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ORIGINAL RESEARCH ARTICLE

Borna disease virus-specific circulating immune complexes, antigenemia, and free antibodies—the key marker triplet determining infection and prevailing in severe mood disorders

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Borna disease virus (BDV), a unique genetically highly conserved RNA virus (*Bornaviridae; Mononegavirales*),[1] preferentially targets neurons of limbic structures[2] causing behavioral abnormalities in animals.[3,4] Markers[5–10] and virus[11–13] in patients with affective disorders and schizophrenia have raised worldwide interest.[3] A persistent infection was suggestive from follow-up studies,[5,14] but inconstant detectability weakened a possible linkage.[15] This study for the first time discloses that detection gaps are caused by BDV-specific circulating immune complexes (CIC), and their interplay with free antibodies and plasma antigens (p40/p24). Screening 3000 sera each from human and equine patients over the past 4 years by new enzyme immunoassays (EIAs) revealed that BDV-CICs indicate 10 times higher infection rates (up to 30% in controls, up to 100% in patients) than did previous serology.[16,17] Persistence of high amounts of CICs and plasma antigens correlates with severity of depression. Even BDV RNA could be detected in plasma samples with strong antigenemia. Our discovery not only explains the course of persistent infection, but offers novel easy-to-use diagnostic tools by which new insights into BDV-related etiopathogenesis of disease and epidemiology are possible. *Molecular Psychiatry* (2001) **6,** 481–491.

CICs had predominantly been studied for their pathogenic role, namely vascular damage, in autoimmune diseases[18] and chronic viral infections (CMV, EBV, HBV, HIV).[19,20] In contrast low replication and infection rates of BDV in blood cells[21] concealed possible CIC formation.

Longitudinal studies[5,11,14] led to the discovery of BDV-specific CICs and their general occurrence during the human and animal infection described here, which plausibly resolves previous diagnostic discrepancies.[15] Unlike non-specific CIC-assays,[22] an easy enzyme immune assay (EIA) with specifically immobilized anti-p40/p24 monoclonal antibodies (moAbs)[23] trapping antigen of BDV-CICs from plasma, was developed. The antibody-part was identified by enzyme-labeled anti-species IgG.

This assay was evaluated over 4 years by screening 3000 plasma samples each from human and equine patients/controls, parallel to previous methods, like antibody detection by immunofluorescence (Ab-IF),[17] and cell-antigen (cAg) by EIA in corresponding PBLs.[11] Surprisingly, p40/p24-CICs represented the most prevalent markers in humans and animals. In patients with different severity and types of depression ($n = 290$), randomly selected from a total of 787 from independent university clinics (Figure 1), CICs were present in 62% and 52%, as compared to 11% and 20% of Ab-IF, and to 15% and 23% of cAg (infection rate ~70%) (Figure 2). CIC determination implicated a considerably higher infection prevalence than previous serology.[16,17] In healthy controls ($n = 100$), CIC rates (24%) exceeded the Ab-IF-rates (2%) 10-fold, whereas cAg was lacking (Figures 1, 2).

Sensitivity and specificity of BDV-CICs were further proven by standard methods.[20,22,24] PEG-treated CICs gave EIA results concordant with untreated samples, but required 10 times higher initial concentrations. No BDV-CICs were obtained from EIA-negative samples (data not shown). By the above procedure, BDV p40 and p29 (which is part of the p24-dimer[1]) could be demonstrated (Figure 3a). After CIC-isolation from strongly positive patients with either depression (patient DR) or Obsessive Compulsive Disorder (OCD; patient WK) using protein G-affinity-chromatography, BDV p40 and p24 was demonstrated by Western-blotting (Figure 3b and c).

In that chronically depressed patient, individual CICs detected either by anti-N(p40) or anti-P(p24) moAbs revealed weekly oscillations (Figure 4a). In practice, both CIC-types could be assayed in one single test, with similar or higher sensitivity. Long-term maintenance of CICs, as representatively shown over half a year (Figure 4b), seemed to be frequent in chronic courses of depression. The same was true in some OCD

Overview of patients and samples

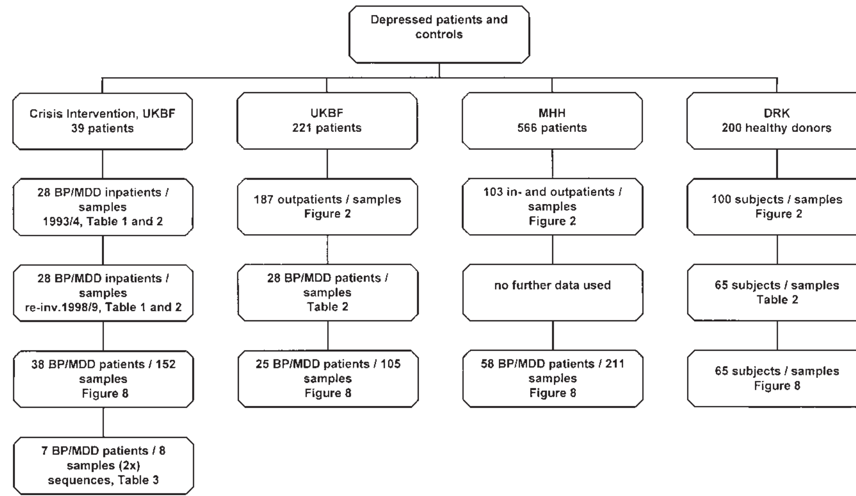


Figure 1 Diagram of patients and samples enrolled in the study. UKBF = University Hospital Benjamin Franklin of the Free University Berlin; MHH = Medical School of Hanover; DRK = German Red Cross, Berlin; BP = Bipolar Disorder; MDD = Major Depressive Disorder.

BDV infection monitoring by previous markers and CICs

Depressed vs. healthy subjects

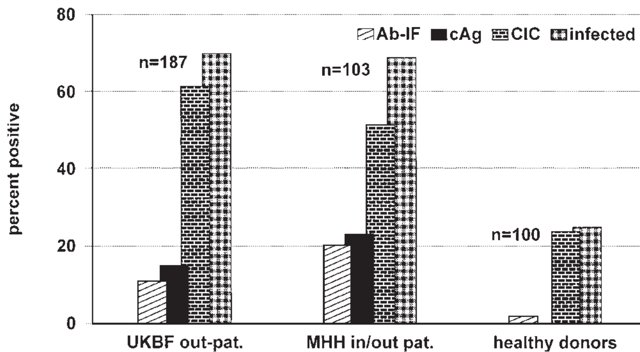


Figure 2 Comparison of BDV-CICs with previous infection markers. Percent prevalence (one sample per patient) of randomly selected patient groups from two different clinics, presenting with different severity and types of depression (see Figure 1; abbreviation of markers, see Table 1); statistical significance by χ^2-test: CIC prevalence in UKBF-patients *vs* controls: $P < 0.0001$, in MHH-patients *vs* controls: $P < 0.001$.

patients (data not shown). The source for continuous CIC formation remained unclear, because of few samples with antigen-positive PBMCs (cAg; Figure 4b). Therefore, cell-produced antigen must have been released into the blood stream. By progressively mounted antibody response, in turn, CICs were formed and antibodies disappeared. Production of free plasma antigen (pAg) could, indeed, be proven by an EIA test used earlier to demonstrate viral proteins in patients' cerebrospinal fluids (CSFs).[3,25] The pAg assay may include the detection of naturally formed N/P heteromers. Likewise, an EIA-based antibody assay completed this test triplet, showing the dynamic of BDV infection (Figure 4b) which seems to be in line with other persistent virus infections.[19]

The test triplet showed striking similarities (high CIC- and pAg-values) in horses with severe Borna disease (BD) and humans with severe depression (representative cases; Figure 5). To further elucidate the significance of both markers, we re-investigated a group of 28 patients with Major Depressive Disorder (MDD) or Bipolar Disorder (BP), hospitalized due to acute severe depressive crisis[26] (Figure 1, Table 1). They were compared with 28 moderately depressed, age- and sex-matched outpatients (group II) with the same disorders (Figure 1, Table 2). In both groups, CIC formation exceeded 90%, approaching 100% infection, whereas Ab-IF and cAg[11,17] remained at the expected 20% and 40%, respectively. In healthy controls (65 blood donors, group III), CIC rates (32%) represented the most prevalent, often only sign of infection (Figure 1, Table 2). Despite a mismatch in age and sex compared to patients, these controls perfectly matched with 3% worldwide BDV seroprevalence (Ab-IF)[17] (Table 2, Figure 2). With respect to CIC rates, the previously assumed prevalence is exceeded ten times, reflecting the background infection in humans.

Low CIC levels (4.8% with high amounts), low rates (6%) and amounts of free pAg, with no cAg, seemed characteristic for latent sub-clinical infections, whereas high CIC levels, and high rates and amounts of pAg significantly paralleled severe depression in MDD and BP patients. Unlike the same prevalence of >90% CIC-positives, group I, presenting with acute depressive crisis, harbored 30% with high values, *vs* only 8% in group II with moderate depression. Likewise, an exorbitantly high rate of antigenemia (40%) and pAg-

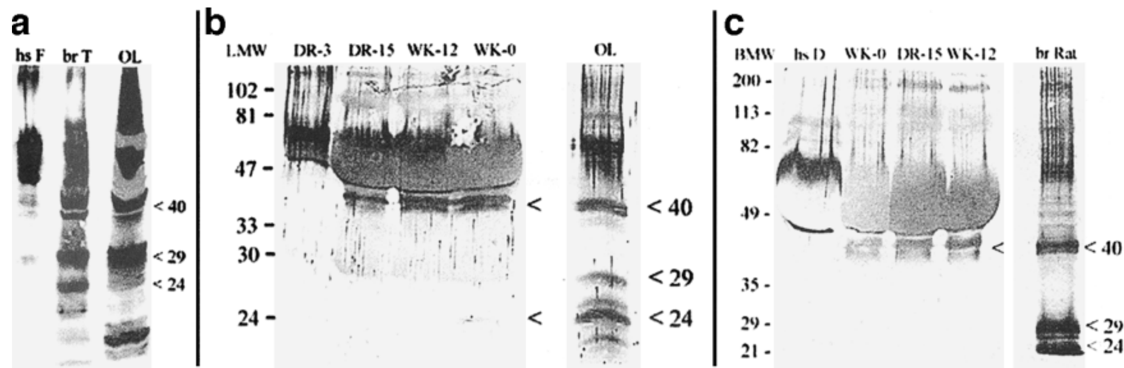


Figure 3 Demonstration of BDV-specificity of CICs. (a) Western-blot (WB) analysis (4–20% gel) of PEG-precipitated and acid-dissociated CICs from plasma of an infected horse (lane hs F), compared with 1:5 diluted brain suspension (20%) from a horse with BD (lane br T), and ultrasonicated, reference strain V-infected oligodendroglia (OL)[11] cell suspension (lane OL). *Note:* BDV proteins p40 and p29 (part of p24) could only partially be dissociated from the higher molecular complex. (b) WB analysis (12% gel) of protein G-Sepharose-isolated CICs from follow-up plasma samples of a chronically depressed patient (DR), hospitalized 3 and 15 weeks (lanes DR-3, DR-15, compare also asterisks in Figure 4b), and an OCD patient (WK), hospitalized 0 and 12 weeks (lanes WK-0, WK-12), compared with 1:5 diluted str. V-infected OL cell suspension. (c) WB analysis (10% gel) of the same samples as in (b), in addition sample hs D, from a horse with BD, but all samples treated with 7 M urea prior to SDS-PAGE; positive control: 1:5 diluted str. V-infected rat brain suspension (10%). *Note:* separation of BDV proteins p40 and p24 was hindered by high amounts of heavy chain antibody parts of the isolated CICs. LMW = low range, BMW = broad range molecular weight markers, pre-stained (BioRad Labs, Hercules, CA, USA). Immune-stain (a)–(c) by rabbit hyper-immune serum EV 3, diluted 1:100;[23] arrows indicate major BDV proteins.

amount (>80% positives with high values) corresponded with severe MDD or BP (group I), and low frequencies (11%) and amounts (no pAg-positives with high values) with moderate MDD or BP (Table 2).

To estimate pAg-concentrations, a calibration curve of purified native p40 and p24 showed (Figure 6) that the detection limit ranged from 0.3 to 0.5 ng of N- and P-protein (3–5 ng ml^{-1}), representing sufficiently sensitive levels compared to other systems.[20] Detectable quantities ranged from 3 to 5000 ng ml^{-1}. Estimated high antigen levels (>100 ng ml^{-1}) were reached in severely impaired patients, as well as in horses with BD (Figure 5; see patients S-29, S-12, horses Sc and Ba). Vice versa, high antibody titers (Ab-EIA; 1:50000) of such a recently investigated patient 'neutralized' 50% of a medium pAg amount of 1 ng per 50 μl present in horse plasma (Figure 7), thus supporting the assay-specificity.[20]

High antigenemia together with high CIC values indicate continuous, abundant production of viral components not complexed by antibodies. Such infection states, predominantly found in severely depressed patients, together with an almost 100% infection rate, strengthen the idea that BDV is etiopathogenetically linked to mood disorders. Further support came from viral RNA, present in 16.5% (25/152) of plasma samples and 47.4% (18/38) severely depressed patients, but only in 0.95% (3/316) of plasma samples and 3.6% (3/83) moderately depressed patients from two independent clinics (UKBF and MHH), and in none of the samples (0/65) from healthy controls (0/65) (Figures 1 and 8a). Based on matched means of samples (four per patient), age, sex, and approximately matched BP/MDD ratios, viral RNA was 17 times as frequent in samples, and 13 times as frequent in patients with severe than with moderate depression. This could be correlated with different frequencies of plasma antigen, present in 38.7% (58/150) of samples from severely depressed, *vs* 16.1% (50/310) from moderately depressed patients, and 6.2% from healthy donors (Figure 8b).

Moreover, repeated RNA amplification from aliquot specimens was only successful in plasma harboring high antigen levels, derived from seven patients of the former group (Table 1, Figure 1). Any molecular work was performed with double-coded samples, at different time points and locations other than for immune assays (Tables 1 and 2). Surprisingly, sequencing of 35.3% of ORF-p40 (genomic nucleotides [nt] 320–711) and 36.7% of ORF-p24 (nt 1571–1791) revealed identical mutations, with 0.77% and 2.26% divergencies to strain V,[27] respectively. According to other human- and animal-derived gene bank sequences ($n = 30$, ORF-p40; $n = 44$, ORF-p24), two mutations each were rare[28] (nt 464 and 542, nt 1668 and 1721) (Table 3), but they were also found in brain samples of a horse which had died from natural BD. Speculative explanations might attribute these mutations to predominant RNA quasi species, or to commonly virulent strains.[3]

This first report on CICs, plasma antigens, and plasma RNA in BDV infection allows new interpretations of human infections and disease, explaining previous discrepant results.[28,29] The rapid and easy CIC-assay does not require PEG concentration. Interference with free plasma antigen is negligible, due to the 10 times difference in dilutions. Thus, the novel test triplet (CICs, pAg, Abs) represents a powerful tool for BDV diagnostic.

The results indicate BDV-CICs as general phenomena in infected humans and animals, representing pre-

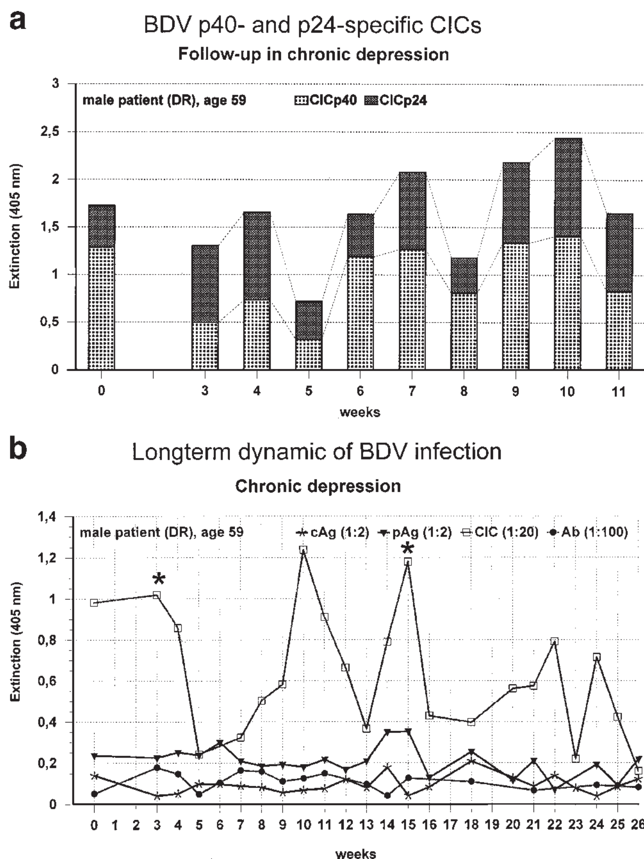


Figure 4 Long-term follow-up of BDV-CICs in a representative case of chronic depression (patient DR). (a) Weekly determined ratios of individual p40- and p24-CICs during a 3-month-period. (b) Dynamic of CIC development in relation to other infection markers (abbreviations, see Table 1). Values are given as extinction at 405 nm, measured in the starting dilutions for each test (see Materials and methods).

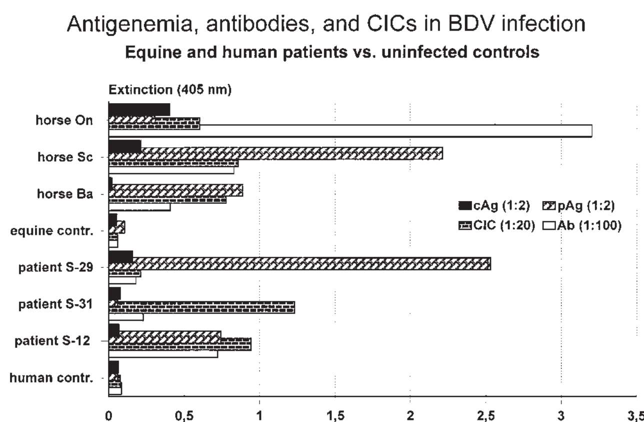


Figure 5 Comparative analysis of antigenemia, antibodies, and CICs determined in representative samples of human patients and diseased horses (one sample per subject). Horses presented with clinical symptoms of BD (mainly apathy, somnolence), sample of horse On taken close to death, of horse Sc taken 5 days after onset of acute disease, and of horse Ba during disease development; samples of severely depressed patients S-29, S-31, and S-12 (see Table 1) were from days 4, 7, and 14 at hospital, respectively.

dominant markers. CIC formation appears to be the consequence of alternately replicating BDV, leading to short-term antigen-positive PBLs, antigen release into the blood (cell sources unclear), and induction of antibodies, the latter two forming CICs. This first discovery of BDV-CICs allows realistic estimations of infection prevalence which is ten times higher than previously assumed,[17] fitting in with the concept of BDV as a well-adapted old virus.[3] A low pathogenicity (3–4%) in vulnerable human subjects[30] can be postulated, considering a world-wide life-time prevalence of recurrent affective disorders of at least 1%, and almost 100% infection found in such patients, *vs* up to 30% of asymptomatic carriers. An etiopathogenic role of BDV in mood disorders seems considerably strengthened, given the significant coincidence of severe disease and antigenemia, in the presence of high CIC values. High amounts of free antigens, also detectable in CSFs of MDD patients,[25] are prerequisites for the hypothesized pathomechanism of an interference of viral components with neurotransmitter receptors,[2] thus disturbing the sensitive balance of neuronal circuits.[11] Viral RNA accompanying high antigenemia (possibly as ribonucleoproteins [RNPs][1]), appeared to be inconsistent and rare. Whether long-term maintenance of BDV-CICs could account for known kinds of IC-diseases,[19] remains elusive.

Materials and methods

Patients

Between June 1996 and April 1999, 3063 psychiatric patients were screened, of whom 787 either consulted the Psychiatric Clinic of the Free University Hospital Benjamin Franklin in Berlin (UKBF; $n = 221$), or the Psychiatric Department of the Medical School of Hanover (MHH; $n = 566$), as potential participants of clinical studies. Here, randomly selected sub-cohorts of period January 1997 to October 1998 were presented, namely 187 outpatients from the UKBF and 103 in/outpatients from the MHH, both groups diagnosed as having affective disorders. In addition, serial plasma samples (stored −20°C) from clinically intensely studied patients with severe MDD or BP ($n = 39$), who had been admitted to the Crisis Intervention Center at UKBF between June 1993 and August 1994,[26] were re-investigated. We also included 100 out of 200 randomly selected blood donors (collected June 1998) from the German Red Cross (DRK, Berlin), who were tested negative for hepatitis B- and C-virus and HIV. Finally, BDV-RNA analyses of 533 plasma samples from 121 patients of the above groups and of 65 healthy donors were performed between May 1998 and April 2000 (overview in Figure 1).

Animals

Between August 1995 and July 1999, blood samples of 3282 horses, the majority sent from practitioners and horse clinics, were screened to exclude or confirm BDV infection (Ab-IF, cAg, CIC). From this background experience of 4-years-evaluation of CIC- and additional

Table 1 BDV infection markers in severely depressed inpatients with either Major Depressive Disorder (MDD) or Bipolar Disorder (BP)

Patient	*Initials*	*Age/sex*	*Clin. diag.*	*DSM-III-R*	*Hospitalized*	*Year*	*Sample*	*Ab-IF*	*Ab-EIA*	*cAg*	*pAg*	*CIC*	*pRNA-1*	*pRNA-2*
S-1	MG	74/F	rMDD	296.3	20 days	1993	1368st	1+	(+)	(+)	neg	2+	P295-neg	P661-neg
S-3	HW	27/F	BP	296.7	13 days	1993	2267st	neg	2+	**1+**	**4+**	2+	**P274-pos**	P640-neg
S-4	GB	42/F	BP	296.53	7 days	1994	3525st	neg	1+	neg	neg	1+	P310-neg	P676-neg
S-6	DW	38/M	BP	296.7	10 days	1994	3022st	neg	?	**neg**	**4+**	4+	**P307-pos**	**P673-pos**
S-7	MG	36/M	rMDD	296.3	7 days	1993	1920st	neg	2+	neg	neg	3+	P297-neg	P663-neg
S-8	SK	47/F	rMDD	296.3	4 days	1994	3548st	neg	neg	1+	neg	1+	P313-neg	P679-neg
S-10	HZ	42/F	seMDD	296.22	1 days	1993	1934st	neg	1+	**neg**	**1+**	4+	**P298-pos**	P664-neg
S-11	AI	35/M	BP	296.5	13 days	1993	2693st	1+	1+	**1+**	**neg**	2+	**P287-pos**	P653-neg
S-12	BG	53/F	seMDD	296.2	14 days	1993	1803st	1+	3+	neg	3+	4+	NA	NA
S-14	K-EH	57/M	rMDD	296.3	5 days	1993	2691st	neg	1+	?	neg	2+	P288-neg	P654-neg
S-15	HF	81/F	rMDD	296.3	15 days	1993	2290st	neg	1+	(+)	neg	neg	P277-neg	P643-neg
S-17	ES	38/F	seMDD	296.23	10 days	1994	1848st	neg	1+	neg	neg	2+	P302-neg	P668-neg
S-20	BK	28/F	seMDD	296.21	10 days	1993	2661st	neg	?	1+	neg	1+	P285-neg	P651-neg
S-21	DB	42/F	rMDD	296.33	16 days	1993	2620st	neg	?	1+	neg	neg	P283-neg	P649-neg
S-23	HO	83/F	rMDD	296.3	7 days	1993	1353st	neg	neg	neg	neg	2+	P292-neg	P658-neg
S-24	GC	52/M	BP	296.5	21 days	1994	3549st	neg	(+)	**(+)**	**4+**	1+	**P314-pos**	**P680-pos**
S-26	DM	56/M	BP	296.7	1 days	1994	5998st	1+	neg	1+	(+)	1+	P773-neg	ND
S-27	US	32/F	BP	296.7	13 days	1994	5985st	neg	neg	**neg**	**4+**	1+	**P319-pos**	**P685-pos**
S-28	AD	64/M	rMDD	296.34	13 days	1994	2550st	neg	neg	**neg**	**neg**	1+	**P325-pos**	P691-neg
S-29	BE	43/F	BP	296.7	4 days	1994	2089st	neg	1+	**1+**	**5+**	1+	**P323-pos**	**P689-pos**
S-31	RH	58/M	rMDD	296.33	7 days	1994	2035st	neg	1+	neg	neg	4+	P320-neg	P686-neg
S-33	SF	52/M	rMDD	296.3	4 days	1994	1822st	neg	1+	**1+**	**4+**	(+)	**P331-pos**	**P697-pos**
S-34	GN	41/F	seMDD	296.2	11 days	1994	1815st	neg	2+	neg	neg	3+	P332-neg	P698-neg
S-35	ES	70/F	seMDD	296.2	5 days	1994	2592st	neg	1+	**neg**	**4+**	(+)	**P327-pos**	**P693-pos**
S-36	HB	59/F	seMDD	296.23	11 days	1993	1458st	NA	2+	**neg**	**neg**	4+	**P291-pos**	P657-neg
S-37	LQ	68/F	rMDD	296.3	11 days	1993	2027st	neg	(+)	**neg**	**4+**	(+)	**P279-pos**	**P645-pos**
S-38	EB	84/F	rMDD	296.3	0 days	1994	1839st	neg	2+	**1+**	**neg**	3+	**P333-pos**	P699-neg
S-39	BK	73/F	rMDD	296.33	25 days	1993	2663st	1+	3+	(+)	neg	2+	P286-neg	P652-neg

Abbreviations: MDD: r = recurrent, se = single episode; Ab = antibody, IF = immunofluorescence (endpoint titration), EIA = enzyme immunoassay; cAg = antigens (p40/p24) in peripheral blood leucocytes (PBLs), pAg = antigens (p40/p24) in citrated blood plasma; CIC = circulating immune complexes, specific for BDV antigens p40/p24, in blood plasma; pRNA = BDV-RNA in plasma (amplification of ORF-p40 fragment, see Table 3), pRNA-1 and pRNA-2 = aliquot samples, taken from the same plasma and processed at different time points, samples therefore double-coded. Clinical data and primary tests (Ab-IF, cAg): 6/1993–8/1994; further investigation (Ab-EIA, pAg, CIC) of same samples (stored at −20°C): 7/1998 to 6/1999; pRNA: code P274–336: tested 10/1998, P640–702: tested 6/1999; P704–806: tested 4/2000. Semiquantitative scoring of EIAs: neg = negative (cut-off ≤0.1 extinction); ? = questionable; (+) = low-positive; + and ++ = medium-positive; ≥ +++ = high-positive; NA = sample not available; ND = not done.

Table 2 Prevalence of BDV infection in patients with different severity of depression and healthy subjects

Group	*I* Inpatients with severe depression (acute crisis)	*II* Outpatients with moderate depression	*III* Healthy subjects	*Statistical significance Group I vs III*	*Statistical significance Group I vs II*
Sample collection and testing Ab-IF, cAg	6/1993–8/1994	3/1997–10/1998	6/1998		
Testing CIC, pAg, Ab-EIA	7/1998–6/1999	3/1997–10/1998, 6/1999	6/1998, 7/1999		
Testing pRNA	10/1998, 4/2000	7/1998–2/1999	3/1999		
Repeat	6/1999				
Sequencing	1999 and 2000				
No. of subjects and samples	28	28	65		
No. of BP/MDD	8/20	9/19			
Mean age (range)	52.68 ± 16.53 (27–84)	51.29 ± 12.19 (23–72)	38.60 ± 11.64 (18–64)		
No. of males/females	9/19	10/18	50/15		
Ab-IF pos. (%)	5* (18.5)	6 (21.4)	2 (3.1)	$P = 0.011$	ns
Ab-EIA pos. (%)	20 (71.4)	4* 14.8)	13** (20.3)	$P < 0.001$	$P < 0.001$
cAg pos. (%)	13 (46.4)	10 (35.7)	0 (0)	$P < 0.0001$	ns
pAg pos. (%)	11 (39.3)	3* (11.1)	4 (6.2)	$P < 0.001$	$P = 0.017$
High amounts of pAg (%)	9 (81.8)	0 (0)	0 (0)	$P < 0.001$	$P = 0.0017$
CIC pos. (%)	26 (92.9)	26 (92.9)	21 (32.3)	$P < 0.0001$	ns
High amounts of CIC (%)	8 (30.8)	2 (7.7)	1 (4.8)	$P = 0.0021$	$P = 0.036$
pRNA pos. (%)	13* (48.2)	1 (3.6)	0 (0)	$P < 0.0001$	$P < 0.001$
Repeat pos. (%)	7 (53.9)	ND	ND		
Infection rate (%)	28 (100)	28 (100)	22 (33.8)	$P < 0.0001$	ns

Patients' groups I and II were from the Crisis Intervention Centre[26] and the Psychiatric Clinic of the Free University Hospital Benjamin Franklin in Berlin (UKBF) (see also Figure 1); data were based on values of the first or second sample taken within 1–2 weeks; healthy subjects' group III were randomly selected blood donors from the German Red Cross; *($n = 27$), **($n = 64$), BP = Bipolar Disorder, MDD = Major Depressive Disorder; for explanation of BDV infection markers, see Table 1. Statistical significance by χ^2-test, ns = no significant difference.

Note: Given the above infection prevalence determined by at least one positive of six different assays, the CIC assay alone detected 92.9% (26/28) of infections in acutely depressed patients, and 95.5% (21/22) of (latent) infections in healthy donors, by one sample per subject analysis.

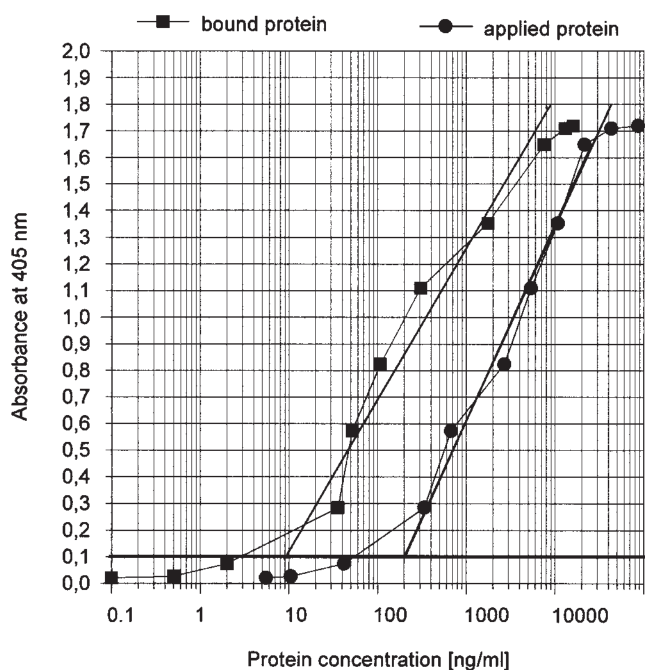


Figure 6 Sensitivity of EIA test to determine native BDV antigens in plasma. Calibration diagram plotting protein concentrations of 0.1 ng ml^{-1} up to 10 000 ng ml^{-1} of isolated native, equally mixed BDV p40 (N) and p24 (P) proteins (see Methods), *vs* EIA extinction values (at 405 nm) determined before and after test performance.

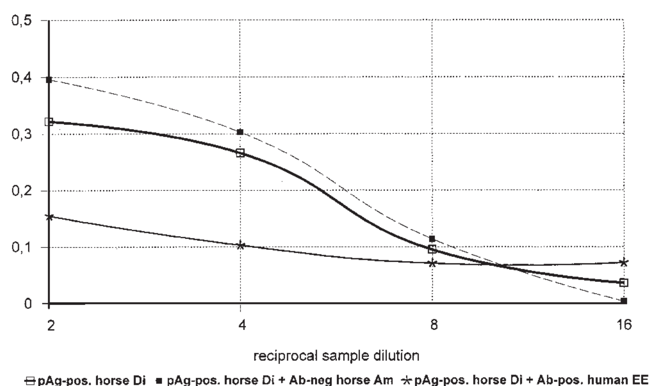
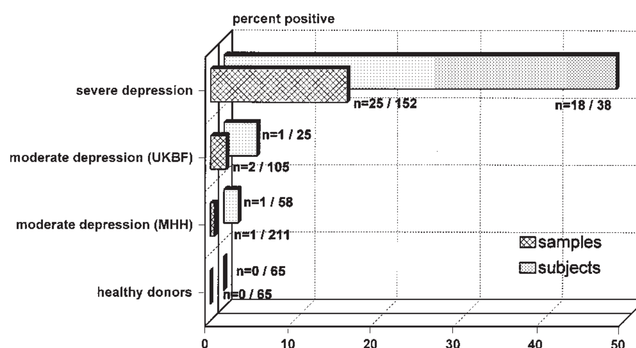


Figure 7 Competitive inhibition of BDV plasma antigens by natural antibodies. EIA values of indicated dilutions of plasma antigens from horse Di, untreated and after treatment with either undiluted antibody-positive human plasma from patient EE (Ab-titer by EIA: 1:50 000), or with antibody-negative plasma from horse Am (dotted line).



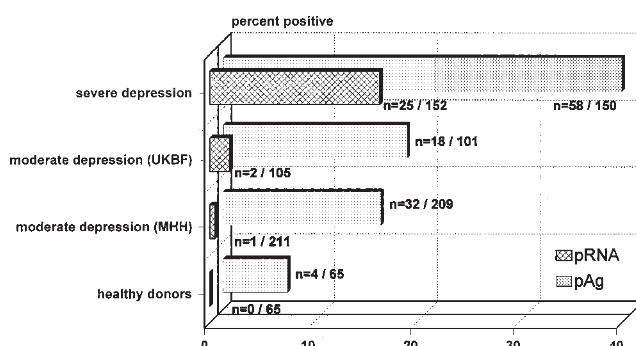


Figure 8 Frequency of BDV-RNA in plasma correlated with severity of depression and antigenemia. (a) Comparison of percent amplified RNA (ORF-p40 fragment, see Methods) from follow-up plasma samples of severely and moderately depressed patients, as well as corresponding percent RNA-positive patients (see Figure 1): mean number of samples were 4.0 ± 2.4 (severe group), 4.2 ± 2.4 (moderate group, UKBF), 3.6 ± 1.4 (moderate group, MHH); in the same sequence: mean ages were 50.5 ± 17.0 years, 51.9 ± 11.3 years, 50.1 ± 12.1 years; male to female ratios were 26.3–73.7%, 28–72%, 37.9–62.1%; BP to MDD ratios were 23.7–76.3%, 40.9–59.1%, and 30.8–69.2%, respectively. Controls were 65 healthy donors (one sample per subject), as described in Table 2. (b) Comparison of percent amplified RNA from plasma samples with percent antigen-positive plasma samples; patients and controls as described in (a). Statistical significance by χ^2-test, pRNA, severe *vs* moderate UKBF: $P < 0.001$; pRNA, severe *vs* moderate MHH: $P < 0.0001$; pAg, severe *vs* moderate UKBF: $P < 0.001$; pAg, severe *vs* moderate MHH: $P < 0.0001$.

pAg-testing in animals, the results of which would be beyond the scope of this paper, only data from single but representative cases were included, because of the striking analogy to human patients (see Figures 3, 5).

Blood samples
Citrated blood samples (10 ml), collected at the above clinical institutions and mailed to our laboratory at the Robert Koch-Institut, were separated by density-gradient centrifugation (Ficoll–Paque) 1.077 (human blood)[5] and 1.09 (equine blood) into plasma and PBLs, coded prior to any assay described here, and stored at –20°C.

Isolation and analysis of CICs
Polyethylene glycol (PEG) precipitation of immune complexes was performed as described,[24] with minor modifications. Briefly: 100 μl of patient plasma was added to 3.9 ml of a 3.5% (wt/vol) solution of PEG 8000 (Sigma, St Louis, MO, USA) in 0.1 M borate buffer (pH 8.4), and incubated at 4°C for 18 h. The precipitate

Table 3 Sequence analysis of repeatedly amplified BDV-RNA from aliquots of blood plasma samples of patients with severe depression (Table 1)

(a) Mutations in ORF I (p40) nt 320–711 (392 base pairs = 35.3% of ORF I) with respect to BDV reference strain V

Genome position	368	464	542
ORF I—position	315	411	489
Amino acid	105	137	163
Strain V	GTC Val	TTC Phe	AAG Lys
S-6, S-24, S-27, S-29, S-33, S-35, S-37	GT**T** Val	TT**T** Phe	AA**A** Lys

(b) Mutations in ORF II (p24) nt 1571–1791 (221 base pairs = 36.7% of ORF II) with respect to BDV reference strain V

Genome position	1649	1668	1673	1694	1721
ORF II—position	378	397	402	423	450
Amino acid	126	131	134	141	150
Strain V	GAT Asp	ATC Ile	CTC Leu	CTA Leu	GAG Glu
S-6, S-24, S-27, S-29, S-33, S-35, S-37	GA**C** Asp	**G**TC Val	CT**T** Leu	CT**G** Leu	GA**A** Glu

was pelleted (1.800 × *g*, 30 min) and washed twice in 2 ml each of 3.5% PEG. After the final wash, pellets were dissolved in 200 μl of 5 mM Tris/HCl buffer (pH 7.2) containing 0.05% Tween 20, and used undiluted in the CIC-EIA. For further analysis of CICs, a different protocol was used. From 5 ml serum or plasma, CICs were precipitated at 4°C overnight by adding 20 ml of 3.5% PEG in 0.1 M borate buffer (pH 8.4). The precipitate was washed twice in borate buffer (1952 × *g*, 10 min), the pellet dissolved in 300 μl of 1.5 M glycine (pH 1.85) (1 h at 37°C) as described,[20] and concentrated to half the volume in Centricon YM3 tubes (Millipore Inc, Bedford, MA, USA; cut off 3 kDa (5422 × *g*, 30 min)). A 30-μl aliquot of these dissociated CICs was neutralized with the same volume of 1.5 M Tris/HCl (pH 9.0), immediately boiled in 60 μl Lämmli buffer (5 min), and subjected to SDS-PAGE (ready-to-use gradient gels 4–20%, Novex, San Diego, CA, USA), followed by Western-blot analysis.[11] Alternatively to PEG-precipitation, CICs were isolated by protein G-affinity-chromatography. Plasma samples (0.5 ml each) were mixed with equal volumes of sample buffer (PBS, pH 7.0 + 5 mM EDTA) and applied to a column with protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Buckinghamshire, UK) (bed volume 1 ml). After removing unbound sample components (10 ml wash with sample buffer), bound CICs were eluted with 3 ml and additional 2 ml elution buffer (0.1 M glycine-HCl, pH 2.5). Collected fractions of 1 ml, neutralized with 1 M Tris/HCl, pH 9.0 and optionally treated with 7 M urea, were size-fractionated by SDS-PAGE (10% or 12% gels) and analyzed by Western-blotting.[11] For both CIC-isolation-methods, blotted BDV-specific antigens were identified by the same rabbit hyper-immune serum (EV 3).[23]

Enzyme immuno assays (EIAs)
Following the concept of maximum versatility, all EIAs including the CIC test used the same solid phase support, volume per well (100 μl), and buffers, and the same initial coating steps (1 and 2), described in detail below.

CIC assay
CICs were determined as follows: (Step 1) polystyrene microtiter formate Maxisorp Immuno Modules (Nunc, Roskilde, Denmark) were coated with 1.8 μg ml^{-1} of AffiniPure Goat Anti-Mouse IgG, FcFragment-specific (adsorbed against human, bovine, equine serum proteins), in 10 mM sodium phosphate and 250 mM sodium chloride, pH 7.6, for 1 h at 37°C (or overnight at 4°C). (Step 2) after washing (three times in 0.9% sodium chloride + 0.05% Tween 20, Ultrawash Plus, Dynatech Labs, Chantilly, VA, USA), BDV p40 and p24 mouse monoclonal antibodies (moAbs) (W1, Kfu2, hybridoma supernatants IF-Titer 1:2000),[23] diluted 1:500 in PBS (pH 7.2) + 0.05% Tween 20 (PBS-T), were incubated for 1 h at 37°C (or overnight at 4°C). (Step 3) after washing, plasma (or serum) samples, diluted 1:20 and serially two-fold in PBS-T, were incubated for 1 h at 37°C. (Step 4) after washing, Alkaline Phosphatase (AP)-conjugated AffiniPure Goat Anti-Human IgG, Fc Fragment-specific (adsorbed against mouse, bovine, equine serum proteins), diluted 1:3000 in 20 mM Tris-buffered saline pH 8.0 + 0.05% Tween 20 (TBS-T), was incubated for 1 h at 37°C; for horse CICs: AP conjugated AffiniPure Goat Anti-Horse IgG, FcFragment-specific, in the same dilution). (Step 5) after washing, freshly prepared substrate *p*-nitrophenylphosphate (pNPP) (1 mg ml^{-1}) in 1 M diethanolamin buffer (pH 9.8) + 0.5 mM magnesium chloride was incubated for 5 min at room temperature. (Step 6) the enzymatic reaction was stopped by the addition of 50 μl of 3 M sodium hydroxide, and read at 405 nm in Dynatech Microplate Reader MRX. An extinction value of ≤ 0.1 was scored negative (all EIAs). All second antibodies and conjugates are from Jackson Immuno Research Labs, West Grove, PA, USA (Supplier in Germany: Dianova, Hamburg).

Antigen assays
BDV antigens p40 and p24 expressed inside PBLs (cAg)[5,11] or present in blood plasma (pAg; p40/p24 heteromers included) were determined by the following EIA-protocol: (Steps 1 and 2) as CIC assay. (Step 3) after washing, ultrasonicated PBL[11] or native plasma samples, diluted 1:2 and serially two-fold in PBS-T, were incubated for 2 h at 37°C or overnight at 4°C. (Step 4) after washing, polyclonal rabbit anti-BDV serum (IF-titer 1:10 000),[23] diluted 1:1000 in PBS-T, was incubated for 2 h at 37°C. (Step 5) after washing, AP-conjugated AffiniPure Goat Anti-Rabbit IgG, FcFragment-specific (adsorbed against human serum proteins), diluted 1:3000 in TBS-T was incubated for 1 h at 37°C. After washing, steps 5 and 6 of CIC assay.

Antibody assays
BDV antibodies in blood plasma were determined by immunofluorescence (Ab-IF) as described previously[17] and/or by an EIA-protocol as follows: (Steps 1 and 2) as CIC assay. (Step 3) after washing, ultrasonicated BDV strain V[23] infected oligodendroglia (OL) cells[11] (titer 10^6 focus forming units ml^{-1}), diluted 1:300 in PBS-T, were incubated overnight at 4°C. (Step 4) after washing, plasma (or serum) samples, diluted 1:100 and serially two-fold in PBS-T, were incubated for 2 h at 37°C. After washing, steps 4, 5, and 6 of CIC assay follow.

Isolation and quantitative determination of BDV-specific antigens
Native N- and P-proteins were purified from 20% (w/v) brain suspension of a horse with BD by stepwise centrifugation at 4°C (10 min at $1000 \times g$, 10 min at $15\,000 \times g$, 1 h at $100\,000 \times g$). From the 10-fold concentrated supernatant (10 h, $5000 \times g$, 4°C; Centricon 10-tubes, Millipore Corporation, Bedford, MA, USA), the proteins were isolated by affinity chromatography, using the above moAbs (see CIC assay) which were cross-linked to protein G-Sepharose 4 Fast Flow beads (1 mg ml^{-1}) (Amersham Pharmacia Biotech, Buckinghamshire, UK) by dimethyl suberimidate (22 mM in 0.2 mM disodium tetraborate/NaOH, pH 9.0). Elution was done by a linear concentration gradient from 0 mM to 2000 mM of aqueous $MgCl_2$ solution. Purified N- and P-proteins were used to determine the binding capacity of the antigen assay. Protein concentrations of geometrical dilutions of 0.1 to 90 000 ng ml^{-1} were measured by spectroscopy at 205 nm (peptide linkage) and 595 nm (Bradford assay) *vs* serum albumin standards, before and after incubation in microtiter modules coated with moAbs (see step 3, EIAs). The specifically bound protein amount was calculated by subtraction analysis of two corresponding values. The resulting calibration diagram (Figure 6) allows an estimation of pAg concentration in blood samples.

Competitive inhibition of plasma antigen by natural antibodies
Plasma samples from an acutely sick horse, with an EIA-determined antigen content of 1–2 ng per 100 μl were diluted (1:2, 1:4, 1:8, 1:16) in either plasma from an uninfected antibody-negative horse or from an acutely depressed antibody-positive (EIA-determined titer of 1:50 000) human patient. After incubation for 2 h at 37°C, followed by 4°C overnight, the mixtures and the untreated sample were assayed for antigen by EIA (Figure 7). This basic experiment has been repeated using different dilutions of either antibody samples.

Genetic analysis of plasma samples
Samples had been stored at −20°C for up to 7 years. Of group I (Table 2) which underwent considerable reinvestigation by EIA methods described here, samples had been repeatedly defrosted and frozen prior to molecular analysis.

Total RNA was extracted from 100 μl plasma by TRIZOL reagent according to instructions of the manufacturer (Life Technologies, Paisley, Scotland, UK), and re-suspended in 50 μl RNase-free water. Reverse transcription (RT) of RNA samples, PCR reactions, and primers to amplify ORF p40 and p24 fragments were modifications of a previously published protocol.[5] RNA (2 μg in 8 μl) was denatured for 4 min at 75°C and reverse transcribed by 200 U M-MLV revertase (Life Technologies) in first strand buffer for 1 h at 37°C, in the presence of 10 mM DTT, 0.2 mM dNTPs (MBI Fermentas), 40 U RNasin (Promega), and 2 μM specific (external) primer, in a total volume of 20 μl. RT reaction was stopped at 90°C for 5 min. External primers for ORF p40 were 5′GTCACGGCGCGATATGTTTC3′ (242F; 38A) with anti-genomic polarity and 5′CTTCTTACTCCAGTAAAACGC3′ (989R; 38B) with genomic polarity, corresponding to nt 242–989 of the BDV genome.[27] Primers for ORF p24 were 5′AGACACTACGACGGGAACGA3′ (1327F; 24A) with anti-genomic polarity and 5′TGGGAGCTGGGGATAAATGC3′ (1838R; P2) with genomic polarity, corresponding to nt 1327–1838 of the BDV genome[27] (Roth GmbH, Germany). In the first PCR (50 μl volume), 2 μl of the RT product were amplified in the presence of 0.2 mM dNTPs, 0.4 μM external primers each (anti-genomic + genomic), and 1.25 U Taq polymerase (Perkin Elmer) in PCR buffer mix, by the following cycling protocol: 1 cycle at 95°C for 3 min, then 33 cycles of 94°C for 1 min, 54°C (ORF p40) or 52°C (ORF p24) for 1 min, 72°C for 1.5 min (synthesis), and finishing with 1 cycle at 72°C for 10 min, yielding PCR products of 748 base pairs (bp) and 463 bp of ORF p40 and p24, respectively. In the nested-PCR (50 μl volume), 0.5 μl of the first PCR were amplified using 0.4 μM internal primers and the following alternative cycling protocol: 1 cycle at 95°C for 3 min, then 40 cycles of 94°C for 1 min, 52°C (ORF p40) or 58°C (ORF p24) for 1 min, 72°C for 1.5 min, and finishing with 1 cycle at 72°C for 10 min, yielding nested PCR products of 441 bp and 270 bp of ORF p40 and p24, respectively. Internal primers for ORF p40 were 5′-GCCTTGTGTTTCT ATGTTTG3′ (277F; 186) with anti-genomic polarity and 5′ATTCTTTACCTGGGGACTCA3′ (717R; 606) with genomic polarity, corresponding to nt 277–717 in

the BDV genome.[27] Internal primers for ORF p24 were 5′GCATGATCGAGGCTGAGGAG3′ (1528F; P3) with anti-genomic polarity and 5′GCAACATGGGTGCA-GAGGTC3′ (1797R; P4) with genomic polarity, corresponding to nt 1528–1797 in the BDV genome.[27]

RNA quality controls were done by amplification of the house-keeping gene for β-actin as described.[5] BDV-specific nested products detected by ethidium bromide staining were isolated from the reaction mixture by the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A mixture of 10 μl cDNA (500 ng) and 1 μl primer solution (10 pmol μl^{-1}) was used for direct sequencing analysis by ABI377A-Dye Terminator. For each sample the sequence was independently determined in triplicate using the computer program ABI PRISM Model 377 Version 2.1.1. The range of analyzed sequences was 392 bp for the N protein (nt 320–711) and 221 bp for the P protein (nt 1571–1791).

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