detected, there was no difference among patients and healthy controls (73). Thus, the data so far obtained by different groups are very contentious. In this regard, some of the latter groups proposed that there is no association between BDV infection and psychiatric patients, and that BDV RNA signals in blood samples by RT-PCR could be derived from artificially contaminated materials during RNA extraction as well as RT-PCR procedures. Nevertheless, many of the data from the former groups showed significantly higher prevalence of BDV infection in patients than those in controls, indicating the possibility that some unknown factor(s) might have affected the results.

6.1.2. Chronic fatigue syndrome

Higher prevalence of serum BDV antibodies and viral RNA in the PBMC's as compared with those from

controls have been detected in blood samples from Japanese patients diagnosed with CFS (66, 68) (table 1). Since various viral antibodies including those reactive to the Epstein Barr (EB) virus have been reported to be high in patients with CFS, the antibody elevations are thought to be caused by nonspecific B-lymphocytic responses (155). However, the elevation of antibodies to BDV seen in CFS might be unrelated to such nonspecific polyclonal B-lymphocytic response, because there was no correlation between the antibody titer to BDV proteins and the antibody titers to the EB virus or human herpesvirus-6 in these patients (68). Further, all five members in each of the two family clusters (four members in one, and three in the other had CFS) were shown to be positive for BDV by serological as well as molecular analyses in follow-up studies (67). In addition, one BDV RNA-positive case of CFS was reported by another group in Austria (82). When retested four months later, during a chronic stage of the disease, no BDV nucleic acid was detected in the PBMC's of this patient. In contrast, several studies employing IFA (156), ECLIA (48), and ELISA as well as WB (79) reported no association between BDV and CFS.

Patients with CFS frequently exhibit neuropsychiatric symptoms, including severe depression. The etiology of CFS is still unknown, but evidence suggests the involvement of viral infection(s) (157). In fact, there have been reports for the association of CFS with several viruses such as enterovirus (158, 159), and herpesvirus such as EB virus (160) and human herpesvirus-6 (161, 162). Fibromyalgia also is a disorder that shares many clinical features with CFS (163). A Danish study failed to detect an association of BDV with fibromyalgia (164). Clinical signs of CFS are quite heterogeneous. Hence, results from the different epidemiological studies could have been affected by the different patient groups tested.

6.1.3. Immunosuppressive disorders

Significantly increased BDV seroprevalences have been documented in patients with human immunodeficiency virus (HIV) infection (13.9% in patients with lymphadenopathy), EB virus acute infection (2.7% in adults and 8.8% in children), and parasitic infections including malaria and schistosomiasis (6.9% in adults and 18.8% in children) (39, 135). In addition, BDV seroprevalence was found to be very high (about 40%) in HIV-1 clade E-infected patients with sexually transmitted diseases (STD) in Thailand, compared to the prevalence found in clade E-infected prostitutes (8.3%), and HIV-1-negative blood donors (1.9%) (165). BDV seropositives were not found among clade B-infected intravenous drug

Table 3. Detection of BDV nucleic acids in subjects with various diseases

Reference	Disease	Tissue	Comments
J Kishi <i>et al.</i> , 1995 (64)	Psychiatric	PBMC	67% vs 0% ^a (0%-3.6% divergence at 'P' gene)
Kishi <i>et al.</i> , 1995 (44)	Psychiatric	PBMC	37%
Sierra-Honigmann <i>et al.</i> , 1995 (170)	Blood donor	PBMC	5%
Kishi <i>et al.</i> , 1996 (181)	Schizophrenia	Brain/CSF/PBMC	0% vs 0%
	Schizophrenia	PBMC	3 positive cases (4.2%-9.3% divergence at 'P' gene)
Sauder <i>et al.</i> , 1996 (46)	Psychiatric	PBMC	42% vs 0% (0%-4% divergence at 'P' gene)
	Affective disorders	PBMC	33% vs 0%
	Schizophrenia	PBMC	64% vs 0%
Bode <i>et al.</i> , 1996 (177)	Psychiatric	PBMC	9% vs 0%
de la Torre <i>et al.</i> , 1996 (178)			(0.07%-0.83% divergence at 'P' gene)
Igata-Yi <i>et al.</i> , 1996 (65)	Affective disorders	PBMC	17% vs 0%
	Schizophrenia	PBMC	10% vs 0%
Nakaya <i>et al.</i> , 1996 (66)	CFS	PBMC	12% (6.0%-14% divergence at 'P' gene)
Kitani <i>et al.</i> , 1996 (68)	CFS	PBMC	12.3% vs 4.7%
de la Torre <i>et al.</i> , 1996 (171)	Hippocampal sclerosis	Brain	80%
Czygan <i>et al.</i> , 1999 (174)			Confirmed the data of de la Torre <i>et al.</i> , 1996 (171)
Kubo <i>et al.</i> , 1997 (72)	Psychiatric	PBMC	2% vs 0%
Richt <i>et al.</i> , 1997 (74)	Psychiatric/Schizophrenia	PBMC	0% vs 0%
Lieb <i>et al.</i> , 1997 (153)	Psychiatric/Schizophrenia	Whole blood/PBMC	0%
Salvatore <i>et al.</i> , 1997 (173)	Schizophrenia	Brain	53%
	Affective disorders	Brain	40%
	Alzheimer's disease	Brain	0%
	Parkinson's disease	Brain	0%
	Multiple sclerosis	Brain	0%
	No neurological control	Brain	0%
Haga <i>et al.</i> , 1997 (172)	Schizophrenia	Brain	33%
	Parkinson's disease	Brain	16.7%
	Healthy control	Brain	6.5%
Haga <i>et al.</i> , 1997 (176)	Healthy control	Brain	6.7%
Planz <i>et al.</i> , 1998 (75)	Psychiatric	PBMC	one positive case
Iwata <i>et al.</i> , 1998 (73)	Schizophrenia	PBMC	4% vs 2% (0%-5.1% divergence at 'P' gene)
Chen <i>et al.</i> , 1999 (77)	Schizophrenia	PBMC	13.5% vs 1.4%
Nakaya <i>et al.</i> , 1999 (67)	CFS	PBMC	86% in 2 family clusters
Plantz <i>et al.</i> , 1999 (76)	Psychiatric (3 patients)	Whole blood	100% vs 0%
		Granulocytes	100% vs 0%
		PBMC	67% vs 0%
Kim <i>et al.</i> , 1999 (78)	Affective disorders	PBMC	0%
Evengard <i>et al.</i> , 1999 (79)	CFS	PBMC	0%
Backmann <i>et al.</i> , 1999 (80)	HIV-positive	PBMC/CSF	0% in patients with various neurological diseases
			0% in patients with AIDS dementia complexes
Czygan <i>et al.</i> , 1999 (174)	Affective disorders	Brain	0%
	Schizophrenia	Brain	0%
Vahlenkamp <i>et al.</i> , 2000 (83)	Psychiatric	PBMC	37% vs 15.4%
Tsuji <i>et al.</i> , 2000 (81)	Schizophrenia	PBMC	1.8% vs 0.6%
Nowotny <i>et al.</i> , 2000 (82)	CFS	PBMC	One positive case (3.8% divergence at 'P' gene)
Wittrop <i>et al.</i> , 2000 (164)	Fibromyalgia	Serum/CSF	0%
Nakamura <i>et al.</i> , 2000 (175)	Schizophrenia	Brain	25% vs 0%

Abbreviation used: CFS=chronic fatigue syndrome; PBMC=peripheral blood mononuclear cell; CSF=cerebrospinal fluid. Controls.

^a

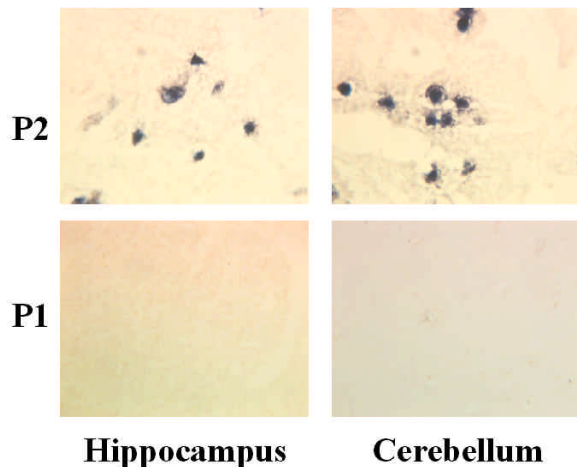


Figure 6. Detection of BDV RNA by ISH in brain tissue from patient P2. Sections from hippocampus and cerebellum from P1 and P2 cases, which were negative and positive for BDV 'P' RNA by RT-PCR, respectively, were subjected to ISH using a BDV 'P' antisense riboprobe. Magnification, x600. Modified from figure 3 in Nakamura *et al.* (175).

users and pregnant women analyzed in the same study (165). HIV-1 clade E-infected patients with STD appear to rapidly progress to AIDS (166, 167), suggesting that clade E may be more virulent than clade B. These findings suggest that strong immunosuppressive conditions, such as infection with clade E, may favor BDV replication. This hypothesis is supported by the finding of a high rate of BDV RNA prevalence in brain tumors from patients with malignant glioblastomas at grade IV (glioblastoma multiforme), but not in patients at grades I to III (168). Glioblastoma multiforme are usually associated with general and severe immunosuppressive states (169). A study by use of ECLIA showed no apparent increase of BDV prevalence in HIV-1-seropositive individuals, although there was no description about HIV clade in the subjects (48). Backmann *et al.* (80) focused on the association with AIDS dementia complex and the results were negative: 12.5% in HIV-positive patients with various neurological disorders versus 8.0% in HIV-seropositive patients with AIDS dementia complex by serological assay. There also was no positive case of BDV RNA in PBMCs and CSF detected by RT-PCR.

6.2. Molecular approach using autopsied brains

Molecular examination of human autopsied brain samples was negative for BDV RNA in one study (170). However, BDV antigen and/or RNA were detected in autopsied brain samples from patients with a history of various mental disorders in five studies (171-175), and in one study in normal controls as well (176). The presence of BDV RNA was first detected in brains having hippocampal sclerosis and astrogliosis (171). Only five of 600 cases from patients with mental disorders were identified as having hippocampal sclerosis and astrogliosis, and BDV antigen and RNA was detected in the brains from four of the five patients by immunohistochemistry (IHC), RT-PCR and ISH. Subsequently, the presence of BDV RNA in three

of the same four brain samples, previously shown to be positive by de la Torre *et al.* (171), was confirmed by Czygan *et al.* (174), although the latter group could not detect any further positive cases in the 86 autopsied brains from patients with various psychiatric diseases [including schizophrenia, affective disorders, and Alzheimer's disease], or from suicide victims or in 52 brains from healthy controls.

Two groups in the USA (173) and Japan (172, 176) examined for the presence of BDV RNA in the limbic system, including the hippocampus, in the autopsied brains from patients or healthy controls by RT-PCR. The results in affective disorders and schizophrenia were high in both groups, while the results in healthy controls were different: no positive case (173) and several positive cases, as well as in patients with Parkinson's disease (172, 176).

A very recent report described the presence of BDV in one of four autopsied brains from schizophrenic patients with a very recent (two years) onset of disease, but no BDV signal was detected in two autopsied brains from healthy individuals (175). In this study, the autopsies were performed within eight to 12 hr of death. Examination to detect BDV RNA by RT-PCR and ISH in a total of 12 different brain regions from the above autopsied brains revealed the presence of BDV in four brain regions [the hippocampus, the cerebellum, the pons, and the temporal lobe of the cerebral cortex] of a BDV-seropositive schizophrenic patient (P2 in figure 6), but not in any brain regions from the other three patients and two normal controls. Several neurons in the hippocampus formation were stained by IHC with a polyclonal serum from a BDV-infected mouse.

Data on BDV infection from autopsied brain samples might be greatly affected by a long post mortem interval before processing for examination. In addition, the duration of disease, frequency of relapses, the clinical course of disease prior to death and autopsy could be very important factors as well. Most of the examinations for BDV were performed on autopsied brains from old patients.

6.3. Isolation of human BDV

An initial report suggested that a BDV-like virus might be isolated from cell cultures inoculated with CSF samples from patients with schizophrenia or acute meningoencephalitis (41). These co-cultures were found to have a few foci of BDV antigens by IFA in initial passages, but the signal disappeared with further passages. A rabbit inoculated with the same CSF sample developed anti-BDV antibodies, although no virus was isolated from this rabbit.

The detection of BDV RNA and antigens in the PBMC's from psychiatric patients prompted studies to investigate the possibility of isolating infectious BDV from such PBMC's. In one study, PBMC's from serial blood samples, collected during acute disease episodes from 32 randomly selected hospitalized psychiatric patients, were analyzed for the expression of BDV antigen and RNA, and used for co-cultivation with a human oligodendroglia cell line (OL cells) (177). BDV was rescued from PBMC's of

three patients. Two of the patients had bipolar disorders, and the third developed an obsessive-compulsive disorder at age 23 and has been hospitalized for the past 14 years. RT-PCR studies showed that the expression of viral antigens correlated with the presence of BDV RNA in the PBMC's (178). Isolation of human BDV required the use of PBMC samples with strong viral antigen expression and at least 11 passages of the initial co-culture (177).

The second BDV isolation from human samples was reported by Plantz *et al.* (76). Infectious BDV could be isolated from the peripheral blood granulocyte fraction of a psychiatric patient. Cells (10^7 cells) in the granulocytic fraction were sonicated and the supernatant was incubated with the guinea-pig cell line, CRL 1405. After the seventh passage, the cell line became persistently infected and the titer of BDV was determined to be $3.9 \log_{10}$ focus-forming units/ml. The third isolation of human BDV was performed with the autopsied brain from a schizophrenic patient (175), patient P2 in figure 6. The BDV RNA-positive brain regions, such as the hippocampus and the cerebellum, from patient P2 were homogenized and intracranially inoculated into Mongolian gerbils that are highly sensitive to BDV (179, 180). Subsequent inoculation of OL cells with homogenates from the BDV RNA-positive gerbil brains allowed the propagation and isolation of human BDV. Virus isolation also was successful by use of Vero cells transfected with the ribonucleoprotein complexes prepared from BDV-positive P2 patient and gerbil brain tissues.

6.4. Sequences variability of BDV in natural animal hosts and humans

BDV exhibits a striking sequence stability. The two horse-derived BDV genomic sequences have more than 95% homology at the nucleotide level (7, 8), which is remarkable for RNA virus isolates with different origin and passage history in animals and cultured cells. Partial sequencing data also suggest high level of sequence conservation among isolates from naturally infected horses of different geographic areas (58), and from different species naturally infected with BDV (57). A maximum nucleotide divergence of 3-4% was observed for the p40 and p24 regions (57, 58).

Analyses of the BDV 'N' and 'P' cDNA sequences amplified by RT-PCR from the PBMC's of psychiatric patients in Hamburg, Germany, revealed a high degree of inpatient and interpatient sequence conservation, as well as a close genetic relationship with animal-derived BDV sequences. Inpatient's divergences at the nucleotide level were 0.2-2.05% at 'N' and 0-1.69% at 'P', whereas the corresponding interpatient's divergences were only slightly higher (46). However, in another study, a significantly higher sequence variability was reported in the human BDV p24 sequences amplified by RT-PCR from the PBMC's of neuropsychiatric patients (181). Analyses of the BDV p24 cDNA clones derived from each of the three schizophrenic patients revealed inpatient's divergences at the nucleotide level of 4.2-7.3%, 4.8-7.3%, and 2.8-7.1%, respectively. Interpatient's divergences at the nucleotide level were 5.9-12.7%. BDV p24 nucleotide sequences derived from the PBMC's of patients differed

from the horse derived sequences strain V and He/80 by 4.2 to 9.3%, respectively. The different experimental procedures used in the RT-PCR analyses (181, 46) might have contributed to these differences between the two studies (46).

Sequencing of human BDV (178) isolated by Bode *et al.* (177) showed striking sequence conservation with known animal BDV strains (178). The human BDV isolate from granulocytes of a psychiatric patient also showed comparable sequence stability, but with slightly higher variability in the 'X' region (76). The BDV isolate from the autopsied brain samples made by Nakamura *et al.* (175) was again genetically closely related to, but distinct from, animal-derived BDV's. Thus, the sequence data do not indicate the presence of a human-specific BDV subtype. The high sequence conservation, together with the difficult procedures required to isolate the virus, however, has raised concerns about possible contamination with laboratory virus strains, and stresses the need for further studies on human BDV infection to confirm these findings (90, 92, 182, 183).

Recently, a novel genotype was reported in BDV derived from a horse in Austria (111). This animal had never been to a region endemic for BDV. The animal's clinical and histopathological picture matched that of classical Borna disease. This virus strain differs from all other known strains by about 15% at the nucleotide level. Interestingly, conservation at the amino acid level were very high (93%-98%) for all viral proteins, except for 'X' that was only about 81% identical to its counterparts in other BDV's. Primers at the 'N' and 'P' regions generally used for epidemiological studies in previous reports did not amplify by RT-PCR the sequences of this isolate. Thus, the results suggest that the data accumulated on BDV sequences showing the high conservation could be due to the initial screening by RT-PCR with such primer sets for BDV-positive subjects. In fact, it has been demonstrated that BDV can undergo surprising changes during passage in animals, while persistently infected cells are resistant to superinfection with BDV. It was hypothesized that the BDV genome is mutating more frequently than estimated from its invariant appearance in persistently infected cultures, and that resistance to superinfection might strongly select against novel variants (182, 184).

In general, viruses isolated from the CNS has extremely low genetic variability, and this applies even to HIV isolated from the brain (185, 186). The reason for this is unclear, but can be multi-facet: the CNS is unique in its response to viral infections, having no specialized lymphatic drainage, potentially limiting and delaying viral antigen recognition; no dendritic cells and the use of microglia for the initial recognition and presentation of viral antigens; and no expression of major histocompatibility complex (MHC) molecules by normal neurons and glial cells (187). Thus, such immune limitations could contribute to the fate of non-cytopathic viral infections in the CNS, as either persistent or latent. In the case with BDV, persistent infection in the CNS owes, in part, to its non-cytolytic replication and the inherent limitations in the immune system of the CNS.

6.5. Possible correlation between BDV infection and human diseases

Epidemiological studies in humans mostly reported on the association between BDV infection and neuropsychiatric disorders including unipolar depression, bipolar disorder and schizophrenia. Also, BDV linkage has been focused on CFS, AIDS encephalopathy, multiple sclerosis, motor neuron disease and brain tumors.

In 1985, IFA by use of BDV-infected cells as antigens showed that sera from a significant proportion of psychiatric patients contained antibodies to BDV (6, 134). In contrast, the serum antibodies to BDV were significantly lower in healthy controls. Thus, it was proposed that BDV infection might be associated with human psychiatric disorders. Table 1 shows data accumulated, by use of various serologic techniques, by many groups in support of this hypothesis. However, others have questioned the association of BDV with human diseases because of the low titers of antibodies to BDV seen in humans as compared with those detected in naturally and experimentally infected animals. One group suggests that the low titer is due to the lower avidity of the human antibodies to BDV, and proposes that such antibodies may not be induced by the virus, but by an antigenically related unknown agent, or by a cellular immunogen that is up-regulated during psychiatric disorders (51).

Molecular epidemiological analyses by use of the highly sensitive nested RT-PCR were first performed in 1995 to detect BDV nucleic acids in the peripheral blood samples from psychiatric patients (63, 64). Data on BDV prevalence in patients with schizophrenia, mood disorders, and CFS has been collected from Austria, Germany, Japan, Korea, Sweden, Taiwan and the USA (table 3). The results yielded very divergent percentages in patients and in controls as well. Several groups also reported no positive case in the human samples studied. Autopsied brain samples from humans also were examined for BDV antigens and RNA. The brain samples from patients with a history of various mental disorders and even from apparently normal controls gave positive signals (table 3). Again, the positive percentages also were variable among the different studies. Taken in total, the accumulated results support a possible association between BDV and human diseases.

There might be several possible explanations for the varying BDV prevalence detected in the human populations studied. With the RT-PCR amplification of the BDV RNA's from the peripheral blood samples, the cell populations examined were different among groups, i.e., whole blood, PBMC's, and granulocytes. Also, the starting blood sample volumes were variable. This might be critical, because the number of BDV-positive cells in blood can be extraordinarily low. In addition, the methods of nucleic acid extraction from the blood samples were different among groups, and this variation can impact the results critically. Of course, there is always a possibility of accidental laboratory BDV contamination of the samples. This seems unlikely, because most groups reported that blood samples from patients have higher BDV prevalence than controls. In

the studies with autopsied brains, the postmortem time for sampling might impact the detection of the BDV signals, especially the viral RNA signals. Also, one of the possible effects on the data is the stage of BDV infection: at acute phase or later stages of the disease.

Generally, BDV replicates slowly and establishes persistent infection. BDV transcription and replication have been characterized in several types of actively dividing cell lines, while the major target cells for BDV *in vivo* are predominantly non-dividing neuronal cells. Therefore, we need more information on the status of BDV in such non-dividing cells, especially in those of human origin. If only 'P' is expressed in the non-dividing normal neuronal cells, as a kind of latency, especially in human brains, it will overcome the major critique (51) that argues against the association of BDV with human diseases, i.e., only 'P' protein, its RNA or antibody are detected predominantly as BDV signals in human samples (51). If BDV latency in brains can be demonstrated, it would explain why BDV infection is difficult to detect before disease onset in the patients, and that periodic reactivation from latency may give positive BDV signals.

6.6. Experimental anti-viral therapies of psychiatric patients

Oral application of amantadine was proposed for the treatment of BDV-related psychiatric disorders (188-191), because *in vitro* experiments from one study reported that amantadine exhibited anti-BDV activity (188). Treatment with amantadine showed clearance of BDV from blood, simultaneously with anti-depressant effects in the patients (188). On the contrary, several groups questioned the anti-BDV effect of amantadine, because subsequent experiments showed that it has no apparent impact against BDV in cell cultures and infected animals (192-194). The guanosine analogue, ribavirin, exhibited inhibitory effect on the transcription of BDV in persistently infected cells (195, 196). A likely mechanism for its activity is the reduction of the intracellular GTP pool, resulting in the inhibition of transcription and capping of BDV mRNA's (196). However, there has been no report on its clinical trial in BDV associated diseases.

A new therapeutic perspective in psychiatry is the filtration of CSF in patients with encephalitis related to BDV (197-199). BDV-specific antigens as well as specific IgG's in the CSF together with slight BDV encephalitis were detected in psychiatric patients, suggesting a slight human Borna disease in these cases (200, 201). The symptoms seen in patients with BDV encephalitis-related schizophrenia (197, 199) and depression (198) improved after CSF filtration. About 250-300 ml CSF were filtered daily over a series of 5 days. As this procedure has been performed only in a limited number of cases, additional clinical trials with larger sample sizes are needed to determine its efficacy.

7. BDV-INDUCED PATHOGENESIS IN MODEL SYSTEMS

In this section, we will introduce several results obtained from *in vitro* and *in vivo* model systems. The

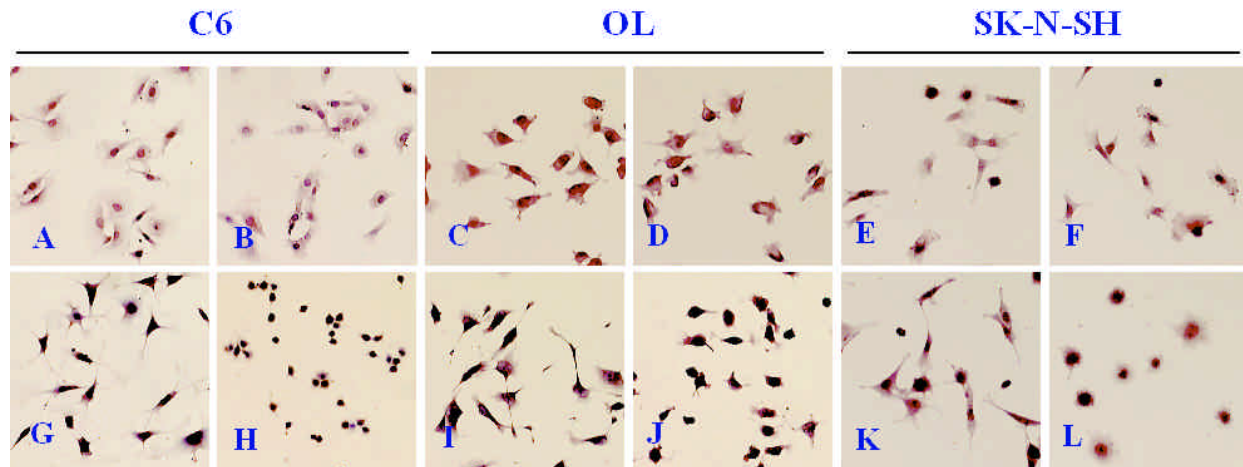


Figure 7. BDV infection inhibits neurite outgrowth of neural cells. Persistently BDV-infected (panels B, D, F, H, J, and L) and uninfected (panels A, C, E, G, I, and K) C6, OL, and SK-N-SH cells were grown under normal conditions (panels A to F) or on plates coated with laminin (2.5 μ g/ml) (panels G to L). The cells were cultured for 3 to 4 hr and were then fixed and briefly stained with hematoxylin. Modified from figure 4 in Kamitani *et al.* (203).

information may help to elucidate BDV pathogenesis in the CNS and provides insight on the possible mechanisms implicated in some human CNS disorders. This information when taken together with those discussed in the epidemiological studies may help to clarify if BDV is associated with human disorders.

7.1. *In vitro* systems

A wide range of host cell types and species can be infected with BDV that usually replicates *in vitro* without cell lysis, and establishes persistently infected cells chronically producing the virus. BDV also is tightly cell-associated. Although BDV infects cells easily through cell-to-cell interaction, the infectivity titers of cell-free virus in the culture medium or even in the cell lysate are usually very low. Interestingly, BDV replication rates are different among cell types. As previously shown, more BDV is produced by neuronal cell lines than by astrocytic cell lines (202). In addition, nerve growth factor treatment can enhance virus production (202).

Recent study has demonstrated that the BDV 'P' protein binds specifically with the host cellular protein, amphoterin (203). The 'P' protein is a nucleus-associated phosphoprotein and is putatively a cofactor for the BDV polymerase during replication and transcription (204, 205). Being one of the abundantly expressed viral proteins in infected cells, it is possible that the binding of 'P' to host factor(s) would induce functional alterations in the infected neural cell environment. Amphoterin, a high-mobility group-1 (HMG-1) protein, is a neurite outgrowth factor of 30-kDa that is present in abundance in developing brains. BDV infection, as well as the purified 'P' protein in culture medium, can significantly inhibit cell process outgrowth of cells maintained on laminin (figure 7). Furthermore, migration activity of the cells to laminin was also decreased by BDV infection. These results suggest that BDV infection causes a functional disturbance of amphoterin in cells by the interaction of the 'P' protein. This functional disturbance may result in

neurodevelopmental damage in the developing brain, as reported in BDV-infected neonatal rats. Thus, the interaction of amphoterin with BDV 'P' may similarly occur in the developing brains of the persistently BDV-infected humans. However, there has been no report on the neonatal infection or the possible vertical transmission of BDV in humans.

7.2. *In vivo* systems

Pathological findings in naturally infected horses and sheep are similar to those in experimentally infected animals such as rats. Immunocompetent adult rats infected with BDV develop severe encephalitis and neural dysfunction because of anti-BDV immunopathogenesis. Initial immune cell infiltrates in the perivascular spaces are CD8-positive and CD4-positive T cells, NK cells, and macrophages (206-208). The encephalitic reactions in the CNS correlate to strong immune responses, especially CD8-positive T-cell-mediated immune responses against the 'N' proteins (209-211). Also, experimentally infected rats have a hyperactive movement disorder because of abnormal dopamine activity (212, 213).

Some animals such as adult black-hooded rats and adult BALB/c and SJL mice have limited susceptibility to BDV-induced encephalitis (214, 215). However, such animals exhibit significant behavioral abnormalities. Similar behavioral abnormalities are also seen in experimentally BDV-infected animals such as newborn rats, adult rats with suppressed immune systems due to thymectomy or administration of immunosuppressive drugs, and in certain species or strains of animals (216).

Neonatal rats infected with BDV develop persistent infection and show developmental disturbances affecting specific areas in the brain (217-221). Neonatal BDV infection also results in a variety of behavioral abnormalities and neuroanatomical disturbances without generalized meningitis or encephalitis (219, 220, 222, 223). A chronic upregulation of proinflammatory cytokines such

as IL-1 beta has been observed in the hippocampus and cerebellum of the neonatally infected rats (224, 225). Recent studies have demonstrated that neonatal BDV infection directly alters the concentrations of neurotransmitters in the brain, including norepinephrine and serotonin (226). Furthermore, BDV infection displays a progressive decrease in synaptic density and plasticity, especially in the cortex and hippocampus, which precedes a significant dropout of the cortical neurons in the infected rats (227). Reduced mRNA expression levels of neurotrophin-3, brain-derived neurotrophic factor and nerve growth factor are found in the hippocampus of newborn infected rats (228). These observations indicate that BDV infection has direct effects on the microenvironment of neuronal cells in the infected brain.

8. CONCLUDING REMARKS

Accumulated evidence indicate that the host range and geographic distribution of BDV are widespread, including a significant number of asymptomatic cases in healthy domestic animals and possibly humans. BDV gene products and antibodies can be readily detected in the CNS and serum, respectively, of experimentally infected animals and naturally occurring cases of Borna disease in animals. In contrast, expression of BDV in humans appears to be highly restricted. It is possible that factors, such as strong immunosuppression could provide an environment that facilitates increased expression levels of BDV. Alterations in lymphocyte function, elevated membrane glycoconjugate (Le^x), and altered natural killer activities have been reported in schizophrenic (229, 230) and CFS (231) patients, respectively. It remains unknown to what extent these immune dysfunctions may contribute to the higher prevalence of BDV in these patients. Stress, a common finding in neuropsychiatric patients, can also contribute to immunosuppression by altering glucocorticoid levels (232). This, in turn, could enhance susceptibility to infectious agents, including BDV.

BDV RNA is not always detected in the PBMC's from BDV-seropositive individuals and *vice versa* (44, 46, 61, 64, 66, 76, 84, 85, 88, 175). Seropositive but RNA-negative cases may represent BDV infection that has been cleared by the host immune responses. Conversely, detection of BDV RNA in the absence of viral antibodies could reflect a recent infection with a delayed humoral response. Follow-up studies of these cases monitoring the presence of BDV RNA in PBMC's and antibodies to BDV in serum will indicate whether seroconversion occurs at later times, and how it relates to virus load in the PBMC's. In fact, follow-up studies often observed such phenomenon (63, 67, 76, 82). Immune complexes, frequently formed during virus persistent infections, may also mask the detection of serum antibodies to BDV, as recently shown by CIC's (50).

The identification of the virus source and routes of BDV infection in humans are germane issues to the potential role of BDV in neuropsychiatric disorders. Accumulated evidence suggests that the nose is a main site of virus entry in animals (114). Sporadic evidence suggests the possibility of transmission from BDV-infected animals to humans (71, 137, 233). The close genetic relationship

between BDV sequences derived from human and animal strains supports this hypothesis. Recently, vertical transmission of BDV has been demonstrated in a pregnant mare (234). Her brain, with multiple neuronal degeneration, necrosis and hemorrhage, and the histologically normal brain of the fetus were both positive for BDV RNA by ISH. Further, BDV RNA and proteins were detected in placenta samples from 3 mares infected with BDV, encouraging the possibility of vertical transmission (Okamoto *et al.*, personal communication). On the other hand, studies on BDV infection in wild rodents did not indicate natural infection (119, 235). Thus, the significance of rodents as a reservoir for natural animal hosts as well as for humans remains inquisitive. Recently, wild birds were proposed to be natural reservoirs of BDV (133).

Many questions arise needing exact answers. For example, how could the same infectious agent play a role in the complex CNS disorders in these different species? How the same infectious agent could play a role in these different and complex CNS disorders? For the former question, we must wait for the answer from more extensive studies with more samples from humans as well as naturally infected animals, including subclinical cases. One possible explanation for the latter question is the location of BDV persistent infection in the CNS. Different locations for BDV infection could affect the subsequent clinical manifestations. The differences in the transmission route may affect the persistent infection site of BDV. Another point is the involvement of immune responses to BDV in the pathogenicity in the CNS. The BDV 'N' protein, as well as 'P' protein, is the first viral specific antigen expressed early after infection as the predominant viral proteins in the infected cells. In addition, 'N' is the major target for both humoral as well as cell-mediated immune responses in horses naturally infected with BDV (209, 210). According to the data obtained from experimentally infected rats (211), such immune responses against 'N' are related to the pathogenicity in the CNS (111). Also, the MHC class I-restricted recognition of BDV 'N' protein may represent the key event in the different pathogenicity in naturally infected animals: immunopathology of Borna disease development or immunoprotection (211). Variability in cytokine expression in the CNS over the course of Borna disease may also be important for the modulation of the immune response (236). Also, the timing of initial BDV infection in naturally infected animals could affect the subsequent differences in the disease, because BDV infection could give different effects in mature and immature neuronal cells in the CNS. The presence of subclinical cases in naturally infected animals may suggest the importance of the mechanism for persistent infection in non-dividing neuronal cells. The characterization of this point may lead to the understanding of the difference in virulence in different animals, which may be related to certain viral factor(s).

Recent finding of the association between BDV 'P' protein and amphotericin (203) may suggest that BDV persistent infection could induce abnormal neuronal function in the CNS, even in the absence of immunopathological brain damage. In addition, major

human mental disorders likely involve a whole concert of genes, with varying effectiveness among individuals, and interacting with exogenous factors to generate a variety of clinical phenotypes. BDV persistence in the CNS may represent an exogenous factor contributing to these disorders. Viral variants, even single amino acid substitutions, may exhibit remarkably different disease phenotypes in the same host. Similarly, genetic differences among individuals play a critical role in the outcome of their interaction with infectious agents. Thus, depending on the individual's particular hereditary vulnerability and the properties of specific viral variants, BDV infection could have diverse clinical consequences, manifested after a variable period of incubation, and thus contribute to distinct types of mental disorders. This hypothesis is supported by the observation that BDV-infected animals display a heterogeneous range of neurobehavioral alterations that are associated with diverse neuropathological findings. Thus, variable symptomatology and pathological manifestations observed in BDV-infected animals could be influenced by both host and viral factors. Recently, the polymorphisms of host genes such as the HIV coreceptor and cytokine were shown to significantly affect AIDS pathogenicity (237-239). Thus, studies on the genetic polymorphisms in patients with neuropsychiatric disorders may help to understand the mechanism for the possible role, if any, of BDV pathogenesis in the CNS. Several studies have already been reported in patients with Schizophrenia: association of the IL-1 beta genetic polymorphism (240) and association of the genetic polymorphisms at the dopamine receptor, the serotonin receptor and neurotrophic factors, such as the brain-derived neurotrophic factor (241).

The molecular analysis of human biological samples to detect BDV poses considerable technical difficulties. Comprehensive molecular epidemiological studies aimed at determining the prevalence of BDV in neuropsychiatric patients from different geographic areas will help to evaluate the significance of BDV as a potential human pathogen. These studies will require the use of standardized methods for the detection of viral antibodies and RNA in human biological samples, as well as uniform criteria for the clinical diagnoses.

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Abbreviations: BDV:Borna disease virus; NNS: nonsegmented, negative-, single-stranded; ORF: open reading frame; NES:nuclear localization signal; NES: nuclear export signal; ISH:*in situ* hybridization; CSF: cerebrospinal fluid; IFA:immunofluorescence assay; MAb:monoclonal antibody; WB:Western blotting; RK:rabbit kidney; ELISA:enzyme-linked immunosorbent assay; RT-ELISA:reverse-type ELISA; ECLIA: electrochemiluminescence immunoassay; CIC:circulating immune complex; RT-PCR:reverse transcriptase-polymerase chain reaction; PBMC:peripheral blood mononuclear cell; CNS:central nervous system; CFS: chronic fatigue syndrome; TCPR:T-cell proliferative response; MRI:magnetic resonance imaging; EB: Epstein Barr; HIV:human immunodeficiency virus; STD :sexually transmitted disease; IHC:immunohistochemistry; MHC:major histocompatibility complex; HMG:high-mobility group

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