JOURNAL OF VIROLOGY, Nov. 1996, p. 7713–7724 Vol. 70, No. 11
0022-538X/96/$04.00+0
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Detection of Borna Disease Virus (BDV) Antibodies and BDV RNA in Psychiatric Patients: Evidence for High Sequence Conservation of Human Blood-Derived BDV RNA

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Received 25 April 1996/Accepted 6 August 1996

In several vertebrate species, Borna disease virus (BDV), the prototype of a new group of animal viruses, causes central nervous system disease accompanied by diverse behavioral abnormalities. Seroepidemiological data indicate that BDV may contribute to the pathophysiology of certain human mental disorders. This hypothesis is further supported by the detection of both BDV antigens and BDV RNA in peripheral blood mononuclear cells (PBMCs) of patients with psychiatric disorders and the isolation of BDV from such PBMCs. Here we describe serological and molecular epidemiological studies on psychiatric patients and healthy individuals from the area of Homburg, Germany. Using a novel Western blot (immunoblot) assay, we found a BDV seroprevalence of 9.6% among 416 neuropsychiatric patients, which is significantly higher than the 1.4% found among 203 healthy control individuals. Human sera displayed a prominent immunoreactivity against the virus nucleoprotein, the p40 antigen. Reverse transcriptase-mediated PCR analysis of RNA extracted from PBMCs of a subset of 26 of the neuropsychiatric patients revealed that 50% were BDV RNA positive. Three of the 13 BDV RNA-positive patients also had BDV-positive serology, whereas one patient with serum antibodies to BDV p40 antigen did not harbor detectable BDV RNA in PBMCs. BDV p40 and p24 sequences derived from human PBMCs exhibited both a high degree of inter- and intrapatient conservation and a close genetic relationship to animal-derived BDV sequences.

Clinical and epidemiological evidence together with virological studies indicate that viruses can contribute to the pathophysiology of neuropsychiatric disorders whose etiology remains elusive (35, 56, 60).

Borna disease virus (BDV) is an enveloped nonsegmented, negative-stranded RNA virus (8, 13, 14). Replication and transcription of the BDV genome take place in the nuclei of infected cells (7, 13), and RNA splicing contributes to BDV gene expression regulation (15, 48). These features, unique among the members of the *Mononegavirales*, signal BDV as the prototype of a new group of animal viruses (16, 46). The BDV genome contains five open reading frames (ORFs), I to V (8, 13). As yet, viral polypeptides with predicted molecular masses of 40, 24, and 16 kDa corresponding to ORFs I, II, and III, respectively, have been detected in BDV-infected cells and tissues.

In several vertebrate species, BDV causes central nervous system disease characterized by behavioral abnormalities and diverse pathological manifestations, depending on the species, age, and immune status of the host, as well as the particular virus strain and route of infection (32, 33, 40, 41). This wide host range and the observation that BDV-induced behavioral disturbances in animals resemble some types of affective disorders in humans (52) prompted studies aimed at determining a possible association between BDV and human mental disorders.

Seroepidemiological studies show a significantly higher seroprevalence of BDV in patients with neuropsychiatric disorders than in healthy individuals (2, 5, 18, 43, 57). The detection of BDV RNA in peripheral blood mononuclear cells (PBMCs) from neuropsychiatric (6, 26) and chronic fatigue syndrome (39) patients, together with the recent isolation of infectious BDV from PBMCs of three psychiatric cases (17), provides additional support for the hypothesis that BDV may contribute to certain human mental disorders. However, little is known about which viral antigens are recognized by BDV-positive human sera and the correlation between detection of anti-BDV antibodies in serum and viral RNA in PBMCs. Moreover, there is only very limited information on BDV sequences derived from PBMCs of psychiatric patients from different geographic areas and on the level of divergency between human- and animal-derived BDV sequences (4, 6, 17, 24, 47).

Here we describe the development of a new Western blot (immunoblot) assay for the detection of human anti-BDV antibodies, using as target antigens recombinant BDV proteins expressed in insect cells. Using this assay, 40 (9.6%) of 416 serum specimens from psychiatric patients from the area of Homburg, Germany, were found to be BDV seropositive, compared with only three (1.4%) of 203 serum specimens of a control group from the same area. The majority of these human sera positive for BDV recognized only the BDV p40 antigen. Reverse transcriptase (RT)-mediated PCR (RT-PCR)

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TABLE 1. Assignment of psychiatric patients' diagnoses to seven main categories and diagnoses of seropositive patients

Diagnostic category according to ICD-9	No. of patients[a]	No. of seropositive patients
1 (mental disorders due to organic factors)		
290.0–290.8[b]		
293.0–293.9	34	7
294.0–294.9		
310.0–310.9		
2 (schizophrenia and other psychotic disorders)		
295.0–295.9		
297.0–297.9	114	16
298.0–298.9		
3		
Affective psychoses		
296.1		
296.8		
296.9	52	6
Manic and bipolar affective disorder		
296.0		
296.2–296.6		
4 (neurotic, personality, and adjustment disorders)		
300.0–300.9		
301.0–301.9	54	8
308.0–308.9		
309.0–309.9		
313.0–313.9		
5 (mental disorders related to drugs and alcohol)		
291.0–291.9		
292.0–292.9	22	1
303.0		
304.0–304.9		
305.0–305.9		
6 (mental retardation)		
315.0–315.9	2	0
317.0		
318.0–318.2		
7 (Not otherwise specified mental disorders)		
306.0–306.9		
307.0–307.9	20	2
333.6		
333.8		

[a] Diagnoses from 298 of 416 patients were available.
[b] Four-digit ICD-9 code for diagnosis.

analysis of RNA extracted from PBMCs of a subset of 26 psychiatric patients revealed 13 of them to be BDV RNA positive. Among the BDV RNA-positive patients, only three had serum antibodies to BDV antigens detectable by Western blotting. In contrast to a recent report (24), we found that BDV p40 and p24 sequences derived from PBMCs of neuropsychiatric patients exhibit a high degree of inter- and intrapatient conservation and also are genetically very closely related to BDV sequences of animal origin.

MATERIALS AND METHODS

Subjects. Serum specimens from 416 unselected and consecutively admitted psychiatric in patients from the Department of Psychiatry of the University Hospital in Homburg, treated for a variety of psychiatric diseases (Table 1), as well as from 203 surgery patients (matched by age and sex to the first 203 psychiatric patients) from the same hospital, were evaluated for BDV serology. The group of psychiatric patients consisted of 203 males and 213 females. The mean age was 44.85 years (standard deviation, 40.01 years; range, 18 to 85 years). Diagnostic classification was made at discharge according to ICD-9 (10) and was blind regarding BDV data. The four-digit ICD-9 subclasses were condensed to seven main diagnostic categories (Table 1). All categories had comparable numbers of patients.

RT-PCR studies were performed on 28 blood samples from 26 of the psychiatric patients (21 males and 5 females) and 23 blood samples from healthy volunteers from Homburg. Patient blood samples used for RT-PCR and serology were taken at admission.

Generation of recombinant baculoviruses. BDV p40, p24, and p16 genes were amplified by PCR from the respective cDNAs (14). The primers used for PCR amplification were p40 (5′-ATGCCACCCGGGAGACGCCTGATTGAT-3′, 5′-CGGGATCCCGGGCTAGTTTAGACCAGTCACTCC-3′), p24 (5′-GGCCATATGCGCCCGGGCCCATCGAGTCTGGTCGACTCCCTG-3′, 5′-CTCGAGCCCGGGTTATGGTATGATGTCCCACTCATC-3′), and p16 (5′-CGAATCCCCGGGAATTCAAAGCATTCCTA-3′, 5′-TCCCCCCGGGCAGTATTGCAACTAACGG-3′). Underlined nucleotides indicate BDV-specific sequences. Amplified DNA fragments were digested with *Xma*I and ligated into the baculovirus transfer vectors pAC409 (p40 and p16) and pAC401 (p24) (54) linearized with *Xma*I. Recombinant baculoviruses were generated by cotransfection of *Spodoptera frugiperda* SF158 insect cells with the respective recombinant baculovirus transfer vectors and linearized BaculoGold DNA (Pharmingen, La Jolla, Calif.) as a source of baculovirus DNA, using Lipofection as described previously (21, 23). SF158 cells were maintained in TC100 medium supplemented with penicillin (40 IU/ml) and streptomycin (50 μg/ml) (54). The expression of the different recombinant proteins in insect cells was verified by Western blot analysis using polyclonal rabbit sera specific for each protein (see below).

Generation of specific rabbit antisera. BDV p40, p24, and p16 polypeptides were expressed as TrpE fusion proteins in *Escherichia coli* BL21/DE3 (53), using the pATH vector system (28). An N-terminal 826-bp fragment of the BDV p40 gene was excised from pCRII-p40 with *Xba*I-*Hin*dIII and ligated into *Xba*I-*Hin*dIII-digested pATH2 DNA. The complete p24 ORF was excised from pCRII-p24 with *Eco*RI and ligated to *Eco*RI-digested pATH1 DNA. *Xma*I-digested PCR-amplified DNA corresponding to full-length p16 ORF was inserted into *Xma*I-digested pATH2 DNA. Protein extracts from bacteria expressing the TrpE fusion proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fusion proteins were recovered from the gel by using 50 mM NH_4HCO_3–3% β-mercaptoethanol as the elution buffer. Freeze-dried proteins (25 to 50 μg) were emulsified in 1 ml of phosphate-buffered saline (PBS) and 1 ml of complete or incomplete Freund's adjuvant. For each BDV protein, two rabbits were immunized by intradermal injection and subsequently boosted subcutaneously at 3-week intervals with 25 to 50 μg of antigen in Freund's incomplete adjuvant. Blood was collected three times during weeks 3 and 14, and the serum was analyzed for the presence of BDV-specific antibodies by Western blotting.

Detection of BDV-specific antibodies. Lysates of insect cells infected with either recombinant baculoviruses or wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus) as a negative control were separated by SDS-PAGE (12 or 15% gel) as described previously (20). After electrophoretic transfer, the nitrocellulose membrane (0.2-μm pore size; Schleicher & Schuell Inc., Keene, N.H.) was preincubated at 25°C for 30 min in blocking solution (5% nonfat dry milk in PBS). After being cut in strips, the membranes were incubated with the human sera (diluted 1:20) or the rabbit sera (diluted 1:100) in blocking solution overnight at 4°C. Membranes were washed three times in PBS, incubated for 1 h at 25°C with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-human or goat anti-rabbit immunoglobulin G [Sigma, Munich, Germany]), diluted 1:500 in blocking solution. After three washes with PBS, bound antibodies were visualized either with diaminobenzidine in the presence of hydrogen peroxide or by the enhanced chemiluminescence method, using an ECL kit (Amersham Buchler, Braunschweig, Germany).

For competition experiments, approximately 10^7 baculovirus-infected SF158 cells were washed twice in ice-cold PBS and resuspended in 1 ml of lysis buffer (100 mM Tris-HCl [pH 9.0], 100 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin). After 15 min on ice, debris were pelleted (30 min, 4°C, 10,000 × *g*), and 300 μl of clarified lysate was added to 100 μl of serum. The mixture was kept at 4°C for 8 to 10 h, mixed with blocking solution, and incubated overnight with the nitrocellulose membrane strip. The membrane was then processed as described above.

Preparation of PBMCs. Blood samples (15 to 20 ml) collected in the presence of EDTA as anticoagulant were used to purify PBMCs, using Ficoll-Paque Plus (Pharmacia, Freiburg, Germany). PBMC samples were washed twice with RPMI and finally resuspended in 3 to 4 ml of 90% fetal calf serum–10% dimethyl sulfoxide on ice. Aliquots of 1 to 1.5 ml were stored frozen in liquid nitrogen until use.

Preparation of RNA. Total RNA extraction from PBMCs was performed by using either an RNA Easy Purification kit (Qiagen, Hilden, Germany) in the Homburg laboratory as instructed by the manufacturer or TRI reagent (MRC Inc., Cincinnati, Ohio) in the La Jolla laboratory. Briefly, cell pellets were lysed in 1 ml of TRI reagent, and 20 μg of oyster glycogen was added as carrier prior to extraction of the RNA. RNA samples were dissolved in 10 μl of chelexed diethylpyrocarbonate-treated double-distilled H_2O and stored at −70°C until use.

TABLE 2. Primers used for nested PCR for detection of BDV p40 and p24 cDNAs

Gene	PCR round	Primer[a]	Sequence	Nucleotide positions in BDV RNA genome
p40	1st	BV259F	5′-TTCATACAGTAACGCCCAGC-3′	259–278
		BV829R	5′-GCAACTACAGGGATTGTAAGGG-3′	829–808
	2nd	BV277F	5′-GCCTTGTGTTTCTATGTTTGC-3′	277–297
		BV805R	5′-GCATCCATACATTCTGCGAG-3′	805–766
p24	1st	BV1387F	5′-TGACCCAACCAGTAGACCA-3′	1387–1405
		BV1865R	5′-GTCCCATTCATCCGTTGTC-3′	1865–1847
	2nd	BV1443F	5′-TCAGACCCAGACCAGCGAA-3′	1443–1461
		BV1834R	5′-AGCTGGGGATAAATGCGCG-3′	1834–1816

[a] F and R indicate the antigenomic and genomic, respectively, polarities.

Detection of BDV RNA sequences in PBMCs by RT-PCR. Total RNA (1 to 2 μg) was reverse transcribed by using 200 U of Superscript II RNase H^- RT (Gibco BRL, Gaithersburg, Md.) in a total volume of 20 μl using either random hexanucleotides at 14 pmol/μl or a BDV p40-specific primer, BV829R (Table 2), at 10 μM. Reverse transcription reactions were also primed with oligo(dT) primers at 2.5 μM in the Homburg laboratory as described previously (29).

PBMC cDNAs were screened for the presence of BDV p40 and p24 sequences by nested PCR. Primer pairs used for first and second rounds of BDV p40 and p24 PCR are indicated in Table 2. The conditions for both BDV p40 PCR rounds were as follows: 94°C for 2 min (1 cycle) and 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min (40 cycles in the La Jolla laboratory; 50 cycles in the Homburg laboratory). The conditions for both BDV p24 PCR rounds were as follows: 94°C for 3 min (1 cycle) and 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (50 cycles). PCRs were completed by a final extension round of 10 min at 72°C. For the first round of p24 and p40 PCR, 4 to 10 μl of the cDNA product was used; for the second round of p24 and p40 PCR, 2 μl of the first-round PCR product was used. Each PCR used primers at 0.2 μM, deoxynucleoside triphosphates at 200 μM, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 U of *Taq* polymerase (Boehringer Mannheim) in a final volume of 50 or 100 μl.

Total RNA was also used to generate cDNAs with either a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primer or oligo(dT) primers, followed by PCR amplification with specific primers to generate a 192-bp GAPDH fragment as described previously (9).

PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and then subjected to Southern blot hybridization using a BDV p40- or BDV p24-specific probe. BDV p40 sequences (528 bp) were detected with a ^{32}P-labeled probe corresponding to an internal 415-bp p40 fragment (nucleotides 345 to 760 in the BDV RNA genome). BDV p24 sequences (391 bp) were detected with a p24-specific oligonucleotide (nucleotides 1627 to 1647 of the BDV genome) ^{32}P labeled with terminal deoxynucleotidyl-transferase. BDV p40 and p24 sequences were also detected with a p40 fragment (nucleotides 277 to 805 of the BDV genome) or a p24 fragment (nucleotides 1443 to 1834 of the BDV genome), both amplified by PCR in the presence of digoxigenin-dUTP for nonradioactive detection using a nonradioactive DNA labeling and detection kit (Boehringer Mannheim).

In vitro transcription of RNA. A plasmid containing a full-length p24 ORF (strain C6BV) cloned in pCRII was linearized with *Bam*HI and in vitro transcribed to generate a sense p24 RNA, using an mMESSAGE mMACHINE-T7 kit (Ambion Inc., Austin, Tex.). In vitro-transcribed p24 RNA was analyzed by agarose gel electrophoresis, and its concentration was estimated by ethidium bromide staining and comparison with calibrated markers.

Nested p24 RT-PCR using r*Tth* DNA polymerase. Approximately 10^4 RNA molecules of in vitro-transcribed p24 RNA mixed with 5 μg of total RNA prepared from the human histiocytic lymphoma cell line U-937 (ATCC CRL 1593 [55]) was used for a reverse transcription reaction, followed by an amplification reaction of p24 cDNA sequences by using recombinant *Tth* (r*Tth*) DNA polymerase and an EZ buffer pack (Perkin-Elmer, Branchburg, N.J.) with p24-specific primers BV1387F and BV1865R (Table 2) and conditions as described previously (26). A second-round PCR using 5 μl of the RT-PCR product, also using r*Tth* DNA polymerase, the EZ buffer pack, and primers BV1443F and BV1834R (Table 2), was performed as described previously (26). The same amount of in vitro-transcribed p24 RNA, also mixed with 5 μg of U-937 total RNA, was used for a p24 RT-PCR using the conditions described above for the analysis of PBMCs.

Cloning and sequencing of PCR products. p24 and p40 PCR products were cloned in the pCRII vector by using a TA cloning kit (Invitrogen, San Diego, Calif.), in a pMOS vector by using a pMOSBlue T-Vector kit (Amersham Buchler), or in a pNOTA/T7 vector by using the Prime PCR Cloner cloning system (5prime/3prime Inc., Boulder, Colo.). One to three clones of each cloned product were sequenced with Sequenase version 2.0 (U.S. Biochemical Corporation, Cleveland, Ohio). Sequencing of uncloned PCR product was performed by using a Abi Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer).

RESULTS

Establishment of a Western blot assay to detect antibodies to BDV antigens. To detect anti-BDV antibodies present in human sera, we developed a Western blot assay using as target antigens BDV p40, p24, and p16 proteins expressed in insect cells by using the baculovirus system. The expression of these proteins was verified by using rabbit antisera raised against each of the three BDV proteins synthesized in *E. coli* as TrpE fusion proteins (Fig. 1). The p40 and p24 proteins migrated with their expected electrophoretic mobilities, although in the case of p24 protein, we observed a doublet. Whether the lower p24 band represents a degradation product or differences in posttranslational modifications is unknown. The p16 protein encoded by BDV ORF III has recently been shown to be glycosylated in vivo, thus migrating with an apparent molecular mass of 18 kDa (27). However, in our system, p16 exhibited an apparent molecular mass of about 22 kDa, probably as a result of a glycosylation pattern characteristic for insect cells (23, 36). The specificity of the immunoreactivity exhibited by the sera from rabbits immunized with recombinantly expressed BDV proteins was supported by results obtained in assays using a serum of a rabbit acutely infected with BDV. This serum recognized both p40 and p24 antigens, but it did not react with

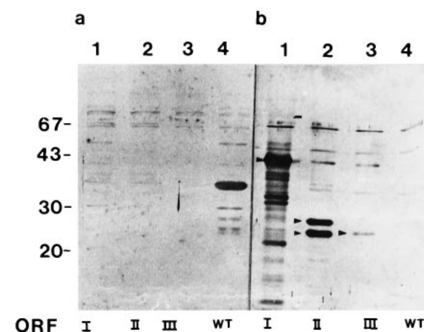


FIG. 1. Expression of BDV p40, p24, and p16 proteins in insect cells. ORFs I to III of BDV, corresponding to proteins with predicted molecular masses of 40, 24, and 16 kDa, respectively, were expressed in insect cells by using the baculovirus system. Lysates of infected SF158 insect cells were separated by SDS-PAGE (12% gel) and transferred to nitrocellulose, and the recombinant proteins were visualized by using specific polyclonal rabbit antibodies at a dilution of 1:100. (a) Preimmune rabbit sera were mixed and incubated with a membrane containing extracts of cells infected with a wild-type (WT) baculovirus (lane 4) or with recombinant baculoviruses expressing the indicated BDV proteins. (b) In parallel, a second membrane strip containing the same set of samples was incubated with a mixture of specific polyclonal rabbit antisera directed against BDV p40, p24, and p16 proteins. The positions of the different recombinant BDV proteins are indicated by arrowheads. The electrophoretic mobilities and sizes (in kilodaltons) of molecular mass marker proteins (Pharmacia) are indicated on the left.

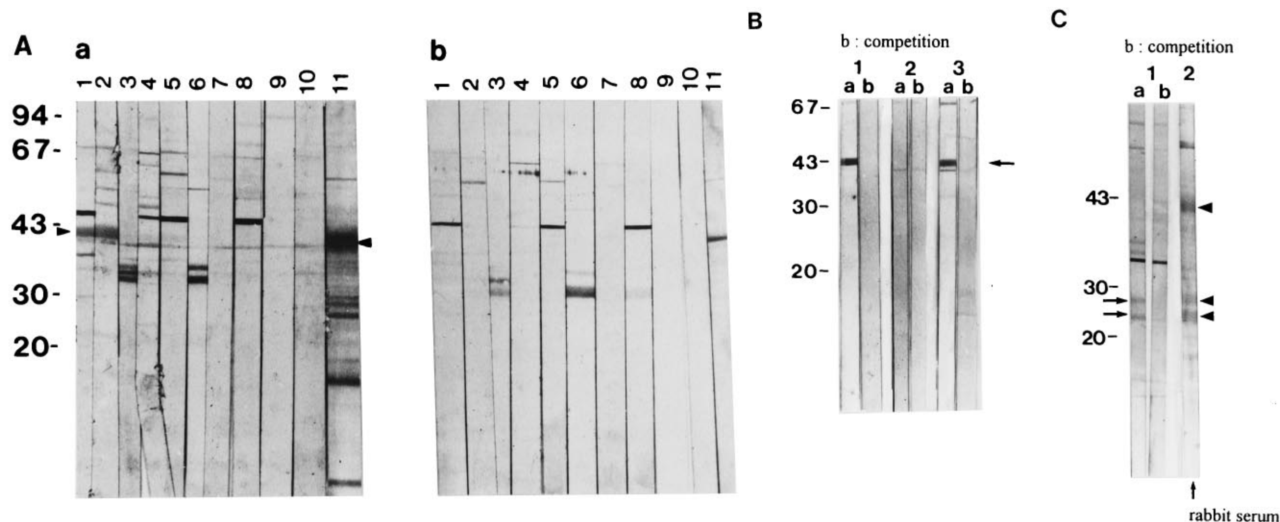


FIG. 2. Detection in human sera of antibodies directed against BDV proteins. (A) Extracts from wild-type (b) and BDV p40 recombinant (a) baculovirus-infected cells were separated by SDS-PAGE (12% gel) and analyzed by Western blotting. Pairs of membrane strips, corresponding to extracts from wild-type and p40 recombinant baculovirus-infected cells, were reacted in parallel with human sera at a dilution of 1:20. Lanes 1 to 11 show immunoreactivities of 10 representative human sera (lanes 1 to 10) and an anti-p40 rabbit serum (lane 11). Lanes 1 and 2 illustrate representative immunoreactivities of p40-positive human sera; lanes 3 to 10 illustrate BDV-negative human sera. Electrophoretic migration of p40 is indicated by arrowheads. (B) Specificity of human sera p40 immunoreactivity. The specificity of the human sera was demonstrated by competition experiments using soluble p40 antigen. Lanes 1 and 3 show the reactions of two positive sera; lane 2 shows the reaction of a negative serum. The position of p40 is indicated by the arrow. Lanes a and b show the immune reactivity before and after, respectively, preincubation of the sera with soluble p40 antigen. Preincubation of the sera with extract from wild-type virus-infected insect cells did not compete for the binding of the human antibodies to p40 (data not shown). (C) Human serum p24 immunoreactivity. A mixture of extracts from BDV p40 and p24 recombinant baculovirus-infected cells was separated by SDS-PAGE (12.5% gel) and analyzed by Western blotting. The positions of the recombinantly expressed p40 and p24 proteins were detected by using a mixture of p40- and p24-specific rabbit antisera (lane 2). Lanes 1a and 1b show the immunoreactivities of a human serum with recombinantly expressed p24 before and after, respectively, preincubation of the serum with soluble p24 antigen. Preincubation of the serum with extract from wild-type virus-infected insect cells did not compete for the binding of the antibodies to p24 (data not shown). Incubation of the human serum with a membrane strip containing electrophoretically separated extract from wild-type baculovirus-infected cells did not show any immunoreactivity with a protein of 24 kDa (data not shown). Molecular mass marker proteins (positions indicated in kilodaltons) were the same as used for Fig. 1. Positions of BDV p24 (double band) and p40 proteins are indicated by arrows on the left and arrowheads on the right, respectively.

p16 (data not shown). In addition, recombinant p40 and p24 BDV antigens were recognized by a serum from a mouse infected with BDV (data not shown).

Detection of antibodies to BDV proteins in human sera. We tested a total of 416 serum specimens from patients treated at the Department of Psychiatry of the University Hospital in Homburg for a variety of mood disorders (Table 1) for the presence of BDV-specific antibodies. Serum specimens were scored as positive only when their corresponding immunoreactivities in Western blots could be competed against by preincubation with extracts from insect cells infected with a recombinant baculovirus expressing the respective viral antigen (Fig. 2). Extracts from insect cells infected with wild-type baculovirus were used as a negative control for the competition experiments (data not shown). Of the 416 serum specimens tested, we found 40 (9.6%) positive for BDV. One serum reacted exclusively with p24, two sera reacted with both p40 and p24, and 37 sera showed reactivity only with p40. No serum specimen with antibodies to p16 was detected. Of the 40 seropositive patients, 24 were male and 16 were female. This sex ratio difference is not significant ($\chi^2 = 0.136$). Figure 2 shows representative examples of Western blot analyses. As a control group, we tested sera of 203 age- and sex-matched control patients treated in the University Hospital in Homburg for surgical procedures or diseases unrelated to viral infections or mood disorders. Among these sera, we detected only three (1.4%) BDV-positive samples, and these reacted exclusively with p40. The diagnoses of all seropositive psychiatric patients and all diagnoses available from the seronegative patients are listed in Table 1. The sevenfold-increased BDV seroprevalence found in psychiatric patients is statistically significant ($\chi^2 = 0.001$), but there was no correlation between BDV seropositivity and any specific diagnosis.

Detection of BDV RNA sequences in human PBMCs. To investigate the prevalence of BDV RNA in PBMCs from psychiatric patients, we used RT-PCR procedures to detect BDV p40 and p24 RNA sequences. Twenty-eight blood samples from 26 patients with various psychiatric diseases (Table 3) were collected. From one patient, we examined three blood samples taken at admission and 16 and 19 weeks after hospitalization. In addition, blood samples from 23 healthy volunteers from the Homburg area were also collected. PBMCs from all 51 blood samples were prepared in the Department of Virology of the University of the Saarland in Homburg and coded, and aliquots from each PBMC sample were independently analyzed by RT-PCR in Homburg and at The Scripps Research Institute in La Jolla. Total RNA isolated from PBMCs was reverse transcribed and then analyzed by nested PCR for the presence of BDV p40 and p24 RNA sequences (Table 3). As a control for RNA quality, we analyzed all PBMC samples for the presence of GAPDH RNA.

After performance of the p40-specific RT-PCR, a predicted 528-bp DNA fragment was detected by ethidium bromide staining in 8 of the 28 PBMC samples analyzed in the La Jolla laboratory (Fig. 3A) and in six of 25 samples analyzed in the Homburg laboratory (Table 3). Southern blot hybridization confirmed the specificity of the RT-PCR products (Fig. 3A and data not shown). RT-PCR for p40 on sample 30 resulted in amplification of a p40-specific 528-bp DNA fragment detectable only by Southern blot hybridization (Fig. 3A, lower panel,

TABLE 3. Summary of RT-PCR and Western blot analyses of 28 blood samples from 26 psychiatric patients

Patient code (sex[a])	RT-PCR[b]				Western blot[c]	Diagnosis[d]
	La Jolla		Homburg			
	p40	p24[e]	p40	p24		
1 (M)	+	NA	+	+	+ (p40)	1 (310.1)
2 (M)[f]	+	NA	NA	+	+ (p40)	3 (296.1)
3 (M)	+	NA	+	+	−	2 (295.3)
4 (M)	+	NA	NA	NA	−	4 (300.4)
5 (F)	+	NA	+	+	−	2 (297.0)
6 (M)	−	NA	−	−	−	2 (295.3)
7 (M)	−	NA	−	−	−	7 (333.8)
8 (M)	−	NA	−	−	−	3 (296.1)
9 (M)	−	NA	−	−	−	4 (300.4)
10 (M)	(+) del	NA	−	−	−	4 (300.4)
11 (M)	+	NA	−	+	−	4 (300.0)
12 (M)	−	NA	−	−	−	4 (300.5)
13 (M)	−	NA	−	−	−	4 (309.1)
14 (M)	−	NA	−	+	−	4 (300.4)
18 (M)[f]	+	NA	+	+	+ (p40)	3 (296.1)
19 (M)	−	−	+	+	−	2 (295.9)
20 (M)	−	NA	−	−	−	4 (309.1)
21 (F)	−	−	−	+	+ (p24)	2 (297.8)
22 (M)	−	NA	−	−	−	4 (300.4)
23 (F)	−	NA	−	−	−	2 (295.3)
24 (M)	−	−	−	+	−	2 (295.6)
25 (F)	−	−	−	−	+ (p40)	3 (296.1)
26 (M)	+	−	+	+	−	2 (295.3)
27 (M)	−	NA	−	−	−	2 (295.3)
28 (M)	−	NA	−	−	−	4 (300.0)
29 (M)	−	NA	−	−	−	2 (295.3)
30 (F)	(+)	NA	−	−	−	2 (295.3)
31 (M)[f]	+	+	NA	NA	+ (p40)	3 (296.1)

[a] M, male; F, female.
[b] (+), signal visible only after Southern blot hybridization; del, deleted p40 DNA fragment; NA, not analyzed.
[c] Antibody reaction against BDV p40 or p24 is indicated in parentheses.
[d] Diagnosis as assigned to the seven main categories (Table 1) and, in parentheses, ICD-9 code number.
[e] In six of the PBMC samples analyzed for p40 in La Jolla, random hexamers were used to prime the RT. These RT products were reanalyzed in a p24-specific nested PCR.
[f] Samples taken from the same patient at different time points.

lane 30). In case of sample 10, shown as p40 negative in Fig. 3A, lane 10, additional independent RT-PCR assays detected by Southern blot hybridization a specific signal that corresponded to a deleted form of p40 (Table 3). In addition, repetition of the p40 nested PCR with the RT product of sample 1 (Fig. 3A) in La Jolla, using 50 instead of 40 cycles, amplified a 528-bp fragment (Table 3) confirmed to be p40 specific by Southern blot hybridization (data not shown). Only two PBMC samples, 11 and 19, were found p40 positive solely in one of the two laboratories (Table 3).

The p24-specific RT-PCR yielded a predicted 391-bp DNA fragment detected by ethidium bromide staining in 11 of 26 PBMCs analyzed in the Homburg laboratory (Fig. 3B). All signals were shown to be p24 specific by Southern blot hybridization (Fig. 3B). All seven PBMCs samples found p40 positive by ethidium bromide staining in La Jolla were also found to be positive for p24 in Homburg. In addition, all six p40-positive samples analyzed in Homburg were also p24 positive at the same location. PBMC samples 2, 18, and 31, obtained at different times from the same patient, were positive for both BDV p40 and p24 (Table 3).

In all RNA samples analyzed by RT-PCR, we could amplify a 192-bp GAPDH fragment (Fig. 3A and data not shown). Some minor discrepancies found between RT-PCR results obtained in the two laboratories (Table 3) are likely related to differences in experimental procedures and quality of the RNA samples analyzed.

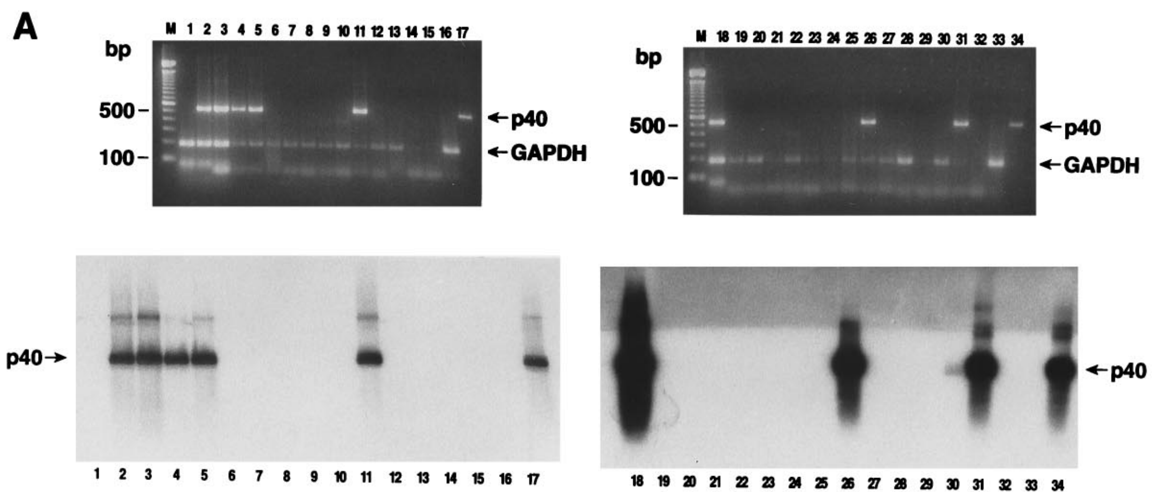
As a control group, we analyzed by RT-PCR 23 PBMC samples from healthy volunteers. All samples were analyzed for p40 in both laboratories and also for p24 in the Homburg laboratory. All samples were found to be negative for both p40 and p24 by ethidium bromide staining of the gel, whereas we could amplify the 192-bp GAPDH fragment in all RNA samples analyzed (data not shown).

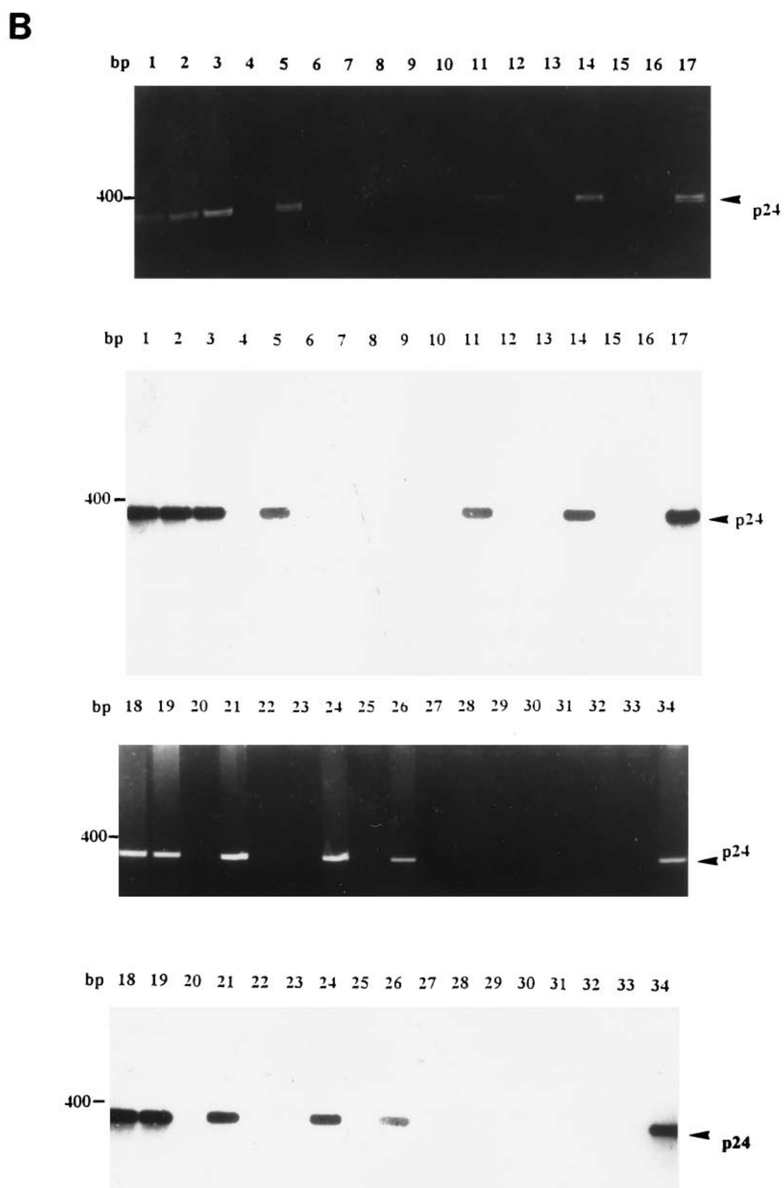
Sera from the 28 blood samples from the psychiatric patients investigated by RT-PCR were also analyzed in the Western blot assay for the presence of antibodies against BDV p40 or BDV p24 (Table 3). Only 6 of the 28 samples had anti-BDV antibodies. All three blood samples from the same patient positive by RT-PCR were also positive for anti-p40 antibodies. We found that a high proportion (9 of 13) of the patients found to be positive for BDV RNA did not have detectable serum antibodies to BDV (Table 3).

The diagnoses of the 26 patients could be mainly assigned to three (Table 3) of the categories indicated in Table 1. BDV RNA-positive patients were found in all three groups. A high percentage (7 of 11) of patients diagnosed with schizophrenia (category 2) were BDV RNA positive. There was no significant difference in the sex ratio among RT-PCR-positive patients.

Sequence analysis of the p40 and p24 RT-PCR products derived from human PBMCs. To determine the sequences of the amplified 528-bp p40 and 391-bp p24 fragments derived from human PBMCs, PCR products were cloned and the nucleotide sequences of one to three clones of each PCR product were determined. Results of sequence analyses are presented in Fig. 4. We sequenced 21 different p40 clones derived from eight different PBMC samples and 13 different p24 clones derived from seven different PBMC samples. In addition, one p24 sequence was derived by sequencing an uncloned PCR product (Fig. 4B). BDV sequences derived from human PBMCs displayed a high degree of conservation with respect to sequences of BDV strains V (8) and C6BV (14), two previously characterized viral sequences derived originally from two naturally BDV-infected horses (Table 4). In cases in which two or more clones of the same PCR product were sequenced, single-clone-specific substitutions and changes that affected all clones derived from the same PCR product were found (Fig. 4), with intrapatient divergencies shown in Table 4. In case of p40, no common mutation between two or more patient samples was found. We identified a mutation in p24 at nucleotide position 1579 in the BDV genome which was common to many but not all patients. Interpatient divergencies for p40 and p24 were only slightly higher than the respective intrapatient divergencies (Table 4). Substitutions in PBMC derived p40 and p24 sequences appeared randomly distributed, with no detectable clusters. To rule out the possibility of mutations introduced via nested PCR, p40 plasmid DNA of the C6BV sequence was amplified via nested PCR and cloned, and three individual clones were sequenced. PCR experimental conditions were identical to those described above for the analysis of PBMC samples. All three sequences were 100% identical to the expected C6BV sequence; thus, mutations due to experimental procedures seemed unlikely.

Compared with C6BV, 21 (72.4%) of 29 and 8 (80%) of 10 nucleotide substitutions found in PBMC p40 and p24 sequences, respectively, involved amino acid substitutions (Table 5), whereas compared with strain V, 45.7% of the 46 and 38.1% of the 21 nucleotide substitutions found in PBMC-derived p40 and p24 sequences, respectively, corresponded to


A
bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
500
100
p40
GAPDH
M 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
p40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34


B
bp 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
400
p24
bp 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

amino acid exchanges. Strains V and C6BV are identical at the amino acid level within the regions of p40 and p24 analyzed in this study. Hence, PBMC-derived sequences exhibited the same amino acid divergency with respect to both strains V and C6BV. The determined intra- and interpatient divergencies of the deduced amino acid sequences were slightly higher than those at the nucleotide level for both p40 and p24 (Table 4). Some of the amino acid exchanges found are nonconservative. The mutation in p24 at nucleotide position 1579 in the BDV genome found in the sequences of many patients corresponded to a conservative amino acid exchange (Arg to His). Amino acid substitutions common to all sequenced clones indicative of a specific human BDV strain could not be observed. Intra- and interpatient divergencies determined for both p24 and p40 did not reveal significant differences in divergence rates between the two BDV genes. p40 RT-PCR products of samples 2, 18, and 31, and p24 RT-PCR products of samples 18 and 31, all from the same patient, were sequenced. These samples were taken in intervals of 4 months (samples 2 and 18) or 3 weeks (samples 18 and 31). Comparison of the different sequences revealed minor fluctuations in the distribution of sequences present at a given time within the patient PBMCs. However, during the period of time separating the first and last sample analyzed, the consensus sequences of both p40 and p24 remained unchanged. Thus, no evolution of BDV p40 sequence was observed during 19 weeks of infection in the same patient.

DISCUSSION

Seroepidemiological studies indicate a significantly higher prevalence of BDV antibodies in neuropsychiatric patients than in healthy individuals (5). However, these studies have relied mostly on immunofluorescence assays (IFA) using BDV-infected cells (5), impeding the identification of the viral antigens recognized by the human sera. Moreover, very low BDV antibody titers in human sera (2, 5, 42, 43), together with the frequent finding in neuropsychiatric patients of autoantibodies that often have a staining pattern very similar to that characteristically associated with BDV infection (19, 30, 51), have contributed to misleading results in studies using IFA (58). Although there is a lack of consensus on a reliable serological method, a recent report suggested that Western blot assays may be more reliable than IFA for evaluating BDV serology (58). Therefore, we developed a Western blot assay using recombinant BDV p40, p24, and p16 polypeptides as sources of target antigens to detect antibodies in human sera directed against these viral proteins.

We detected antibodies that recognized BDV antigens in 40 9.6%) of 416 serum specimens from neuropsychiatric patients, whereas only 3 (1.4%) of 203 samples from the control group had BDV-positive serology. These findings are consistent with the previously reported higher seroprevalence of BDV among neuropsychiatric patients (5). Most of the BDV-positive human sera (37 of 40) recognized only BDV p40. Two serum specimens recognized both p40 and p24 antigens, whereas only one serum sample recognized exclusively p24, and none of the sera examined recognized BDV p16. Consistent with previously documented studies (2, 5, 18, 42, 43), our results indicated that positive human sera had very low titers of BDV antibodies. The possible contribution of immune complexes to this phenomenon cannot be excluded (11). The low serum dilutions (1:20) used in the Western blot assay probably contributed to the nonspecific staining of some proteins present in the extracts used as sources of target antigens. However, in all cases, the immunoreactivity of human sera with p40 and p24 BDV antigens could be blocked in competition experiments using soluble BDV antigens (Fig. 2 and data not shown). The very low number of BDV antibody-positive sera found in the control group provides additional support for the specificity of the reaction of the human antibodies with BDV p40 and p24 proteins.

In contrast to our findings, two other studies also using a Western blot assay documented that 6.5% and 14.4% of sera from patients with affective disorders (18) or schizophrenia (58), respectively, recognized more than one BDV antigen. Nevertheless, these studies also showed that a high percentage of control sera recognized a single BDV antigen. Sera from BDV-infected animals recognize most frequently both p40 and p24 BDV antigens and less often also p16 (33, 34, 44, 58). The reasons for the prominent immune response against BDV p40 in neuropsychiatric patients described here are unknown. It should be noted that p40 is the BDV counterpart of the nucleoprotein (NP) in other nonsegmented, negative-stranded RNA viruses (8, 14, 16, 46). In many cases, the NP is the virus gene product expressed at the highest levels during infection, and the infected host normally mounts a good antibody response against these viral NPs. We could not detect p16 immunoreactivity with any of the human sera analyzed. However, we cannot rule out the possibility that abnormal glycosylation of the p16 protein produced in the baculovirus expression system could alter or mask epitope recognition by human sera. Nevertheless, detection of human anti-p16 antibodies in a Western blot assay has been reported only in one study (58).

Our results did not reveal a distinct association of BDV seropositivity with a specific neuropsychiatric diagnosis. The diagnoses from 118 seronegative patients were not available; however, assuming that the distribution of diagnoses among these 118 patients would be similar to that found for the 298 characterized patients, we could then estimate a BDV seroprevalence of approximately 3% among patients with mental disorders related to drugs and alcohol abuse, which was not significantly different from that found in the control group (1.4%). The percentage (10%) of BDV-seropositive patients that we estimated among schizophrenic patients is similar to that recently reported by others (58).

FIG. 3. Detection of BDV p40 and p24 RNAs in PBMCs of psychiatric patients. Twenty-eight PBMC samples from 26 patients from the Homburg area with diverse psychiatric diseases were independently analyzed for the presence of BDV p40 RNA sequences in La Jolla (A) or BDV p24 RNA sequences in Homburg (B). RNA was extracted from PBMCs and reverse transcribed, and nested PCR was performed with BDV p40- or p24-specific sets of primers (Table 2). As a control for RNA quality, RT-PCR analysis for detection of the housekeeping cellular GAPDH mRNA was performed. RT-PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining (A, upper panels; B, first and third panels). PCR products were analyzed by Southern blot hybridization with a ^{32}P labeled p40 probe (A, lower panels) or a p24 probe labeled with digoxigenin (Boehringer Mannheim) (B, second and fourth panels). PCR products derived from patient PBMCs were applied in lanes 1 to 14 and 18 to 31, respectively. Lane numbers correspond to patients code numbers in Table 3. In lanes 4 and 31 (B), no PCR products were loaded, since the corresponding PBMC samples were not analyzed in the Homburg laboratory. Lanes 15 and 32 show negative RT-PCR controls, for which water instead of RNA was used. RT-PCR products obtained by using total RNA prepared from BDV-negative C6 cells (lanes 16 and 33) or from BDV persistently infected C6BV cells (lanes 17 and 34) were loaded as controls. In the upper panels of panel A, p40 nested RT-PCR products were mixed with the GAPDH RT-PCR products prior to agarose gel electrophoresis. The 528-bp p40 specific, 391-bp p24-specific, and the 192-bp GAPDH-specific fragments are indicated by arrows. Lanes M, (A, upper panels), 100-bp DNA ladder (Gibco BRL). Positions of coelectrophoresed DNA markers are indicated.

A

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C6BV        TAATCCCAGGACTGCACGCTGCGTTTGTTCACGGAGGGGTGCCTCGTGAATCTTACCTGTCGACGCCTGTTACGCGTGGGGAACAGACTGTCGTTAAGACTGCAAAGTTTTACGGGGAAAAGACA 125
Strain V    ....................................................C.................C........A...........T...........G....................G 125
P3-3,4      ............................................................................................................................. 125
P4-1        ........................................T.................................................................................... 125
P4-2        ........................................T.................................................................................... 125
P5-3        ..............................C.............................................................................................. 125
P5-4        ..............................C.............................................................................................. 125
P5-6        ..............................C.............................................................................................. 125
P11-2       ............................................................................................................................. 125
P11-3       ............................................................................................................................. 125
P26-1,3     ...................................T......................................................................................... 125
P26-2       ...................................T....................................................................................G.... 125
*P2-2,5     ............................................................................................................................. 125
*P2-6       ............................................................................................................................. 125
*P18-1      ........................................................................................................G.................... 125
*P18-2,11   ............................................................................................................................. 125
*P31-1      ......................................C........................................................................T............. 125
*P31-3      ............................................................................................................................. 125
*P31-4      .............................................................A............................................................... 125

C6BV        ACACAGCGTGATCTCACCGAGCTGGAGATCTCCTCTATATTCAGCCATTGTTGCTCATTACTAATTGGGGTTGTGATAGGATCGTCATCTAAGATTAAAGCAGGAGCCGAGCAGATCAAGAAAAG 250
Strain V    ..G...................................C..........................A....................G........C............................. 250
P3-3,4      ............................................................................................................................. 250
P4-1        ............................................................................................................................. 250
P4-2        ...................................A......................................................................................... 250
P5-3        ............................................................................................................................. 250
P5-4        ............................................................................................................................. 250
P5-6        ............................................................................................................................. 250
P11-2       ............................................................................................................................. 250
P11-3       ............................................................................................................A................ 250
P26-1,3     ............................................................................................................................. 250
P26-2       ............................................................................................................................. 250
*P2-2,5     ............................................................................................................................. 250
*P2-6       ........................................................................................................................G.... 250
*P18-1      ......................................................................C...................................................... 250
*P18-2,11   ......................................................................C...................................................... 250
*P31-1      ......................................T...................................................................................... 250
*P31-3      ............................................................................................................................. 250
*P31-4      ............................................................................................................................. 250

C6BV        GTTTAAAACTATGATGGCAGCCTTAAACCGGCCATCCCATGGTGAGACTGCTACACTACTTCAGATGTTTAATCCACATGAGGCTATAGATTGGATTAACGGCCAGCCCTGGGTAGGCTCCTTTG 375
Strain V    ............................................................C...........................................A.................... 375
P3-3,4      ....................................T........................................................................................ 375
P4-1        ...............................T......T................................................T..................................... 375
P4-2        ...............................T......T................................................T..................................... 375
P5-3        ............................................G................................................................................ 375
P5-4        .............................................................................G............................................... 375
P5-6        .................T.....C..................................................................................................... 375
P11-2       ..............................................................T...................T.......................................... 375
P11-3       ............................................................................................................................. 375
P26-1,3     ............................................................................................................................. 375
P26-2       ............................................................................................................................. 375
*P2-2,5     ............................................................................................................................. 375
*P2-6       ............................................................................................................................. 375
*P18-1      ............................................................................................................................. 375
*P18-2,11   ............................................................................................................................. 375
*P31-1      .......................................................................................................A..................... 375
*P31-3      ............................................................................................................................. 375
*P31-4      .....................................................................................................T....................... 375

C6BV        TGTTGTCTCTACTAACTACAGACTTTGAGTCCCCAGGTAAAGAATTCATGGATCAGATTAAACTTGTCGCAAGTTATGCGCAGATGACTACGTACACTACTATAAAGGAGTAC 488
Strain V    ..............................................T.....C........G.................A................................. 488
P3-3,4      ................................................................................................................. 488
P4-1        ................................................................................................................. 488
P4-2        ................................................................................................................. 488
P5-3        .............T................................................................................................... 488
P5-4        .............T................................................................................................... 488
P5-6        .............T................................................................................................... 488
P11-2       ................................................................................................................. 488
P11-3       ................................................................................................................. 488
P26-1,3     .T............................................................................................................... 488
P26-2       .T............................................................................................................... 488
*P2-2,5     ................................................................................................................. 488
*P2-6       ................................................................................................................. 488
*P18-1      ...................................................................................T............................. 488
*P18-2,11   ................................................................................................................. 488
*P31-1      A................................................................................................................ 488
*P31-3      ................................................................................................................. 488
*P31-4      ................................................................................................................. 488
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B

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C6BV       CCGGAAGGGAGCAGCTATCGAATGATGAGCTTATCAAGAAGCTAGTGACGGAGCTGGCCGAGAATAGCATGATCGAGGCTGAGGAGGTGCGGGGCACTCTTGGGGACATCTCGGCTCGCATCGAG 125
Strain V   ................G..............A.........T.............................................................A..............T...... 125
P1-1       .G........................................................................................................................... 125
P5-1       .....................................................................................................................A....... 125
P5-2       .....................................................................................................................A....... 125
P5-3       .....................................................................................................................A....... 125
P14-1      ............................................................................................C................................ 125
P24-4      .G...........................................................C............................................................... 125
P24-8      .....................................................................................................................A....... 125
P26-1      .....................................................................................................................A....... 125
P26-19     .....................................................................................................................A....... 125
P26-20     .....................................................................................................................A....... 125
*P18-1     .G...................................................................................................................A....... 125
*P18-2,3   .....................................................................................................................A....... 125
*P31-1     ............................................................................................................................. 125

C6BV       GCAGGGTTTGAGTCCCTGTCCGCCCTCCAAGTGGAAACCATCCAGACAGCTCAGCGGTGCGACCACTCCGATAGCATCAGAATCCTTGGCGAGAACATCAAGATACTGGATCGCTCCATGAAGAC 250
Strain V   ..............................................................T........C........G.....C....................A................. 250
P1-1       ............................................................................................................................. 250
P5-1       ............................................................................................................................. 250
P5-2       .............................................................................................G............................... 250
P5-3       ............................................................................................................................. 250
P14-1      ............................................................................................................................. 250
P24-4      ............................................................................................................................. 250
P24-8      ............................................................................................................................. 250
P26-1      ............................................................................................................................. 250
P26-19     ............................................................................................................................. 250
P26-20     ............................................................................................................................. 250
*P18-1     ............................................................................................................................. 250
*P18-2,3   ............................................................................................................................. 250
*P31-1     ............................................................................................................................. 250

C6BV       AATGATGGAGACAATGAAGCTCATGATGGAGAAGGTGGACCTCCTCTACGCATCAACCGCCGTTGGGACCTCTGCACCCATGTTGCCCTCCCATCCTGCACCTC 354
Strain V   .......................................T................................................................ 354
P1-1       ............................................C........................................................... 354
P5-1       ........................................................................................................ 354
P5-2       ........................................................................................................ 354
P5-3       ................................................................T.................................A..... 354
P14-1      ............................................C........................................................... 354
P24-4      ............................................C........................................................... 354
P24-8      ........................................................................................................ 354
P26-1      ..................................................................................C..................... 354
P26-19     ........................................................................................................ 354
P26-20     ........................A............................................................................... 354
*P18-1     ..................................................................................................A..... 354
*P18-2,3   ........................................................................................................ 354
*P31-1     ........................................................................................................ 354
```

FIG. 4. Nucleotide sequence alignment of BDV p40 (A) and p24 (B) among C6BV (14), strain V (8), and cDNA clones derived from psychiatric patient PBMCs. Partial p40 and p24 sequences shown correspond to nucleotides 298 to 785 and 1462 to 1815, respectively, of the C6BV genome (antigenomic polarity). Dots indicate nucleotide identity with respect to C6BV. Numbers on the left indicate the patient code numbers and numbers of the clones sequenced (i.e., p4-1 corresponds to the sequence derived from clone 1 of patient 4). Different clones derived from the same PBMCs with the same nucleotide sequence were summarized in one single sequence (i.e., p3-3 and p3-4 = p3-3,4). The p24 sequence p18-1 was derived from an uncloned PCR product. Sequences derived from different PBMC samples from the same patient are marked with asterisks.

BDV RNA has been detected in PBMCs of naturally and experimentally infected animals (37, 38, 49) and also in humans (6, 17, 26, 39). This finding provides another diagnostic marker to assess whether BDV prevalence is higher in neuropsychiatric patients than in healthy individuals. Therefore, we analyzed by RT-PCR RNA extracted from PBMCs from psychiatric patients and healthy volunteers from the same geographic area (Homburg). PBMCs from 25 psychiatric patients were analyzed in both laboratories (La Jolla and Homburg) for the presence of BDV p40 and/or p24 RNA sequences. Six (24%) of these patients were found to be BDV positive in both laboratories. Six other patients were found to be BDV RNA positive in only one of the two laboratories, and in two of these cases, BDV sequences were detected only by Southern blot hybridization of the RT-PCR product. One additional patient analyzed only in the La Jolla laboratory was also BDV RNA positive. On the basis of the total number of samples found to be BDV RNA positive in both or only one of the laboratories, 50% (13 out of 26) of the neuropsychiatric patients analyzed carried BDV RNA sequences in their PBMCs. In contrast, none of the 23 PBMC samples from healthy volunteers was positive for p40 or p24 by ethidium bromide after RT-PCR. One of them was found to be positive for p40 after Southern blot hybridization in La Jolla. Attempts to clone this PCR product have failed, and independent nested PCRs using the same RT product did not reproducibly score this sample as BDV RNA positive. Reconstruction experiments indicated that under our RT-PCR conditions, reproducible positive RT-PCR for p40 RNA could not be achieved with 300 or fewer RNA target molecules in a background of 5 μg of total RNA (45). It is possible that the p40 RNA-positive sample found among the healthy controls and the two patient PBMC samples found to be p40 positive only after Southern blot hybridization correspond to cases of very low viral load within the PBMCs.

RT-PCR control experiments consisting of the omission of the RT enzyme in the RT step consistently failed to amplify any BDV sequences, ruling out the possibility of contamination with plasmid DNA or amplicons containing BDV sequences analyzed in our studies. In addition, water samples and BDV-negative RNA samples included as controls in each

TABLE 4. Degree of conservation of human BDV p40 and p24 sequences with respect to animal sequences (C6BV and strain V) and percentages of inter- and intrapatient divergencies of human BDV p40 and p24 sequences

Protein	Range (%) of:		
	Sequence divergency in comparison with C6BV (or strain V)	Interpatient divergency	Intrapatient divergency
p40			
Nucleotide level	0–1.03 (3.48–4.57)	0.2–2.05	0–1.43
Amino acid level	0–1.84 (0–1.84)	0–3.68	0–2.45
p24			
Nucleotide level	0–0.85 (3.11–3.95)	0–1.69	0–1.13
Amino acid level	0–2.56 (0–2.56)	0–4.27	0–2.56

RT-PCR assay were also always negative. Moreover, we rigorously observed well-established guidelines (50) to avoid contamination problems during the performance of RT-PCR assays. In addition, no work with BDV-infected cells has been conducted in the Homburg laboratory. Furthermore, the unique nucleotide changes found in the p40 and p24 RNA sequences derived from the patient PBMCs clearly distinguished them from known animal BDV sequences. Taking all of these findings into account together with the good concordance observed between results independently obtained in both laboratories, we can rule out contamination with a laboratory source of BDV sequences.

Our results did not show a correlation between BDV RNA prevalence in PBMCs of psychiatric patients and specific diagnoses. However, it is worth noting that 7 of 11 patients with diagnoses of schizophrenia were found to be positive for BDV RNA. We observed that only a small subset of patients found to be positive for BDV RNA also had serum antibodies to BDV antigens (Table 3). This lack of correlation between RNA and serological markers has also been found recently by others in humans (26, 39) and animals (37, 38). Seropositive but BDV RNA-negative cases may represent BDV infections that have been cleared by the host immune system. Conversely, detection of viral RNA in the absence of antibodies against BDV may reflect a recent infection with a delayed humoral immune response, as described, for instance, in the case of hepatitis C virus infection (12). BDV could also interfere with the host immune response by using strategies similar to those exploited by other viruses with the ability to persist without eliciting a noticeable humoral response (1). These findings suggest that both serology and RT-PCR should be used to diagnose BDV infection.

TABLE 5. Nucleotide mutations involving amino acid exchanges found in p40 and p24 sequences derived from patient PBMCs

Protein	Patient code	Clone(s)	Nucleotide position in BDV genome RNA	Nucleotide change[a]	Amino acid position in BDV ORF	Amino acid change[a]
p40	5	3, 4, 6	328	A → C	92	His → Pro
	26	1, 2, 3	333	G → U	94	Gly → Trp
	31	1	336	G → C	95	Val → Leu
	18	1	402	A → G	117	Lys → Glu
	26	2	418	A → G	122	Lys → Arg
	18	1, 2, 11	493	U → C	147	Val → Ala
	11	3	531	G → A	160	Glu → Lys
	2	6	543	A → G	164	Lys → Glu
	5	6	565	C → U	171	Ala → Val
	5	6	571	U → C	173	Leu → Ser
	4	1, 2	579	C → U	176	Pro → Ser
	4	1, 2	586	A → U	178	His → Leu
	5	3	592	A → G	180	Glu → Gly
	11	2	610	A → U	186	Gln → Leu
	5	4	625	A → G	191	His → Arg
	11	2	630	G → U	193	Ala → Ser
	4	1, 2	636	G → U	195	Asp → Tyr
	31	4	649	G → U	199	Gly → Val
	31	1	651	C → A	200	Gln → Lys
	31	1	673	U → A	207	Val → Glu
	18	1	756	A → U	235	Met → Leu
p24	24	4	1524	A → C	85	Asn → His
	14	1	1554	G → C	95	Gly → Arg
	5	1, 2, 3				
	18	1, 2, 3	1579	G → A	103	Arg → His
	24	8				
	26	1, 19, 20				
	5	2	1680	A → G	137	Asn → Asp
	26	20	1738	U → A	156	Met → Lys
	1	2				
	14	1	1756	U → C	162	Leu → Pro
	24	4				
	5	3	1776	G → U	169	Gly → Trp
	5	3	1810	C → A	180	Ala → Glu
	18	1				

[a] Change found in PBMCs (right position) compared with C6BV and strain V sequences (left position).

TABLE 6. Comparison of two different RT-PCR methods

Method	No. clones sequenced	No. of mutations found (354 bp of each clone sequenced)[a]
Superscript II RT used for RT reaction; *Taq* polymerase used for nested PCR	5	0 in all
EZ r*Tth* DNA polymerase RNA PCR kit (2.5 mM Mn^{2+}) used for coupled RT-PCR and 2nd PCR	10	7 + del, 5, 4, 7, 11 + ins, 18 + ins, 12, 9, 18, 7

[a] ins and del, single-base-pair insertion and deletion, respectively.

Analysis of BDV p40 and p24 RNA sequences derived from patient PBMCs revealed a high degree of sequence conservation compared with known animal BDV sequences. This finding is consistent with our previous results obtained from sequencing of p24, p16, and p56 genes of BDV isolates from three psychiatric patients by cocultivation of patient PBMCs with BDV-susceptible cells (17). Because of the lack of proofreading activity associated with their polymerases, RNA viruses exhibit high mutation frequencies, which provides the potential for the frequently observed rapid RNA virus evolution (22). However, RNA viruses can also display long-term stasis both in nature and in laboratory conditions as a result of selection for fit master sequences in rather constant environments (22, 59). A high level of sequence conservation has been also found among BDV sequences derived from naturally infected horses from different geographic areas and separated by more than 10 years (47), as well as for BDV sequences derived from naturally infected animals of different species (4). Whether this high sequence conservation is due to a hypothetical, as yet unidentified proofreading function of the BDV RNA polymerase, or the result of selective pressure acting on tight structure-function constraints, remains to be determined.

Sequence variability of BDV p24 in PBMCs significantly higher than that here reported has been recently found in Japanese neuropsychiatric patients (24). It is conceivable that different strains of BDV are present in Japan. Differences in the characteristics of patient populations analyzed in Japan and Germany could also account for the differences in the rate of sequence variation. Nevertheless, we have evidence that different experimental procedures used for the RT-PCR analysis of human PBMCs also significantly contributed to these differences. Studies conducted on Japanese patients (26) used the EZ r*Tth* RNA PCR system (Perkin-Elmer). This method is based on the use of r*Tth* polymerase, which allows both reverse transcription and DNA amplification to be conducted in a single buffer system which contains manganese. Because the negative effect of manganese on the fidelity of DNA synthesis is well documented (3, 31), we compared the experimentally induced mutation frequencies found under our RT-PCR conditions and by the EZ r*Tth* RNA PCR method. For this purpose, p24 RNA was generated by in vitro transcription via T7 polymerase from a plasmid containing the C6BV p24 ORF. Equal amounts of p24 RNA were used to amplify the same fragment within p24, using the EZ r*Tth* RNA PCR method as described previously (26) or our RT-PCR conditions. PCR products generated by both methods were cloned, and the sequences of several independent clones were determined (Table 6). Sequence analysis of five clones derived from PCR products obtained with our RT-PCR conditions showed 100% identity with respect to the C6BV p24 plasmid sequence. In contrast, 10 clones derived from PCR products generated with the EZ r*Tth* RNA PCR method showed 4 to 18 nucleotide substitutions per clone with respect to the template C6BV p24 RNA, representing an intrasample divergency as high as 10.2%. Two of these clones had also single-base insertions or deletions. These results indicate that the higher BDV sequence variation previously reported (24) may have been overestimated because of technical procedures.

The route of BDV transmission has not been clearly established, and how humans could be infected with BDV is unknown. The high conservation observed between human and animal BDV sequences suggests the possibility of zoonosis. However, transmission of BDV to humans via blood seems also plausible. In this regard, a prevalence of 4.2 to 5% of BDV RNA in PBMCs has been recently reported among blood donors in Japan (25). Our data provide strong support for the contention that BDV is associated with certain human psychiatric disorders, possibly contributing to the pathophysiology of these disorders. On the basis of such a hypothesis, it would be predicted that similar associations should be found with blood samples from psychiatric patients from different geographic areas. As yet there is only limited information from psychiatric patients from Germany and Japan. Hence, there is a need for comprehensive molecular epidemiological studies to rigorously evaluate the contribution of BDV to human mental disorders.

ACKNOWLEDGMENTS

C. Sauder and A. Müller contributed equally to this work.

We thank Barbara Gärtner, Daniel Gonzalez-Dunia, and Michael Teng for helpful discussions, Dorothee Grässer for analysis of the patients' diagnoses, Anne Bang for the sequence alignments, and Sigrid König for excellent technical assistance. We thank Georg Pauli for the generous gift of serum from a BDV-infected rabbit, Steven Rubin for serum from a BDV-infected mouse, and Peter Staeheli for the generous gift of RNA isolated from BDV-infected and uninfected C6 cells.

This work was supported by a grant from the University of the Saarland to N. Mueller-Lantzsch and by grant RO1 NS/AI 32355 to J. C. de la Torre; C. Sauder is a recipient of a fellowship sponsored by the Bundesministerium für Forschung und Technologie.

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