ELSEVIER

Virus Research 44 (1996) 33–44

Virus Research

Sequence characterization of human Borna disease virus

Juan Carlos de la Torre[a,*], Liv Bode[b], Ralf Dürrwald[c], Beatrice Cubitt[a], Hanns Ludwig[c]

[a] *Viral-Immunology laboratory, The Scripps Research Institute, 92037 CA, USA*
[b] *Department of Virology, Robert Koch-Institut, 13353 Berlin, Germany*
[c] *Institute of Virology, Free University of Berlin, 14195 Berlin, Germany*

Received 19 November 1995; accepted 29 April 1996

Abstract

Borna disease virus (BDV) causes a central nervous system disease in several vertebrate animal species, which is manifest by behavioral abnormalities. Seroepidemiologic data suggest that BDV might infect humans, possibly being associated with certain mental disorders. This is further supported by the detection of both BDV-specific antigens and RNA sequences in peripheral blood mononuclear cells (PBMCs) of psychiatric patients. For the first time the sequence characterization of human BDV is documented here. BDV was recovered by co-cultivation techniques from the PBMCs of three hospitalized psychiatric patients. BDV was unequivocally identified based on sequence identification of BDV open reading frames (ORFs) p24, p16 and p56, as well as of the predicted catalytic domain of the BDV L polymerase. Each human BDV isolate had an unique sequence, but they displayed a high degree of sequence conservation with respect to BDV isolates from naturally infected animals of different species.

Keywords: Human Borna disease virus; Three BDV genes; Peripheral blood mononuclear cells

1. Introduction

Evidence indicates that in addition to a genetic contribution, environmental determinants also play a role in the etiology of psychiatric disorders including schizophrenia and depression (Morozov, 1983). The hypothesis of a viral contribution is suggested by the realization that viruses can induce progressive neurological disorders associated with diverse pathological findings (Morozov, 1983; Kurstak et al., 1987; ter Meulen, 1991).

Borna disease virus (BDV) is a noncytolytic neurotropic virus that infects a wide range of vertebrate species from birds and rodents to pri-

* Corresponding author. Mailing address: Department of Neuropharmacolgy IMM-6, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037. Tel.: +1 619 5549462; fax: +1 619 5549981; e-mail: juanct@riscsm.scripps.edu.

0168-1702/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
PII S0168-1702(96)01338-X

mates. Borna disease has a variable period of incubation and diverse pathological manifestations depending on the species, immune status and age of the host, as well as route of infection and virus strain (Ludwig et al., 1988; Lipkin et al., 1992; Richt et al., 1992; Koprowski and Lipkin, 1995). BDV is a nonsegmented, negative-stranded (NNS) RNA enveloped virus (Briese et al., 1994; Cubitt and de la Torre, 1994; Cubitt et al., 1994; Zimmermann et al., 1994) with a nuclear site for the replication and transcription of its genome (Briese et al., 1992; Cubitt and de la Torre, 1994), indicating that BDV is the prototype of a new group of animal RNA viruses (de la Torre, 1994; Schneemann et al., 1995).

The reproducible and clinically definable behavioral abnormalities accompanying BDV infection of rats and non-human primates (Sprankel et al., 1978; Narayan et al., 1983; Dittrich et al., 1989; Bautista et al., 1994), led to the speculation that BDV could cause similar central nervous system (CNS) dysfunctions in humans. In support of this hypothesis are the results from cross-sectional seroepidemiological studies showing an increased prevalence of antibodies that recognize BDV-specific antigens in subjects with neuropsychiatric disorders compared with the normal healthy population (Rott et al., 1985, 1991; Bode et al., 1988, 1992; VandeWoude et al., 1990; Fu et al., 1993; Bode, 1995). Moreover, prospective studies on acute psychiatric patients have shown a high percentage of BDV seropositives among patients with major depression (Bode et al., 1993, 1994, 1995; Bode, 1995).

Recently, using flow cytometry (FCM), we detected BDV-specific antigens in CD14+ peripheral blood monocytes (PBM) subset within PBMCs from psychiatric patients (Bode et al., 1994). In addition, BDV-specific RNA sequences have been also detected in such PBMCs (Bode et al., 1995; Kishi et al., 1995).

Co-cultivation of PBMCs from hospitalized psychiatric patients with a human oligodendroglia cell line (OL cells), allowed the isolation and biological characterization of human BDV. BDV was isolated from three of the patients, but not from any of the healthy control subjects (Bode et al., 1996). Here we unequivocally identified these isolates as BDV based on the sequence determination of BDV open reading frames (ORFs) p24, p16, p56, and the putative catalytic domain of the BDV L polymerase. Our data indicate that BDV human isolates are genetically very closely related to BDV from naturally infected animals of different species. These results provide support for the hypothesis that BDV could be one of the environmental factors contributing to the pathophysiology of neuropsychiatric disorders whose etiology remains elusive.

2. Materials and methods

2.1. Subjects and samples

Patients H1 and H3 were two out of 32 psychiatric patients with acute mental disorders hospitalized at the Benjamin Franklin Hospital of the Free University in Berlin, Germany. H1 is a 45-year-old female patient and H3 is a 55-year-old male patient, both with a clinical history of a Bipolar Disorder (DSM-III-R: 296.64 and DSM-III-R: 296.70, respectively). Investigations on patients H1 and H3 were conducted during an acute depressive episode. The third human BDV isolate was obtained from patient H2, a 37-year-old male patient suffering from a chronic Obsessive Compulsive Disorder (DSM-III-R: 300.30; 305.00), who has been hospitalized for the last 14 years at the Theodor-Wenzel-Werk and District Hospital Berlin-Zehlendorf, Germany. Further details on these patients are provided elsewhere (Bode et al., 1996). Clinical diagnoses were according to DSM-III-R criteria (American Psychiatric Association, 1987).

PBMCs samples from healthy control individuals were obtained from blood donors officially registered with the Federal Health Office (1988).

Blood samples of 9 ml each were collected at the hospital in the presence of sodium citrate (10 mM final concentration) as anti-coagulant. Samples were coded and separated into plasma and PBMCs by centrifugation on Ficoll-hypaque (Bode et al., 1995). PBMCs were processed for detection of BDV RNA and virus isolation in a double-blind manner.

Table 1
Sequence, polarity and nucleotide positions in the BDV genome of primers used in this study. Sequences and nucleotide positions are based on the C6BV genome RNA sequence (Cubitt et al., 1994; de la Torre, 1994). The accession number for this sequence is L27077

Primer	Sequence	Polarity	Nucleotide positions BDV RNA Genome
2.1	5′-CAGGAGGCTCAATGGCAACG-3′	Anti-genomic	1261–1280
2.2	5′-TTTATGGTATGATGTCCCAC-3′	Genomic	1878–1859
3.1	5′-ATCGAATCACCATGAATTCAAAGC-3′	Anti-genomic	1882–1905
3.2	5′-GTCAGTATTGCAACTAAGGC-3′	Genomic	2334–2315
4.3	5′-CGGTACGGTTTATTCCTGC-3′	Genomic	3756–3738
3030R	5′-CAGTGTAGGCCTAAGCTTGTG-3′	Genomic	3030–3010
2962F	5′-AAGTTGAGAAGGCGGCGTAG-3′	Anti-genomic	2962–2981
4.4	5′-GCACGCAATTAATGCAGC-3′	Anti-genomic	2225–2242
5248F	5′-TGACCATGAGCTCAACGGC-3′	Anti-genomic	5248–5266
5936R	5′-GCATGATGATGTTAAGCAGGC-3′	Genomic	5936–5916

Details about BDV antigen expression in patients' PBMCs, co-cultivation experiments and biological characterization of these human BDV isolates are described elsewhere (Bode et al., 1996)

2.2. Detection and characterization of BDV RNA in patients' PBMCs

Total RNA was extracted from PBMCs using the acid guanidinium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987). Purified RNA (0.5–2.0 μg) was reverse transcribed using 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research laboratories) and random hexamers (2.5 mM) as primers (Krug and Berger, 1987). PBMCs were screened for the presence of BDV p24 sequences by polymerase chain reaction (PCR) amplification of cDNAs using BDV-specific primers 2.1 and 2.2 (Table 1; Fig. 1). cDNAs were also amplified with specific primers to generate a 192 bp GAPDH fragment (Buesa-Gomez et al., 1994). The conditions for PCR analysis were as follows: 94°C for 5 min (1 cycle); 94°C for 1 min, 55°C for 1 min, 72°C for 1 min (35 cycles); and 72°C extension for 10 min (1 cycle). Each PCR reaction used 1/10 of the cDNA product, 50 pmol of each primer, 1 U of *Taq* polymerase, 100 μM of each deoxynucleotide triphosphate, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0) and 0.1% Triton X-100 in a final volume of 50 μl.

BDV-p24 ORF sequences (618 bp DNA fragment) amplified by PCR were detected by southern blot hybridization using a ^{32}P probe corresponding to an internal p24 fragment (nucleotides (nt) 1329–1749 in the BDV RNA genome).

2.3. Cloning and sequencing of BDV ORFs p24, p16, p56, and the putative catalytic domain of the BDV L polymerase present in OL cells co-cultivated with patients' PBMCs

Total RNA was isolated from OL cells co-cultivated with patients' PBMCs, and reverse transcribed as described above. Full-length p24 (603 nt) and p16 (426 nt) BDV-ORFs were amplified by PCR using primer pairs 2.1 + 2.2, and 3.1 + 3.2, respectively (Table 1; Fig. 1). Amplification of full-length p56 BDV ORF (1521 bp) was done using two pairs of primers, 4.4 + 3030R and 2962F + 4.3 (Table 1; Fig. 1), that generated two overlapping PCR fragments with sizes of 805 and 794 bp, respectively, that covered the entire BDV p56 ORF. The putative catalytic domain of BDV L polymerase (de la Torre, 1994) was amplified using primers BV 5248F and BV 5936R (Table 1; Fig. 1). In all cases, PCR products were readily detected by ethidium bromide staining of the gel. PCR products were gel purified and cloned in the PCR II vector through use of the TA cloning system (Invitrogen, San Diego CA). Sequencing was done with sequenase version 2.0 (US Bio-

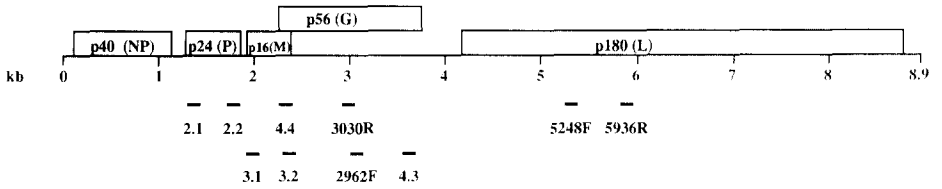

Fig. 1. Diagram of the BDV genome RNA (anti-genomic polarity) and location of primers used in this study. The genome organization presented in the diagram is based on the complete sequence of two BDV RNA genomes as yet reported, strain V and C6BV (Briese et al., 1994; Cubitt et al., 1994; de la Torre, 1994). Accession numbers for strain V and C6BV RNA genome sequences are U04608 and L27077, respectively. Sequence, polarity, and nucleotide positions covered by the primers are summarized in Table 1.

chemical) system according to the manufacturer's instructions. Each ORF sequence presented in Fig. 3 was determined by sequencing of three independent clones obtained from independent PCR events. Only changes found in the three clones for each ORF were considered.

As an internal control for errors introduced by reverse transcriptase (RT) and *Taq* polymerases under the experimental conditions used in our RT-PCR assays, we carried out repetitive sequencing of molecular clones derived from reverse transcription and PCR amplification of RNA from a lymphocytic choriomeningitis virus (LCMV) clone highly adapted to its culture environment. For this purpose, we isolated RNA directly from a single plaque of LCMV Armstrong strain, clone 5 3b. This plaque was grown in Vero cells and originated from a LCMV population that had been serially passaged 30 times in Vero cells using for each passage a multiplicity of infection (m.o.i.) of 0.1 PFU/cell. RNA was reversed transcribed using random hexamers as described above, and PCR conducted using a pair of primers to specifically amplify a 362 bp fragment of the LCMV glycoprotein following procedures described (Evans et al., 1994). The estimated mutation frequency found was less than 2.5×10^{-4} substitutions per nucleotide.

3. Results

3.1. BDV RNA expression in PBMCs from patients H1, H2, and H3

Seventy three coded blood samples randomly selected from serial samples taken during acute disease episodes from 32 hospitalized psychiatric patients, as well as one sample from a chronic psychiatric patient (H2) and five healthy blood donors, were co-cultivated with OL cells and long-term passaged as described (Bode et al., 1996). These studies led to the isolation of BDV from two bipolar patients with acute depression (H1 and H3), and from the chronic patient H2. Patients H1, H2, and H3 harbored relatively high numbers of PBMCs positive for BDV antigens. Here we describe the genetic characterization of these three human isolates.

We first investigated whether detection of BDV antigens in the patients' PBMCs correlated with expression of viral RNA. For this purpose, RNA was extracted from an aliquot of each of H1, H2, and H3 patients' PBMCs, and analyzed by RT-PCR using primers 2.1 and 2.2 (Table 1; Fig. 1), to amplify BDV p24 sequences. PCR products with the expected size of 618 base pairs were obtained with RNA prepared from patients' PBMCs samples, but not with RNA obtained from BDV-antigen negative PBMCs samples of healthy volunteers, as determined by southern blot hybridization using a BDV specific probe internal to the predicted PCR product (Fig. 2). Treatment of RNA samples with DNase-free RNase, but not with RNase-free DNase prior to the RT-PCR assay, as well as the omission of the reverse transcriptase enzyme, prevented amplification of both BDV-p24 and GAPDH sequences.

To molecularly characterize the BDV-specific RNAs present in the patients' PBMCs, we con-

ducted RT-PCR to amplify BDV p24 and p16 full-length ORF sequences, followed by cloning and sequencing of the BDV-specific PCR products. After reverse transcription using random hexamers, cDNAs were subjected to PCR using BDV-specific primer pairs 2.1 + 2.2 and 3.1 + 3.2 (Table 1; Fig. 1), to amplify full-length BDV ORFs p24 and p16, respectively. Products from these PCR reactions, undetectable by ethidium bromide staining, were cloned and transformants screened with ^{32}P probes corresponding to internal p24 and p16 sequences (Briese et al., 1992; Cubitt and de la Torre, 1994; Cubitt et al., 1994).

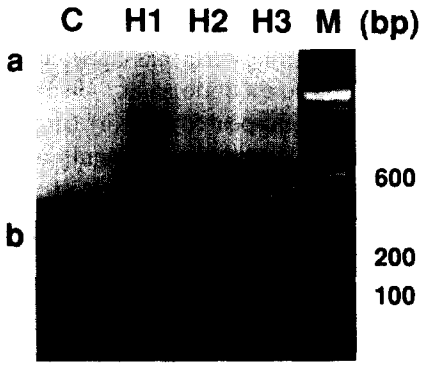

Fig. 2. Expression of BDV RNA in PBMCs of psychiatric patients. Total RNA (1–5 μg) isolated from PBMCs was reverse transcribed by priming with random hexamers and the corresponding cDNAs amplified by PCR as described in Section 2 using: (i) BDV-specific primers to amplify a 618 bp fragment corresponding to full length BDV-p24 ORF, which was detected by southern blot hybridization (a) using a BDV-specific probe internal to the predicted PCR product; (ii) specific primers to amplify a 192 bp GAPDH fragment detected by ethidium bromide staining (b). Samples are: lane 1 (c). PBMCs from a representative healthy control individual negative for BDV antigen; lanes 2–4, PBMCs from psychiatric patients H1, H2, and H3, respectively. The figure corresponds to a composite of the autorad segment showing the results of southern blot hybridization (a) and the part of the gel showing the ethidium bromide staining of GAPDH amplified fragment (b). Track M corresponds to the 1 kb ladder DNA (GIBCO-BRL). Parts (a) and (b) of the composite were lined up with respect to the migration of the 1 kb ladder DNA (track M). Treatment of RNA samples with RNase, but not with DNase, before the RT-PCR assay, as well as omission of the reverse transcriptase, prevented the amplification of both BDV-p24 and GAPDH sequences (not shown).

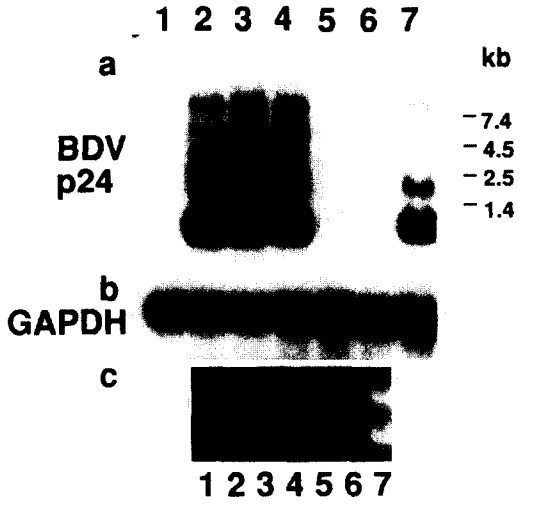

Fig. 3. Expression of BDV RNA in OL cells infected by co-cultivation with PBMCs from psychiatric patients. Total RNA (10 μg) from each sample was analyzed by Northern blot hybridization using specific probes for BDV-p24 (a), and GAPDH (b). Lanes 1 and 5 correspond to RNA from OL cells infected with PBMCs from two representative healthy control individuals negative for BDV antigen; lanes 2–4, correspond to RNA from OL cells infected with PBMCs from patients H1, H2, and H3, respectively. RNA from C6 (lane 6) and C6BV (lane 7) cells, were used as negative and positive controls, respectively, of BDV hybridization. Panel c, shows the ethidium bromide staining of the RNA gel. Experimental procedures were as described in Section 2.

We determined the sequence of three independent clones for each p24 and p16 ORFs derived from each patient's PBMCs.

3.2. Analysis of BDV RNA expression in OL cells co-cultivated with PBMCs from patients H1, H2, H3, and healthy control individuals

BDV persistent infection in a variety of cultured cells is characterized by the absence of cytolysis and lack of cell-free virus production, but relative high levels of viral RNA transcription and replication (de la Torre et al., 1990). Northern blot hybrydization studies revealed that OL cells co-cultivated with the PBMCs of patients H1, H2, and H3 displayed the expected pattern of BDV-specific transcripts detectable with a BDV-p24 probe (de la Torre et al., 1990; Richt et al., 1992; Briese et al., 1994; Cubitt and de la Torre,

1994; Cubitt et al., 1994) (Fig. 3). BDV-specific antigens were also detected in cells expressing BDV-specific RNAs (not shown). OL cells co-cultivated with PBMCs from healthy control volunteers expressed neither viral antigens nor BDV-specific RNAs after more than 20 passages (Fig. 3). These results indicated that infectious BDV, with the ability to replicate in OL cells, was present in PBMCs from patients H1, H2, and H3.

3.3. Sequence characterization of human BDV isolates

To unequivocally identify the isolated infectious agent as BDV, and determine its relationship to the BDV sequences directly derived from PBMCs, as well as to previously known BDV genomic sequences (Briese et al., 1994; Cubitt et al., 1994), we used RNA extracted from OL cells co-cultivated with patients' PBMCs to conduct RT-PCR followed by cloning and sequencing of the amplified products. RNA was reverse transcribed and the corresponding cDNAs subjected to PCR using pairs of primers to amplify three BDV ORFs, corresponding to the p24, p16, and p56 BDV polypeptides, as well as the putative catalytic domain of the BDV L polymerase (Briese et al., 1994; Cubitt et al., 1994; de la Torre, 1994) (Table 1; Fig. 1). RT-PCR assays carried out with RNA extracted from OL cells co-cultivated with BDV-antigen negative PBMCs samples from healthy control individuals failed to amplify any BDV-specific product in several independent experiments.

Sequence analysis confirmed that the infectious agent replicating in OL cells was BDV. Fig. 4 shows the nucleotide sequence alignment for p24, p16 and p56 ORFs among the three BDV human (BDV-Hu) isolates (H1, H2, and H3) and the two previously determined BDV genome sequences (Briese et al., 1994; Cubitt et al., 1994). Direct cloning and sequencing of BDV ORFs p24 and p16 present in the patients' PBMCs (Section 2) revealed that the p16 sequence directly determined in PBMCs of each of the three patients (H1, H2, and H3), was identical to the corresponding p16 sequences obtained after co-cultivation of PBMCs with OL cells. p24 sequences determined before and after co-cultivation of patients' PBMCs with OL cells were also identical in patients H1 and H2, whereas in the case of patient H3, we found one single nucleotide silent change (C → T) in the third base position of codon 127. The nature and total number of amino acid substitutions, as well as total number of nucleotide differences, in ORFs p24, p16 and p56 among BDV-Hu isolates and the horse derived strain V and C6BV isolates are summarized in Fig. 5.

4. Discussion

We have documented the genetic characterization of infectious human BDV isolates. BDV was isolated from three patients with mood disorders by co-cultivation of patients' PBMCs with OL cells, followed by long-term passaging in OL cells as detailed elsewhere (Bode et al., 1996). Successful virus isolation appeared to correlate with the use for the co-cultivation experiments of PBMCs samples expressing the highest levels of virus antigens and RNA.

OL cells co-cultivated with PBMCs from patients H1, H2, and H3, but not from healthy controls, expressed BDV-specific RNAs. Sequence analysis of RT-PCR products obtained using RNA from OL cells co-cultivated with patients' PBMCs and specific primers to amplify BDV ORFs p24, p16, p56, and the catalytic domain of the L polymerase, unequivocally identified these isolates as BDV.

The three human BDV isolates showed a high degree of sequence conservation with respect to BDV strain V and C6BV genome sequences, as well as BDV sequences determined in samples from naturally infected animals of different species. BDV strain V and C6BV sequences had more than 95% homology at the nucleotide level (Briese et al., 1994; Cubitt et al., 1994), which is remarkably high for two RNA virus isolates with different origin and passage history (Holland, 1992; Morse, 1994). Both viruses, BDV strain V and C6BV, were originally isolated from two different naturally infected horse brains and passed several times in rabbits, followed by passages in rats (Schneider et al., 1994). In addition, both viruses

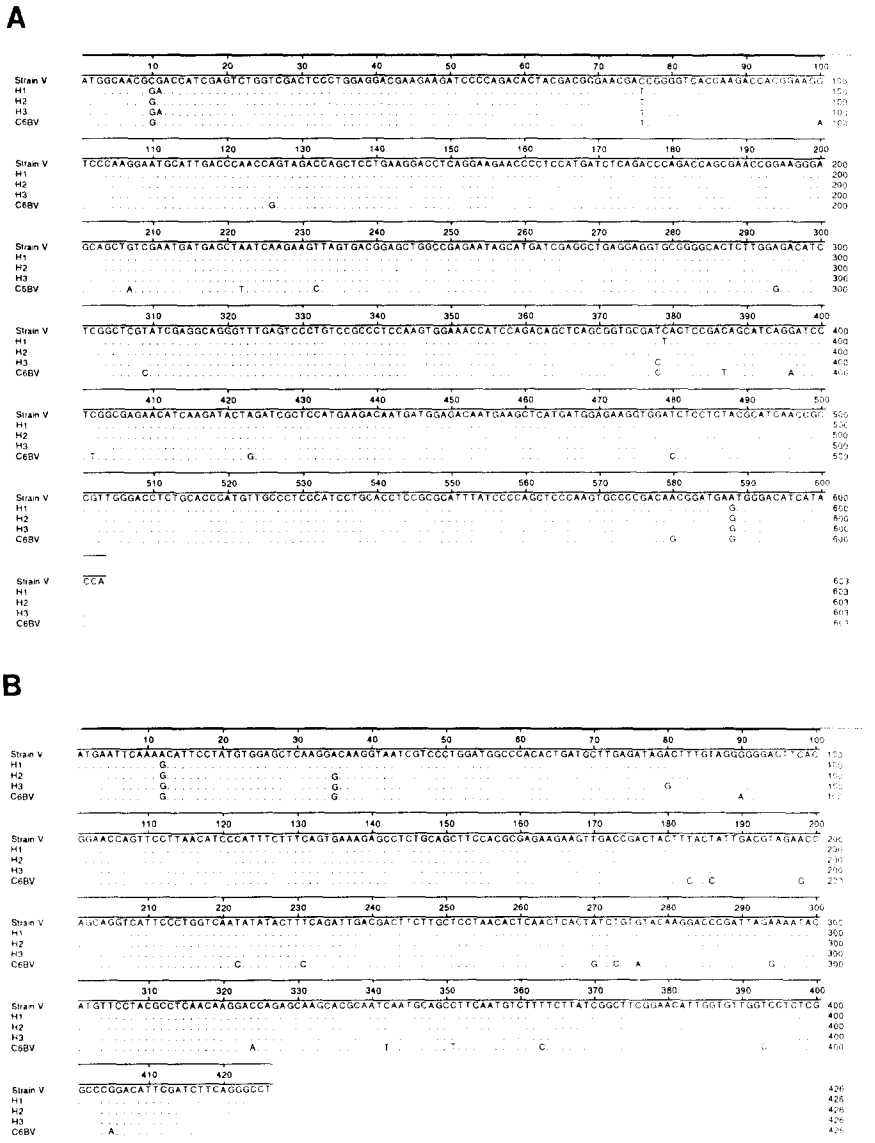

Fig. 4. Nucleotide sequence alignment of ORFs p24 (A), p16 (B) and p56 (C) among BDV-Hu isolates (H1, H2, and H3), C6BV and BDV strain V. Dots indicate the same nucleotide as the one found for that position in the BDV strain V sequence. Numbers on the right correspond to last nucleotide position of each row within the corresponding ORF. The complete RNA genome sequences of strain V and C6BV have been published (Briese et al., 1994; Cubitt et al., 1994; de la Torre, 1994) and have the accession numbers U04608 and L27077, respectively.

were maintained as a persistent infection for more than 20 passages either in OL cells, in the case of BDV strain V, or in the rat astrocytoma C6 cells for C6BV, before their corresponding genome sequences were determined (Briese et al., 1994; Cubitt et al., 1994). Each of the three BDV-Hu isolates had an unique sequence, differing from the other two at one or two nucleotide positions in each of the three ORFs analyzed here, with the exception of identical p56 sequences found for H2

C

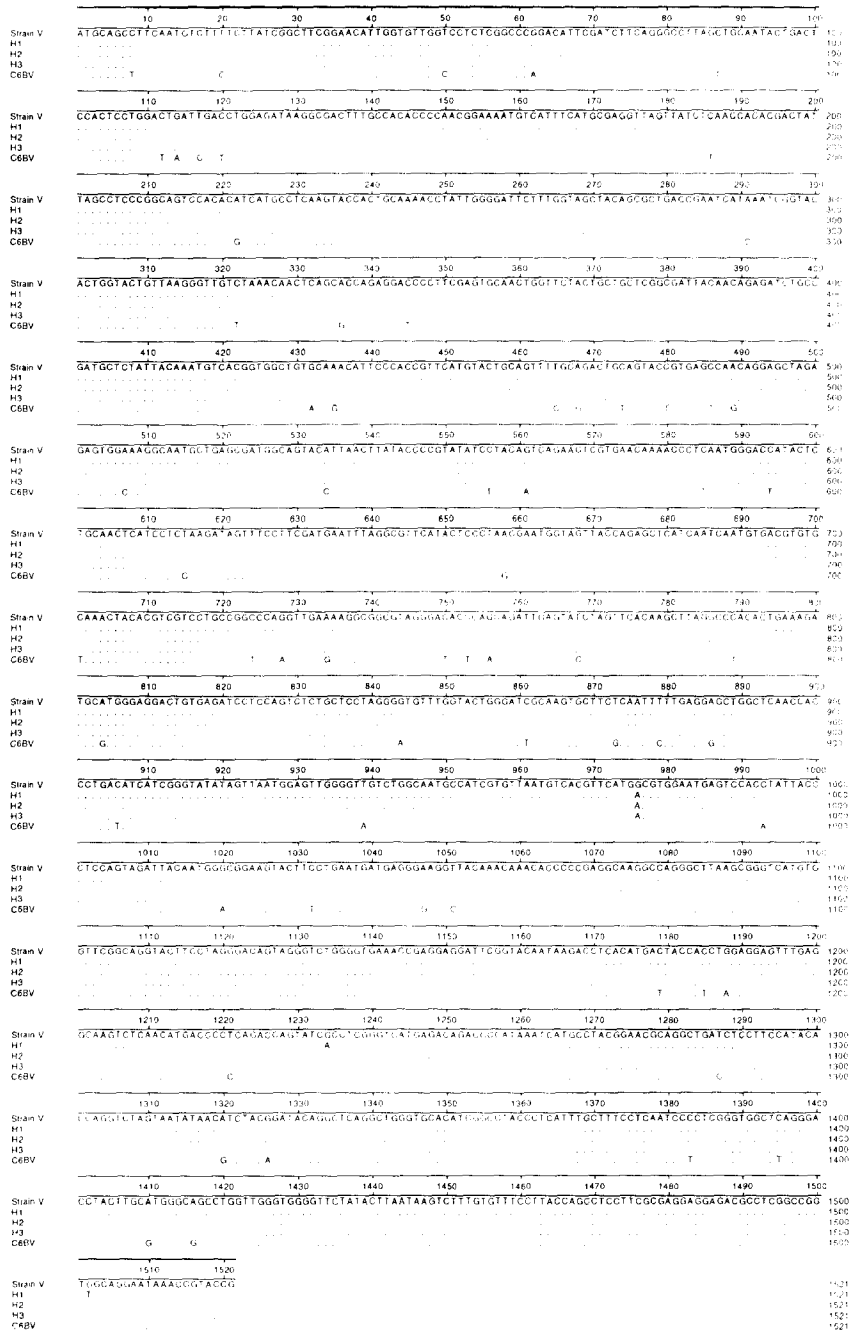

Fig. 4 (C).

A

	p24					p16			p56															
	4	26	34	127	194	12	27	108	3	7	17	21	220	234	242	243	245	282	296	326	412	465	501
C6BV	G	S	I	H	A	G	D	E	L	S	A	Q	A	V	S	L	R	M	G	A	A	L	W
H1	E	-	V	Y	T	D	-	D	P	F	V	R	T	A	P	R	L	V	S	T	T	P	L
H2	-	-	V	-	T	-	-	D	P	F	V	R	T	A	P	R	L	V	S	T	-	P	-
H3	E	-	V	-	T	-	G	D	P	F	V	R	T	A	P	R	L	V	S	T	-	P	-
Strain V	R	P	V	-	T	D	-	D	P	F	V	R	T	A	P	R	L	V	S	-	-	P	-

B

	p24					p16					p56				
	C6BV	H1	H2	H3	Strain V	C6BV	H1	H2	H3	Strain V	C6BV	H1	H2	H3	Strain V
C6BV		16	14	14	17		17	16	17	18		67	65	65	64
H1	4		2	2	5	2		1	2	1	15		2	2	3
H2	2	2		2	3	1	1		1	2	13	2		0	1
H3	3	1	1		5	2	2	1		3	13	2	0		1
Strain V	4	3	2	2		2	0	1	2		12	3	1	1	

Fig. 5. (A) Amino acid differences found in ORFs p24, p16 and p56 among BDV-Hu isolates (H1, H2, and H3), C6BV and strain V. Amino acids are presented in the one single letter code. Numbers on top correspond to the codon position within each ORF. (B) Triangular matrix summarizing the total number of nucleotide (upper right) and amino acid (lower left) substitutions among BDV-Hu isolates (H1, H2, and H3), C6BV and strain V.

and H3 (Fig. 4). Levels of divergence between BDV-Hu isolates and C6BV at the nucleotide level were similar to those found for p24 sequences between BDV isolates from horses separated by more than 10 years and with different history of passages in tissue culture (Schneider et al., 1994). Moreover, high nucleotide sequence conservation, with only 0.3% divergency, has been also reported among p24 BDV sequences from naturally infected horses and sheep (Binz et al., 1994). BDV-Hu isolates displayed a high level of sequence conservation with respect to BDV strain V, with divergencies of 0.5–0.83% for p24, 0.23–0.47% for p16, and 0.07–0.20% for p56 (Fig. 5(B)). In addition, cloning and sequencing of the segment of BDV ORF V corresponding to the putative catalytic domain of the viral L polymerase, revealed a complete amino acid conservation between the three BDV-Hu isolates and BDV strain V (not shown).

No insertions or deletions were observed in p24, p16 and p56 ORFs sequences of BDV-Hu isolates compared with the corresponding reported sequences for BDV strain V and C6BV. When compared with C6BV, most of the nucleotide changes in BDV-Hu isolates were transition events, accounting for 92.8–93.7%, 93.7–94.1%, and 87–88% of the substitutions found in p24, p16, and p56, respectively. Mutations in p24, p16, and p56 ORFs were randomly distributed with non-apparent regions of higher variability. All three BDV-Hu isolates had the substitution Ala→Thr at amino acid position 326 in p56 compared with C6BV and BDV strain V. BDV-Hu H1 isolate had also two amino acids (aa), Thr and Leu at positions 412 and 501, respectively, in p56, that are not found in C6BV or BDV strain V sequences. BDV-Hu H1 and H3 had Glu at position 4 in p24, an amino acid not previously seen at this position in any of the BDV p24 sequences as yet reported (Binz et al., 1994; Briese et al., 1994; Cubitt et al., 1994; Schneider et al., 1994). In addition, BDV-Hu H1 had Tyr at position 127 in p24, instead of the His found in all the other BDV p24 sequences as yet determined. At position 12 in p16, the BDV human isolates H2 and H3 had Gly, whereas H1 had Asp. In addition, at position 27 in p16 BDV-Hu H3 had the non-conservative substitution Gly instead of Asp found in H1 and H2, as well as in strain V and C6BV p16 sequences.

The possibility that the OL cells became BDV positive as result of a contamination with BDV from laboratory sources can be excluded because of: (i) Co-cultivation experiments and later passages of the infected cells were conducted in a tissue culture facility where BDV-infected material had never been previously used. (ii) PBMCs samples were coded and the investigators conducting tissue culture work operated in a double-blind manner. Moreover, in ten independent control experiments OL cells co-cultivated with

BDV-antigen negative PBMCs samples did not express BDV antigen and RNA after more than 30 passages. (iii) Sequence data indicated that each of the three BDV-Hu isolates are genetically more closely related to BDV strain V than C6BV. RT-PCR assays, cloning of amplified products and sequence determination were conducted in the La Jolla laboratory (by JCT) where BDV strain V had not been previously handled. (iv) We did not find changes in p24 and p16 BDV strain V sequences after more than 20 passages in OL cells. This finding probably reflects the adaptation in the laboratory of BDV strain V to grow in OL cells. In contrast, each of the BDV-Hu isolates recovered by co-cultivation of patients' PBMCs with OL cells, differed from the other two BDV human isolates and from BDV strain V in their p24 and p16 sequences (Fig. 4). (v) Direct cloning and sequencing of BDV ORFs p24 and p16 present in the patients' PBMCs, without amplification through co-cultivation with OL cells (Section 2), revealed that p24 and p16 sequences present in PBMCs of patients H1 and H2 were identical to the p24 and p16 sequences determined after co-cultivation of the corresponding PBMCs with OL cells. In the case of patient H3, comparison of sequences derived directly from PBMCs and after co-cultivation with OL cells showed no differences in p16 and one single silent nucleotide change in p24, corresponding to the transition C→T in the third base of codon 127. This result also suggests sequence stability during BDV replication in OL cells.

Control experiments described in Section 2 estimated a mutation frequency lower than 2.5×10^{-4} substitutions per nucleotide for *Taq* polymerase errors, suggesting that it is very unlikely that *Taq* polymerase errors are responsible for the differences found between BDV-Hu isolates and BDV strain V (Fig. 4).

Because the mutation frequencies of RNA viruses exceed, by more than a millionfold, those of their eukaryotic hosts, extremely rapid virus evolution is anticipated and frequently observed (Holland, 1992; Morse, 1994). However, RNA viruses can also exhibit long-term stasis both in nature and in laboratory experiments as result of selection for fit master sequences in rather constant environments (Holland, 1992; Morse, 1994). Although only limited sequence information is presently available, our data indicate a high level of sequence conservation among BDV isolates from PBMCs of the three psychiatric patients and from naturally infected field animals of different species (Binz et al., 1994; Briese et al., 1994; Cubitt et al., 1994; Schneider et al., 1994). This finding suggests that BDV strain V-like sequences may represent prevalent and stable species of BDV in nature, with the ability to infect a number of different animal species including humans. It is worth noting that frequently one single, or very few, amino acid changes can cause drastic phenotypic changes in RNA viruses, including altered tropism and pathogenicity (Holland, 1992; Morse, 1994). Furthermore, the host's genetics also contributes to disease phenotypes caused by BDV infection (Ludwig et al., 1988; Richt et al., 1992; Lipkin et al., 1992). Interestingly, Kishi et al. (1995) have recently reported the detection of BDV p24 sequences in PBMCs of psychiatric patients in Japan of BDV, showing also a high degree of sequence conservation with respect to BDV strain V.

Detection of both BDV antigen and RNA in PBMCs has been also documented in BDV-infected animals (Sierra-Honigmann et al., 1993). Similarly to the situation found in naturally infected horses (Ludwig et al., 1993), BDV-specific antibodies were detected only occasionally in the sera of BDV positive patients, indicating that detection of BDV antigen and RNA in PBMCs reflects the existence of an active BDV infection more accurately than BDV-positive serology.

Our results provide evidence that the three human BDV isolates described here have defined genetic differences among each other and with respect to animal isolates. Nevertheless, both human and animal BDV isolates are genetically very closely related.

BDV was isolated from patients with mood disorders but not from patients with other psychiatric disorders or healthy volunteers, suggesting that BDV could contribute to the pathophysiology of mood disorders. However, hasty conclusions implicating BDV infection as an etiological factor in human mental disorders should not be

drawn at present. However, our data indicate that it should now be possible to conduct comprehensive prospective molecular epidemiological studies to determine rigorously the prevalence of BDV in human populations and psychiatric patients. Information obtained from these studies will facilitate assessment of the possible contribution of BDV to the pathophysiology of major neuropsychiatric diseases whose etiology remains elusive.

Acknowledgements

We thank F.A. Rantam for help isolating BDV-Hu, C. Oldstone, P. Reckwald, and T. Leiskau for indispensable technical assistance, R. Ferszt, and E. Severus for blood samples and clinical diagnoses of patients H1, and H3, W. Schwalbe for blood samples and diagnoses of patient H2. Supported by NS 12428 (B.C. and J.C.T.), and partially by DFG Lu 142/5-2 (H.L.) and an EU contract BMH1-CT94-1791 (H.L.).

References

American Psychiatric Association (1987) Diagnostic and Statistical Manual of Mental Disorders, 3rd edn [revised], Washington, DC, p. 567.

Bautista, J.R., Schwartz, G.J., de la Torre, J.C., Moran, T.H. and Carbone, K.M. (1994) Early and persistent abnormalities in rats with neonatally acquired Borna disease virus infection. Brain Res. Bull. 34, 31–36.

Binz, T., Lebelt, J., Niemann, H. and Hagenau, K. (1994) Sequence analysis of the p24 gene of Borna disease virus in naturally infected horse, donkey and sheep. Virus Res. 34, 281–289.

Bode, L., Riegel, S., Ludwig, H., Amsterdam, J.D., Lange, W. and Koprowski, H. (1988) Borna disease virus-specific antibodies in patients with HIV infection and with mental disorders. Lancet ii, 689.

Bode, L., Riegel, S.,Lange, W. and Ludwig, H. (1992) Human infections with Borna disease virus: seroprevalence in patients with chronic diseases and healthy individuals. J. Med. Virol 36, 309–315.

Bode, L., Ferszt, R. and Czech, G. (1993) Borna disease virus infection and affective disorders in man. Arch. Virol. (Suppl.) 7, 159–167.

Bode, L., Steinbach, F. and Ludwig, H. (1994) A novel marker for Borna disease virus infection. Lancet 343, 297–298.

Bode, L. (1995) in Borna disease, eds Koprowski, H., and Lipkin, W.I. Curr Top Microbiol. Immunol. 190, 103–130.

Bode, L., Zimmermann, W., Ferszt, R., Steinbach, F. and Ludwig, H. (1995) Borna disease virus genome transcribed and expressed in psychiatric patients. Nature Med. 1, 232–236.

Bode, L., Dürrwald, R., Rantam, F.A., Ferszt, R. and Ludwig, H. (1996) First isolates of infectious human Borna disease virus from patients with mood disorders. Mol. Psychiatry 1, 200–212.

Briese, T., de la Torre, J.C., Lewis, A.J., Ludwig, H. and Lipkin, W.I. (1992) Borna disease virus, a negative-stranded RNA virus, transcribes in the nucleus of the infected cells. Proc. Natl. Acad. Sci. USA 89, 11486–11489.

Briese, T., Schneemann, A., Lewis, A.J., Park, Y-S., Kim, S., Ludwig, H. and Lipkin, W.I. (1994) Genomic organization of Borna disease virus. Proc. Natl. Acad. Sci. USA 91, 4382–4386.

Buesa-Gomez, J., de la Torre, J.C., Dyrberg, T., Landin-Olsson, Mauseth, R.S., Lernmark, A. and Oldstone, M.B.A. (1994) Failure to detect genomic viral sequences in pancreatic tissues from two children with acute-onset diabetes mellitus. J. Med. Virol. 42, 193–197.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. Anal. Biochem. 162, 156–159.

Cubitt, B. and de la Torre, J.C. (1994) Borna disease virus (BDV), a nonsegmented RNA virus, replicates in the nuclei of infected cells where infectious BDV ribonucleoproteins are present. J. Virol. 68, 1371–1381.

Cubitt, B., Oldstone, C. and de la Torre, J.C. (1994) Sequence and genomic organization of Borna disease virus. J. Virol. 68, 1382–1396.

de la Torre, J.C., Carbone, K.M. and Lipkin, W.I. (1990) Molecular characterization of the Borna disease agent. Virology 179, 853–856.

de la Torre, J.C. (1994) Molecular biology of Borna disease virus: prototype of a new group of animal viruses [minireview]. J. Virol. 68, 7669–7675.

Dittrich, W., Bode, L., Ludwig, H., Kao, M. and Schneider, K. (1989) Learning deficiencies in Borna disease virus-infected but clinically healthy rats. Biol. Psychiatry 26, 818–828.

Evans, C.F., Borrow, P., de la Torre, J.C. and Oldstone, M.B.A. (1994) Virus-induced immunosupression: kinetic analysis of the selection of a mutation associated with viral persistence. J. Virol. 68, 7367–7373.

Federal Health Office (1988) Germany Bundesgesundhbl 31(7), 286.

Fu, Z.F., Amsterdam, J.D., Kao, M., Shankar, V., Koprowski, H. and Dietzschold, B. (1993) Detection of Borna disease virus-reactive antibodies from patients with affective disorders by Western immunoblot technique. J. Affect Disor. 27, 61–68.

Holland, J.J. (Ed.) (1992) Genetic diversity of RNA viruses. Curr. Top. Microbiol. Immunol. 176.

Kishi, M., Nakaya, T., Nakamura, Y., Zhong, Q., Ikeda, K., Senjo, M., Kakinuma, M., Kato, S. and Ikuta, K. (1995) Demonstration of human Borna disease virus RNA in human peripheral blood mononuclear cells. FEBS Lett. 364, 293–297.

Koprowski, H. and Lipkin, W.I., eds (1995) Borna disease. Curr. Top. Microbiol. Immunol. 190.

Krug, M.S. and Berger, S.L (1987) First-strand cDNA synthesis primed with oligo (dT). Methods Enzymol. 152, 316–325.

Kurstak, E., Lipowski, Z.J. and Morozov, P.V. (1987) Viruses, Immunity, and Mental Disorders. Plenum, New York.

Lipkin, W.I., Briese, T. and de la Torre, J.C. (1992) Borna disease virus: molecular analysis of a neurotropic infectious agent. Microb. Pathogenesis 13, 167–170.

Ludwig, H., Bode, L. and Gosztonyi, G. (1988) Borna disease: a persistent infection of the central nervous sytem. Prog. Med. Virol. 35, 107–151.

Ludwig, H., Furuya, K., Bode, L., Klein, N., Dürrwald, R. and Lee, D.S. (1993) Biology and neurobiology of Borna disease viruses (BDV) defined by antibodies neutralizability and their pathogenic potential. Arch. Virol. (Suppl.) 7, 111–133.

Morozov, P.V. (1983) Research on the viral hypothesis of mental disorders. In: S. Mendlewicz and H.M. Praag (Eds.), Advances in Biological Psychiatry, 12. Karger, New York.

Morse, S.S. (Ed.) (1994) The evolutionary biology of viruses. Raven, New York.

Narayan, O., Herzog, S., Frese, K., Scheefers, H. and Rott, R. (1983) Behavioral disease in rats caused by immunopathological responses to persistent Borna disease virus. Science 220, 1401–1402.

Richt, J.A., VandeWoude, S., Zink, M.C., Clements, J.E., Herzog, S., Stitz, L., Rott, R. and Narayan, O. (1992) Infection with Borna disease virus: molecular and immunological characterization of the agent. Clin. Infect. Dis. 14, 1240–1250.

Rott, R., Herzog, S., Fleischer, B., Winokur, A., Amsterdam, J.D., Dyson, W. and Koprowski, H. (1985) Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. Science 228, 755–756.

Rott, R., Herzog, S., Bechter, K. and Frese, K. (1991) Borna disease, a possible hazard for man? Arch. Virol. 118, 143–149.

Schneemann, A., Schneider, P.A., Lamb, R. and Lipkin, W.I. (1995) The remarkable coding strategy of Borna disease virus: a new member of the nonsegmented negative strand RNA viruses [minireview]. Virology 210, 1–8.

Schneider, P.A., Briese, T., Zimmermann, W., Ludwig, H. and Lipkin, W.I. (1994) Sequence conservation in field and experimental isolates of Borna disease virus. J. Virol. 68, 63–68.

Sierra-Honigmann, A.M., Rubin, S.A., Estafanous, M.G., Yolken, R.H. and Carbone, K.M. (1993) Borna disease virus in peripheral blood mononuclear and bone marrow cells of neonatally and chronically infected rats. J. Neuroimmunol. 45, 31–36.

Sprankel, H., Richarz, K., Ludwig, H. and Rott, R. (1978) Behavior alterations in tree shrews (Tupaia glis, Diard 1820) induced by Borna disease virus. Med. Microbiol. Immunol. 165, 1–18.

ter Meulen, V. (1991). Virus-cell interactions in the nervous system. Seminars in the Neurosciences 3.

VandeWoude, S., Richt, J.A., Zink, M.C., Rott, R., Narayan, O. and Clements, J.E. (1990) A Borna disease cDNA encoding a protein recognized by antibodies in humans with behavioral diseases. Science 250, 1278–1281.

Zimmermann, W., Breter, H., Rudolph, M. and Ludwig, H. (1994) Borna disease virus: immunoelectron microscopic characterization of cell-free virus and further information about the genome. J. Virol. 68, 6755–6758.