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Borna disease virus: implications for human neuropsychiatric illness

W. Ian Lipkin, Anette Schneemann and Marylou V. Solbrig

The cause of Borna disease, a neurological syndrome affecting mammals and birds, has recently been shown to be infection with an RNA virus. Molecular genetic analysis suggests that Borna disease virus represents a new viral taxon. It has a wide host range and is tropic for specific circuits in the central nervous system. There is indirect evidence that links it to diseases of the human central nervous system.

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Borna disease (BD) is a unique neurological syndrome caused by infection with an unclassified RNA virus[1]. It was first described approximately 200 years ago in horses in southeastern Germany as a syndrome of agitated aggressive behavior that progressed over a period of weeks to inanition and death[2]. Although horses in Germany account for most recognized clinical cases of BD, natural infection has also been reported in sheep, cattle and cats in other parts of Europe as well as in North America and Asia[3–7]. In 1929, Zwick prepared a filtered homogenate from the brain of a horse with BD that induced disease in rabbits after intracerebral inoculation[8]. Since that time, investigators have used such homogenates experimentally to infect a wide range of vertebrate hosts including chickens, mice, rats, rabbits, tree shrews and rhesus monkeys. In each species, infection produced prominent disturbances in behavior[3].

A wide variety of cell-culture systems have been established for propagation of virus in the laboratory. In general, only low levels of Borna disease virus (BDV) are produced in cultured cells, with those of neural crest origin being the most productive (1–10 infectious units per cell)[9–11]. Yields of virus from infected animals are similarly low; titers of 10% brain homogenates from experimentally infected rats rarely exceed 10^6 focus-forming units per ml (Ref. 12). These features, coupled with the fact that the virus is cell associated, have hindered its purification and characterization. Viral nucleic acids were isolated only recently by subtractive cDNA cloning[13,14]. Molecular probes facilitated the purification of viral particles[11] and, subsequently, cloning of the viral genome[15,16].

Naturally and experimentally infected animals have antibodies to viral proteins in sera and cerebrospinal fluid (CSF)[3]. Two viral proteins of 40 kDa and 23 kDa form a soluble (S-antigen) complex that accumulates in the nucleus of infected cells[17]. In experimentally infected rats, serum antibodies to the 40 kDa and 23 kDa proteins can be detected before the onset of the clinical syndrome, rapidly reach peak titers and remain elevated throughout the course of disease[18,19]. Neutralizing antibodies have been identified in sera and CSF from naturally and experimentally infected animals[20–22]. This neutralizing activity is due at least in part to antibodies directed against an 18 kDa viral protein[23,24]. In sera from experimentally infected rats, neutralizing antibodies are detected approximately 4 weeks after the

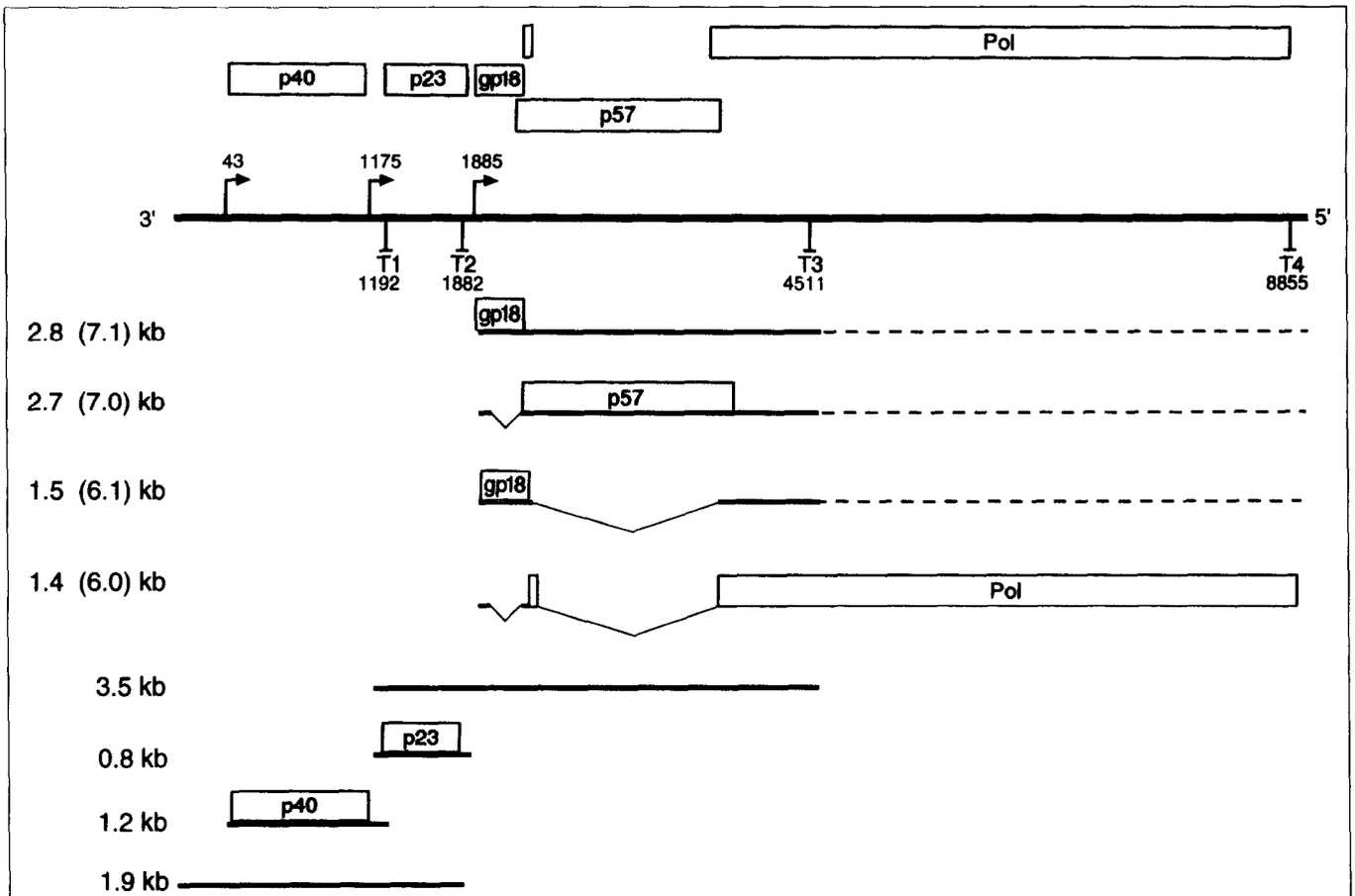

Fig. 1. Organization and transcriptional map of the Borna disease virus (BDV) genome. The 8.9 kb genome, shown in the 3′ to 5′ direction, contains antisense information for five open reading frames (ORFs). ORFs for p40, p23, gp18 and Pol are in the same frame, whereas the ORF for p57 is in the +1/−2 frame relative to the others. Transcriptional initiation sites and their nucleotide position on the genome of BDV strain V are indicated by arrows pointing downstream; transcriptional termination sites are indicated by vertical bars. Location of viral transcripts relative to the genome is shown below. Stippled extension of the 2.8 kb RNA and its splice products indicates readthrough at termination site T3, which results in the synthesis of the longer RNAs. The 1.2 kb RNA and the 0.8 kb RNA have been shown to serve as messages for p40 and p23, respectively[1]. Transcripts predicted to serve as mRNAs for gp18, p57 and Pol are shown[30,31]. It is not known whether the 1.9 kb RNA and the 3.5 kb RNA, both of which represent transcriptional readthrough products, serve as mRNAs for the translation of BDV proteins.

appearance of antibodies directed against the S-antigen[24]. The role of neutralizing antibodies in BD is unclear given that viral titers in brain remain elevated in the presence of neutralizing antibodies in serum and CSF.

Until recently, ante-mortem diagnosis of BD rested on detection of serum antibodies to the S-antigen by immunohistochemistry or western blot. Confirmation of disease required detection of S-antigens in brain and the transmission of infection to animals or cultured cells. These methods have largely been replaced by the use of virus-specific nucleic acid probes for northern hybridization or reverse transcriptase–polymerase chain reaction (RT–PCR)[25]. Furthermore, primary screening for evidence of infection can now be performed by using recombinant viral proteins in enzyme-linked immunosorbent assays (ELISAs)[19].

Biology of the agent

The genome organization and transcription strategy of BDV suggest that it represents a new taxon within the order of nonsegmented, negative-strand RNA viruses (*Mononegavirales*)[15,16]. BDV was considered likely to be an enveloped virus because infectivity is reduced after exposure to organic solvents[26], however, formal proof of this hypothesis came only recently with the identification of 90 nm, enveloped, spherical infectious particles[27]. The 8.9 kb viral antigenome contains five open reading frames (ORFs) (Fig. 1)[15,16]. In the 5′ to 3′ antigenomic orientation, the ORFs encode proteins predicted to correspond to the nucleoprotein (p40), phosphoprotein (p23), matrix protein (gp18), glycoprotein (p57) and L polymerase (Pol) found in nonsegmented negative-strand RNA viruses. To date, only p40, p23 and gp18 have been detected in infected cells and tissues.

Transcription and replication of the BDV genome occur in the cell nucleus[11,28]. The only other negative-strand RNA animal virus known to transcribe in the cell nucleus is influenza virus, which contains a segmented genome. The transcriptional mechanism of influenza virus is unusual in that cellular mRNAs are required to initiate transcription of the viral genes. This is caused by the inability of the influenza RNA transcriptase to prime RNA synthesis and modify the 5′ termini by capping and methylation[29]. In contrast, BDV transcriptional initiation is independent of cellular

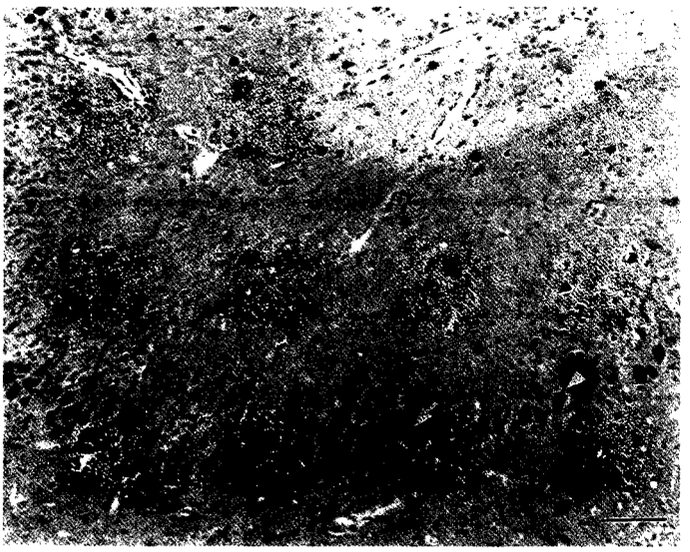

Fig. 2. Borna disease virus (BDV) proteins in CA3/4 pyramidal neurons of hippocampus. The figure is a section from Lewis rat brain showing viral antigens in the nucleus and cytoplasm of a hippocampal pyramidal neuron (arrow). The arrowhead indicates an uninfected neuron. The primary antibody used was polyclonal rabbit anti-BDV serum. Stained with hematoxylin. Scale bar = 62.5 μm. (Courtesy of S.A. Rubin and K.M. Carbone, Johns Hopkins University School of Medicine.)

mRNAs[30]. It is not known yet whether enzymatic activities required for capping and methylation reside within the BDV transcriptase or whether cellular enzymes are recruited for this purpose. Both influenza virus and BDV subgenomic RNAs are post-transcriptionally modified by RNA splicing, which is presumably carried out by the host splicing machinery[29–31].

Regulation of BDV gene expression is complex. The levels of viral subgenomic RNAs and the corresponding protein products decline with the distance between the transcribed gene and the 5′ end of the antigenome[15,16]. Whereas p40 and p23 are translated from separate transcription units, gp18, p57 and Pol are expressed from a single transcription unit by alternative splicing of two introns from primary RNA transcripts and differential use of two transcriptional termination sites (Fig. 1)[30,31]. Such a multilevel system for regulation of viral gene expression could account for such key features of BDV biology as low-level production of infectious virus and persistence in cells of the central nervous system (CNS)[30].

Neurobiology of BD

BD has been extensively studied in experimentally infected adult rats. These animals have an immune-mediated disease that presents clinically as hyperactivity and exaggerated startle responses 10–14 d after intracerebral infection[32]. The acute phase coincides with the appearance of viral proteins in limbic-system neurons (Fig. 2) and infiltration of mononuclear cells into the brain (Fig. 3)[12]. After 2–3 weeks, rats also show stereotyped motor behaviors (the continuous repetition of behavioral elements), dyskinesias, dystonia and flexed seated postures[33]. The extrapyramidal-movement and behavior disorder has been linked to distinct changes in CNS dopamine systems. Infected rats are more sensitive to dopamine (DA) agonists than are normal rats; their movement and behavior disorder is improved following treatment with selective DA antagonists. Neurochemical analysis revealed partial DA depletion and compensatory metabolic hyperactivity in nigrostriatal and mesolimbic DA systems. Quantitative receptor autoradiography revealed reduction of one class of striatal dopamine receptors[33]. Later in the disease, many animals become blind due to an inflammatory retinopathy[18]; 5–10% become obese, achieving body weights of up to threefold normal[3]. Although viral nucleic acids have occasionally been detected in peripheral blood mononuclear cells of persistently infected rats[34] and naturally infected horses (F. Steinbach and H. Ludwig, unpublished), it is unlikely that the blood is a major route for CNS infection. BDV is exquisitely neurotropic[9,12]; following limb inoculation, the virus travels trans-synaptically and centripetally into the CNS. Although the onset of disease is delayed after peripheral infection, the course is otherwise unchanged[12]. Animals infected as neonates do not mount a cellular immune response to the virus (Fig. 3b) and have a different disease, characterized by hyperactivity and subtle learning disturbances[35,36].

Species differences in BD

Disease in naturally infected horses or sheep and experimental infection in rabbits or birds is usually fatal. In contrast, inoculation of rats and primates may lead to persistent infection and a normal lifespan[3]. Chronically infected rats appear inactive and prematurely senescent. In tree shrews (*Tupaia glis*), BDV causes disturbed social and sexual behaviors, which are manifested as abnormal dominance relationships and failure to reproduce[37]. Experimentally infected rhesus monkeys are initially hyperactive and easily distracted, but become apathetic and hypokinetic in the latter phase of the disease[38]. An epidemic infectious meningoencephalitis attributed to BDV was recently described in ostriches in Israel[39]. This epidemic generated concern because of the apparent broad jump in both the species and geographical range of the virus. Unlike BD in mammals, the ostrich disease is focused in the spinal cord and presents as hindlimb paralysis. Although antibodies from these birds react with BDV proteins and, conversely, sera from BDV-infected animals bind to extracts from infected birds, neither infectious BDV nor BDV nucleic acids have been isolated from the ostriches. Therefore, additional experiments will be required to determine whether ostriches are infected with BDV or a similar agent.

BD in humans

Recognition of the broad experimental host range of BDV has led to the proposal that it might cause human neuropsychiatric disease. Because the behavioral disturbances in the rat model were considered to be reminiscent of affective disorders, such as bipolar and monopolar depression, the initial question was whether patients with these disorders might be infected with BDV.

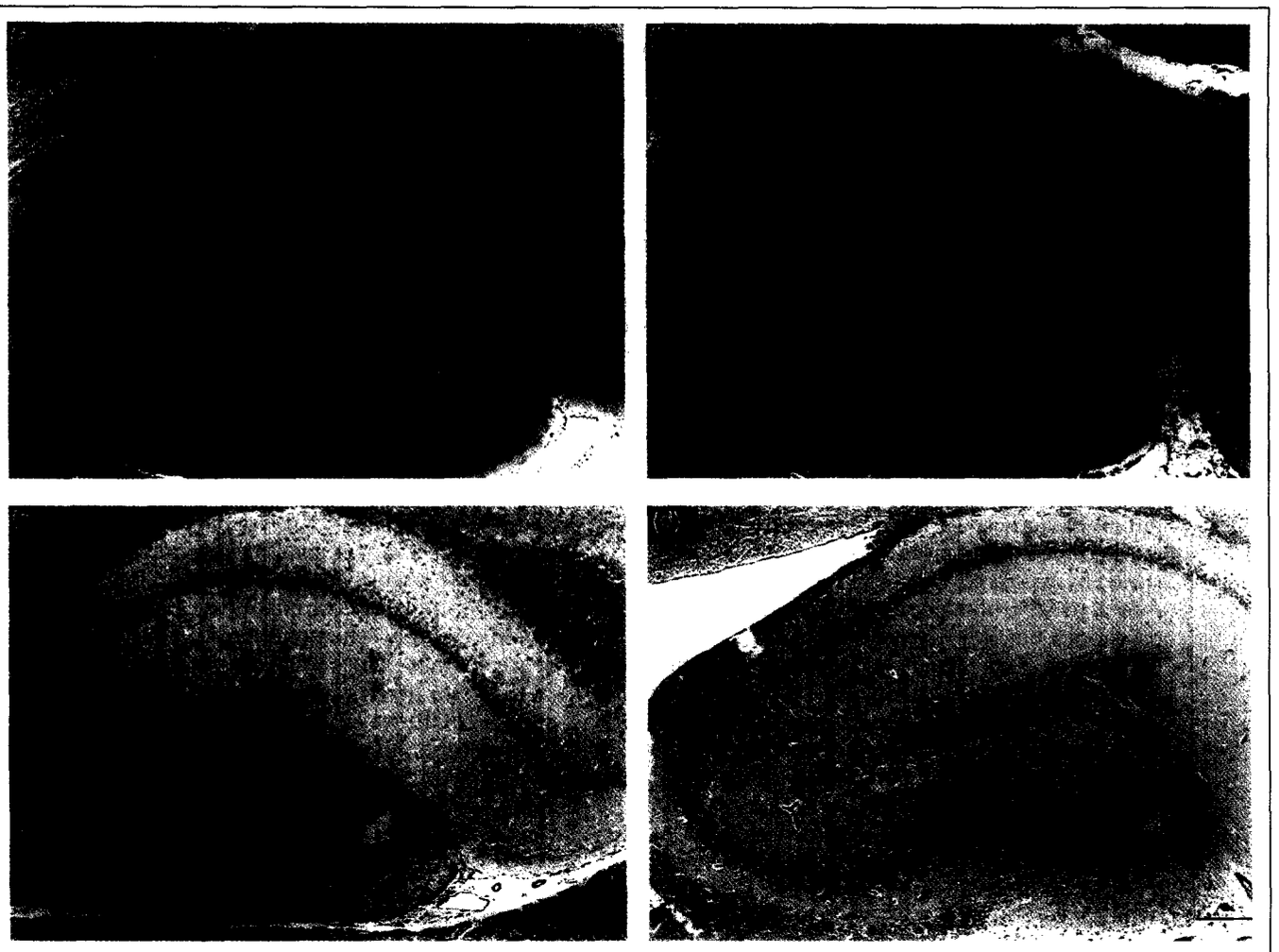

Fig. 3. Sections through the hippocampus of **(a)** uninfected, **(b)** neonatally infected and **(c,d)** adult-infected Lewis rats. **(b)** Rat was infected on postnatal day 1 and killed 60 d after infection. Note the loss of dentate gyrus (arrow) and the absence of inflammation. **(c)** Rat was infected at 5 weeks old and killed 5 weeks after infection. Note the early dissolution of the dentate gyrus (arrow) and the severe inflammation (arrowhead). **(d)** Rat was infected at 5 weeks old and killed 12 weeks after infection. Note the destruction of the dentate gyrus (arrow) and the resolution of inflammation (arrowhead). Stained with hematoxylin and eosin. Scale bar = 0.25 mm and applies to all parts. (Courtesy of S.A. Rubin and K.M. Carbone, Johns Hopkins University School of Medicine.)

Using an indirect immunofluorescence assay on infected cells (immunofocus test; IFT), Rott *et al.*[40] examined sera from approximately 1000 patients with affective disorders and 200 normal control subjects in Germany and the USA for reactivity with viral antigens. Antibodies to viral proteins were detected in 16 patients (1.6% prevalence). Unexpectedly, 12 positive sera were from the USA, where the virus was not known to cause disease in horses and sheep, its natural hosts[40]. A follow-up IFT study of 5000 patients with neurological and psychiatric disease and 1000 normal controls from Germany, the USA and Japan identified 4–7% of patients as seropositive versus 1% of controls. CSF from three seropositive patients was tested for the presence of infectious virus by application to embryonic rabbit brain cells or by intracerebral inoculation into rabbits. Cultured cells transiently expressed proteins that reacted with monoclonal antibodies to BDV proteins. Although no rabbits showed signs of disease (an atypical course in this host, for which infection is usually fatal), all produced antibodies to BDV (Ref. 41). At present, this is the only published report suggesting that BDV has been recovered from human subjects. Using western blot analysis, which allows determination of the specific proteins detected by sera, Fu *et al.*[42] examined sera from a cohort of 138 affective-disorder patients and 117 healthy controls for immunoreactivity to the putative N protein (p40), the phosphoprotein (p23) or both. Antibodies to p40 were detected in 38% of patients versus 16% of controls; to p23 in 12% of patients versus 4% of controls and to both proteins in 6.5% of patients versus 1% of controls[42].

Bode *et al.* studied sera from 3000 European, North American and African adults and children for antibodies to BDV by IFT and immunoprecipitation of the S-antigen complex[43]. Subjects had a wide range of diseases including psychosis, multiple sclerosis (MS), Epstein–Barr-virus (EBV) infection, asymptomatic or symptomatic HIV infection, malaria and schistosomiasis. Approximately 2% of healthy individuals or subjects with psychiatric diseases had antibodies to BDV. In contrast, 13–14% of patients with MS or

HIV-associated lymphadenopathy were immunoreactive to BDV. Antibodies to BDV were found in children with various infectious diseases: 5.6% of children with EBV, 8.3% of children with either malaria or schistosomiasis, and 18.8% of children with both malaria and schistosomiasis. Immunoprecipitation studies indicated that p40 was the antigen detected by sera in IFT. These findings led the authors to propose that BDV may be acquired early in life, latently infect human populations worldwide and reactivate during immune challenge or in association with CNS disease. With the objective of establishing a correlation between immunoreactivity to BDV and the duration and severity of psychiatric disease, serial IFT was performed on sera from 70 patients with a variety of diagnoses including minor or major depression, paranoid psychosis, schizophrenia, anxiety disorder and personality disorder. Overall, the prevalence of immunoreactivity to BDV was greater than 20%, a marked increase from the 2–4% found in the earlier study by assay of each subject at one time point. A total of 37% of major depressives, 25% of paranoid psychotics, but only 6% or less of patients with reactive depression and other neurotic conditions were seropositive by day 17 of illness[44]. This cohort of patients was next examined for the presence of viral antigens by fluorescence-activated cell sorting (FACS) analysis of peripheral blood monocytes using antibodies to p40 and p23. Of these subjects, 40–50% were found to be antigen carriers, twice the proportion predicted by the previous serological survey[45].

In addition to considering affective disorders, some investigators have focused on potential associations between BDV and schizophrenia. Waltrip *et al.* examined the prevalence of antibodies to p40, p23 and gp18 (alternatively described as 14.5 kDa) in 90 schizophrenic subjects and 20 controls by western blot analysis[46]. Over 14% of patients and no controls had antibodies to two or more viral proteins. This definition of immunoreactivity was significantly associated with abnormal brain morphology in magnetic resonance image analysis (MRI) and the clinical diagnosis of deficit syndrome, a schizophrenia subgroup characterized by social withdrawal, neurological dysfunction and neuroanatomical abnormalities. These findings are consistent with a report from Bechter *et al.* that, in psychiatric patients, there is a correlation between the presence of antibodies to BDV and MRI evidence of cerebral atrophy[47].

In summary, BDV has been suggested to cause two psychiatric diseases in human subjects, affective disorders and schizophrenia. There is no general consensus as to which of these diagnostic categories is more likely to contain infected subjects. Although there is experimental evidence that the virus can infect primates, only indirect or preliminary data support a role for BDV in human disease. Schizophrenia, a syndrome of probable mesolimbic DA hyperactivity, is neurochemically more similar to the disease observed in BDV-infected animals than either bipolar depression or monopolar depression.

Three other human CNS diseases of probable infectious origin even more closely resemble natural and experimental BD. Viliuisk encephalomyelitis (VE), affecting the Iakut people of Siberia, is an encephalitic illness with psychiatric or intellectual sequelae, movement and posture disorders, and obesity[48]. Bojinov's encephalitis (BE) is a sporadic encephalitis causing transient psychosis, intellectual dysfunction and movement disorders that was described in Bulgaria in the 1960s by Bojinov[49]. Encephalitis lethargica (EL), reported by von Economo in 1917, caused a parkinsonian syndrome with behavioral disturbances and movement disorders similar to those found in Borna disease[50]. However, unlike VE and BE, EL was pandemic and affected thousands of people worldwide. Because VE and BE are rare diseases, and EL occurred before the advent of modern virology, it has been difficult to assess the role of BDV in these diseases.

Conclusion

BD is an exciting new viral system with unique biology, a wide and perhaps expanding host range, and tropism for specific circuits in the CNS. Although it is likely that BDV could infect humans to cause a disease that would resemble schizophrenia, there is no firm evidence yet that it does so. With the advent of sensitive methods for detecting viral footprints, such as ELISA and RT–PCR, we anticipate that the epidemiology of BDV infection in human and animal populations will be resolved rapidly. Regardless of the outcome, this agent will provide insight into the evolution of RNA viruses, as well as potent models for basic and clinical neuroscience.

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Tissue-culture invasion: fact or artefact?

Virginia L. Miller

Although widely used, tissue-culture assays cannot be exact models of the conditions that are met *in vivo* by pathogenic bacteria. However, recent studies of specific mutants suggest that the model is good for highly invasive bacteria, but it remains to be seen if this is true for weakly invasive bacteria.

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In vitro assays using cultured eukaryotic cells to study entry/invasion, survival and multiplication of pathogenic bacteria have been used for nearly half a century[1], but their use has been even more intensive during the past 15 years. These assays are used not only to study obligate intracellular pathogens, such as *Chlamydia* and *Rickettsia*, but also to study facultative intracellular pathogens, such as *Shigella*, *Listeria*, *Yersinia*, *Mycobacterium tuberculosis* and *Salmonella*. For the purpose of this article, I use the term 'facultative intracellular' pathogen to include all pathogens that have an intracellular phase at any step during the infectious process. Thus, this is perhaps a good time to step back and ask – just how well do these assays reflect events that happen during infection *in vivo*?

The basic tissue-culture invasion assay consists of variations on the following protocol. Subconfluent monolayers of the target cells are grown in microtitre dishes. The bacteria to be tested are then added and allowed to incubate with the eukaryotic cells for between a few minutes and several hours. In some cases, a brief, gentle centrifugation step is included to promote contact between the bacteria and the cell surface. After incubation, the monolayers are washed thoroughly to remove non-adherent bacteria. At this point, fresh tissue-culture medium containing an agent that preferentially kills the extracellular bacteria is added; the antibiotic gentamicin is most commonly used. The monolayer is then washed again to remove the antibiotic. The eukaryotic cells are then lysed and plated for viable bacterial counts, which represent intracellular bacteria. Alternatively, staining protocols or optics that differentiate between extracellular and intracellular bacteria can be used, but these techniques are more difficult to quantify. Tissue-culture invasion assays are sufficiently simple and quantitative to be used to test the effect of potential inhibitors, such as cytochalasin D, which disrupts microfilaments,