

for the p38 BDV-gene. Two samples gave positive RT-PCR results in only the laboratory currently engaged in BDV work. In the other two laboratories not previously exposed to BDV, the outcome of RT-PCR analysis of replicates of these samples was negative (Table 1). In no other sample was a positive reaction yielding BDV-specific sequences obtained (Table 1).

In Giessen, PBLs from 42 seropositive psychiatric patients and four seronegative controls from a BDV endemic area in Germany (Günzburg) were again analyzed in the two classes of laboratories. In this case, the more sensitive nested PCR method was used with primers specific for the BDV p24-gene. Positive results with PBLs from two patients were again obtained in only the laboratory where BDV work was ongoing, but not confirmed in another independent BDV-free laboratory (Table 1). All the other samples were negative (Table 1). In addition, consecutive samples obtained from these individuals over a 9 month period were studied and in no case were BDV-sequences detected (Table 2). We

found that the seropositive patients remained positive for BDV-specific antibodies and the seronegative controls did not seroconvert (data not shown).

Infectivity

In attempts to recover virus from PBLs of seropositive patients, we either cocultivated these with rabbit embryonic brain (REB) cells or injected them into rabbits, *i.c.*, since rabbits and REB cells are highly susceptible to BDV infection (Ludwig *et al*, 1988). Immunofluorescence analysis was used to survey REB cells for evidence of BDV infection and immunoblot techniques used in an effort to detect BDV-antigens in extracts of the cells. Cocultivation of REB cells with PBLs from the Giessen cohort of seropositive patients through either two (47 PBL samples) or 8–10 (eight PBL samples) passages failed to provide any evidence of the presence of BDV.

Furthermore, *i.c.* inoculation of two rabbits with PBLs prepared from the Philadelphia patient exhibiting the highest anti-BDV titer neither caused clinical disease nor the induction of BDV-specific antibodies in the animals during a 2 month observation period.

Discussion

A possible association between BDV-infection and major psychiatric disorders was initially proposed on the basis of finding BDV-reactive antibodies in the sera of relatively minor subsets of such patients (Amsterdam *et al*, 1985; Rott *et al*, 1985, 1991; Bode *et al*, 1988; VandeWound *et al*, 1990; Bechter *et al*, 1992; Fu *et al*, 1993; Waltrip *et al*, 1995). The more recent detection of BDV antigens, and BDV-RNA by RT-PCR, in PBLs of psychiatric patients (Bode *et al*, 1995; Kishi *et al*, 1995a,b) has been taken as evidence of the presence of BDV antigens and, presumably, BDV itself, in these individuals. Surprisingly however, there has been no correlation found between the presence of BDV RNA or BDV antigens and seropositivity to BDV antigens in individual patients. Moreover, only a few patients have been identified whose serum antibodies have reacted with both the p-38 and p-24 antigens of BDV whereas sera of naturally infected horses (unpublished observations) and experimentally infected rats (Rott *et al*, 1991; Fu *et al*, 1993) regularly exhibit antibodies to both antigens. These observations prompted us to further evaluate possible correlations between BDV and human psychiatric illness. Two centers, one in Germany and one in the USA, were involved in this study and aliquots of the human material to be assayed for BDV-RNA were tested independently in both countries in at least two different classes of laboratories; one being routinely engaged in BDV

Table 1 Analysis of human PBLs using RT-PCR with BDV-specific primers

Presence of BDV-specific sequences/Number of individuals tested

(a) Philadelphia cohort ¹	Schizophrenic patients	Normal controls
Lab 1	2/10	0/10
Lab 2	0/10	0/10
Lab 2	0/10	0/10
(b) Giessen cohort ²	Psychiatric patients	Normal controls
Lab 1	2/24	0/4
Lab 2	0/24	n.d.

¹Primers specific for the p38 BDV gene were used

²Primers specific for the p24 BDV gene were used; all psychiatric patients were seropositive for BDV-specific antibodies

Table 2 RT-PCR analysis of PBLs obtained in consecutive bleeds of BDV seropositive and seronegative individuals

Number of subjects ¹ positives ²	Number of bleeds	Number of RT-PCR
(a) seropositive subjects:		
32	1	0/32
6	2	0/12
2	3	0/6
1	4	0/4
1	5	0/5
(b) seronegative subjects:		
4	2	0/8

¹Subjects were selected as described in Materials and methods. The psychiatric diagnoses of these individuals are detailed in Table 1

²Primers specific for the p24 BDV gene were used

work while the second did not have prior exposure to BDV.

We obtained positive results for the presence of BDV-RNA only in laboratories where BDV is routinely studied. Simultaneous or subsequent analysis of replicate samples in other laboratories where there had been no prior work on BDV yielded negative results. In order to rule out the possibility that BDV-specific RNA may appear only transiently in patient's PBL, we obtained samples from the peripheral blood of BDV-reactive, antibody seropositive, psychiatric patients at regular intervals. Even in these serial consecutive bleedings, we failed to detect BDV-sequences. In addition, we could not detect BDV-infectivity in PBLs after as many as 10 serial passages of cocultivation with REB cells. We are therefore forced to conclude that contamination may seriously influence the outcome of studies using RT-PCR methodology to probe for BDV-sequences in PBLs of psychiatric patients, and stringent criteria must be met to control against false negative results. Henceforth, based on the data currently available in the literature, we cannot support the contention that BDV-specific sequences are present in up to 30% of patients with psychiatric illness.

The observation of psychiatric patients in the Giessen and Philadelphia cohorts with serum antibodies reactive with the p-24 and/or p38 BDV-antigens but neither infectious virus nor BDV-specific sequences detectable in their PBLs may pose a question concerning the source of the antigenic stimulus in humans. Is the antigen responsible for the induction of antibodies in humans actually BDV or some other infectious agent, or a self component that cross-reacts with BDV determinants?

One of the major considerations concerning the use of RT-PCR to probe the putative link between BDV-infection and human psychiatric disorders is that the technique, as shown here, is susceptible to 'false' positive reactions, presumably due to its exquisite sensitivity and contamination of the laboratory with RNA. It should be noted that, in our hands, the frequency of positive results obtained in laboratories dedicated to work with BDV, that we were unable to confirm in clean laboratories, approaches that reported for sequence-positive individuals in other studies (Bode *et al*, 1995; Kishi *et al*, 1995a,b).

Like other studies, our investigation suffers from having examined only a small pool of psychiatric patients and an even smaller number of controls. Even though the presence of serum BDV-reactive antibodies, itself a relatively infrequent occurrence, was used to target individuals of interest, we were unable to confirm the presence of BDV-specific RNA sequences in PBLs from any of these. This does not however exclude the possibility that BDV RNA may be present in some psychiatric patients where

virus infection may be restricted to specialized areas of the brain such as the limbic system which are preferentially infected by BDV in naturally and experimentally infected animals (Rott and Becht, 1995). We conclude that the tangible evidence necessary to resolve the issue of whether or not there is any direct association between BDV infection and human psychiatric illness remains elusive.

Materials and methods

US cohort

Subjects were randomly selected from the pool of schizophrenic patients treated at the Thomas Jefferson University Hospital (TJUH) in Philadelphia. Mean age of the seven male and three female subjects was 38.0 ± 13.3 S.D. years and the mean duration of illness was 16.4 ± 13.6 S.D. years. Inclusion criteria for schizophrenic subjects were as follows: (1) DSM-4 diagnosis of schizophrenia or schizoaffective disorder; (2) minimum 18 years of age; (3) provision of informed consent. Exclusion criteria for schizophrenic subjects were the presence of HIV-infection or other medical problems likely to alter the presentation and/or treatment of the psychiatric symptomology. A control group, ethnically and age-matched to schizophrenic subjects, was selected from the pool of volunteers presenting at the TJUH blood donor center.

German cohort

The patients were from the University of Ulm, Department of Psychiatry II, Bezirkskrankenhaus Günsberg. BDV seropositive patients were randomly selected from ongoing studies (positives represented 42 out of a total pool of more than 500 seropositive tested individuals). The ICD-10 system was utilized for psychiatric diagnosis (see Table 3). The range of diagnoses obtained in the study group covers the range found in seropositives from

Table 3 Diagnosis of Giessen/Günzburg Psychiatric Patients

General diagnosis (# patients; total 42)	ICD-10 diagnosis (# patients)
Schizophrenic disorders (16)	F20.xx—most paranoid type in acute or post-acute stages (10); first diseased (3), chronic residua (3)
Depressive disorders (9)	F32.1 (5) F33.1 (4)
Generalized anxiety disorders (4)	F41.1 (4)
Personality disorders (3)	F60.x (3)*
Organic personality disorders (3)	F07.x (3)*
Alcoholic personality disorders (4)	F10.71 (4)
Dementias (2)	F0x.x (2)*
Oligophrenia (1)	F71 (1)

*x and x.x indicates variable numbers (0–9) within the respective codes

ongoing screening investigations of unselected psychiatric patients; however no selection was made on the basis of diagnosis. Further inclusion/exclusion criteria were as detailed for the Philadelphia cohort. Seronegative controls were volunteers from the department's medical personnel.

Isolation of PBL

From each patient and control subject, 10–20 ml of blood was collected into heparinized (Philadelphia) or EDTA-treated (Giessen) tubes. PBLs were purified, according to the manufacturer's protocol, using Ficoll-Hypaque density gradient centrifugation (Pharmacia, Sweden). Blood was collected in the hospital and PBLs were processed in laboratories which had no history of work with BDV or BDV-specific nucleic acids. Replicate aliquots of isolated PBLs were then distributed to the various laboratories for further analysis.

RNA-extraction and RT-PCR reaction

RNA was isolated from PBLs using RNeasy (Qiagen, Crawley, UK). RT-PCR extractions were performed in two classes of laboratories, one which routinely carries out BDV investigations and another which had no prior contact with BDV. In the studies performed in Philadelphia, 1 µg or 50% of the RNA obtained from a PBL sample was subjected to reverse transcription with Superscript RT (Gibco BRL) using the following anti-sense p38-specific primer (Zimmerman *et al*, 1994):

5'-CTTCTTACTCCAGTAAAACGC-3'

After 1 h the cDNA was amplified using 39 PCR cycles employing the following primers:

sense primer:

5'-GTCACGGCGGATATGTTTC-3'

anti-sense primer:

5'-ATTCTTTACCTGGGGACTCA-3'

Amplification of 3-GAPDH was used as an internal control using the following primers:

RT 5'-AAGCAGTTGGRGGRGCAGG-3'

PCR sense

5'-AAGGTGAAGTCCGAGTCAA-3'

anti-sense

5'-TTCTCCATGGTGGTGAAGAC-3'

RNA from BDV-infected rat brain was included in all experiments as a positive control. All samples were coded and analyzed in a blinded fashion.

In the Giessen and Marburg studies, 50% of the isolated RNA was reverse transcribed for 1 h at 42°C using p24-specific sense and anti-sense primers in order to amplify genomic and subgenomic BDV-specific RNA (Richt *et al*, 1993). The cDNA was amplified using 32 PCR cycles as described pre-

viously (Zimmerman *et al*, 1994). The resulting amplification products were subjected to a second 32 cycle PCR amplification using a nested primer set:

nested p24 sense primer:

5'-TCAGACCCAGACCAGCGAA-3'

nested p24 anti-sense primer:

5'-AGCTGGGGATAAATGCGCG-3'

The products of amplification were analyzed in agarose gel with subsequent Southern blot hybridization using an internal oligonucleotide P24 BDV probe (Richt *et al*, 1993), 3' end labeled with digoxigenin-11-dd-UTP (Cat No. 1362376; Boehringer Mannheim, Germany). RNA from BDV-infected rat brain was tested in parallel as a positive control. In the Marburg laboratory, positive control BDV RNA was only used a single time to ensure the quality of the newly synthesized primers and the reaction conditions and was not included in the subsequent assays with test samples.

Since pure BDV RNA is not available, the sensitivity of the RT-PCR techniques employed was tested using total RNA isolated from BDV-infected rat brain containing 10⁷ tissue culture infectious doses of BDV per gm of tissue. Viral RNA could be detected in as little as 0.2 picograms of total rat brain RNA.

Experimental animals

In an attempt to recover BDV from potentially infected human PBL, New Zealand white rabbits were inoculated i.c. with approximately 10⁶ PBL (in 50 µl medium) from a donor seropositive for BDV-reactive antibodies. The rabbits were monitored daily for 60 days for the appearance of clinical signs of Borna disease and bled 40 and 60 days post inoculation.

In vitro infectivity assays

In vitro infectivity assays were performed using viable PBLs cocultivated with rabbit embryonic brain (REB) cells (1:1 ratio). The cells were passaged up to 10 times and then fixed in acetone prior to being analyzed with BDV-convalescent rat serum using the indirect immunofluorescence assay described previously (Rott *et al*, 1991).

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References

- Amsterdam JD, Winokur A, Dyson W, Herzog S, Gonzalez F, Rott R, Koprowski H (1985). Borna disease virus: A possible etiologic factor in human affective disorders? *Arch Gen Psychiatry* **42**: 1093–1096.
- Bechter K, Herzog S, Schuttler R (1992). Possible significance of Borna disease for humans. *Neurol Psychiatry and B Res* **1**: 23–29.
- Bode L, Riegel S, Ludwig H, Amsterdam JD, Lange W, Koprowski H (1988). Borna disease virus-specific antibodies in patients with HIV infection and with mental disorders. *Lancet* **ii**: 687.
- Bode L, Zimmermann W, Ferzt R, Steinbach F, Ludwig H (1995). Borna disease virus genome transcribed in psychiatric patients. *Nature Medicine* **1**: 232–236.
- Fu ZF, Amsterdam JD, Kao M, Shankar V, Koprowski H, Dietzschold B (1993). Detection of Borna disease virus-reactive antibodies from patients with affective disorders by Western blot technique. *J Affect Dis* **27**: 61–68.
- Kao M, Hamir AN, Rupprecht CE, Fu ZF, Shankar V, Koprowski H, Dietzschold B (1993). Detection of antibodies against Borna disease virus in sera and cerebrospinal fluid of horses in the USA. *Vet Rec* **132**: 241–244.
- Kishi M, Nakaya T, Nakamura Y, Kakinuma M, Takahashi TA, Sekiguchi S, Uchikawa M, Tadokoro K, Ikeda K, Ikuta K (1995a). Prevalence of Borna disease virus RNA in peripheral blood mononuclear cells from blood donors. *Med Microbiol Immunol* **184**: 135–138.
- Kishi M, Nakaya T, Nakamura Y, Zhong Q, Ikeda K, Senjo M, Kakinuma M, Kato S, Ikuta K (1995b). Demonstration of human Borna disease virus RNA in human peripheral blood monocytes. *FEBS Letters* **364**: 293–297.
- Ludwig H, Bode L, Gosztonyi G (1988). Borna disease: A persistent virus infection of the central nervous system. *Prog Med Virol* **35**: 107–151.
- Richt JA, Herzog S, Habertzettl K, Rott R (1993). Demonstration of Borna disease virus-specific RNA in secretions of naturally infected horses by the polymerase chain reaction. *Med Microbiol Immunol* **182**: 293–304.
- Rott R, Brecht H (1995). Natural and experimental Borna disease in animals. In: *Borna disease*. Koprowski H, Lipkin WI (eds). pp. 17–30.
- Rott R, Herzog S, Bechter K, Frese K (1991). Borna disease, a possible hazard for man? *Arch Virol* **118**: 143–149.
- Rott R, Herzog S, Fleischer B, Winokur A, Amsterdam JD, Dyson W, Koprowski H (1985). Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. *Science* **228**: 1755–1756.
- Vandewound S, Richt JA, Zink MC, Rott R, Narayan O, Clements JE (1990). A Borna disease virus cDNA clone encoding a protein recognized by antibodies in humans with behavioral diseases. *Science* **250**: 1278–1281.
- Waltrip RW, Buchanan RW, Summerfelt A, Breir A, Carpenter WT, Bryant NL, Carbone KM (1995). Borna disease virus and schizophrenia. *Psychiatry Res* **56**: 33–44.
- Zimmermann W, Durrwald R, Ludwig H (1994). Detection of Borna disease virus RNA in naturally infected animals by a nested polymerase chain reaction. *J Virol Methods* **46**: 133–143.