Journal of NeuroVirology (1997) 3, 174–178

http://www.jneurovirol.com

Case report

Failure to detect Borna disease virus infection in peripheral blood leukocytes from humans with psychiatric disorders

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The presence of antibodies reactive with Borna disease virus (BDV) in the sera of some patients with certain psychiatric illnesses has been taken as evidence that this veterinary neurotrophic virus may occasionally infect and cause psychiatric disorders in humans. In this paper, we report the results of our studies concerning the detection of BDV-specific RNA in blood cells from patients with psychiatric diseases. Contrary to the results obtained by others, we have found no evidence for the presence of BDV-RNA in such cells. Prior work with BDV sequences in the assay environment, together with the exquisite sensitivity of RT-PCR, may account for the sporadic appearance of false positive evidence that BDV-specific RNA is present in human blood cells.

Keywords: Borna disease virus; psychiatric disease; PBLs; RT-PCR

Introduction

Borna disease virus (BDV) has been promoted as a contributor to psychiatric disease in humans. The first reports on the presence of BDV-reactive antibodies in the sera of patients with mood disorders were followed by other serological data showing that BDV-reactive antibodies are present in the sera of a significantly greater proportion of mentally ill patients than normal individuals (Amsterdam *et al*, 1985; Rott *et al*, 1985, 1991; Bode *et al*, 1988; VandeWoude *et al*, 1990; Bechter *et al*, 1992; Fu *et al*, 1993; Waltrip *et al*, 1995). This led to the hypothesis that human BDV infection may contribute to the pathogenesis of certain human psychiatric disorders. Using the RT-PCR technique, several investigators have reported the detection of BDV-specific RNA sequences in peripheral blood leucocytes (PBL) obtained from psychiatric patients (Bode *et al*, 1995; Kishi *et al*, 1995a,b). To further investigate these observations as well as provide preliminary evidence concerning the geographical distribution of psychiatric patients with PBL-borne BDV-sequences, we have conducted a multicenter study in Germany, where Borna disease (BD) is indigenous (Rott and Becht, 1995), and the US, where BD is unknown (Kao *et al*, 1993), to examine, using molecular approaches under rigidly controlled conditions, the prevalence of BDV-sequences in human PBL.

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Received 15 August 1996; revised 29 October 1996; accepted 1 November 1996

Results

RT-PCR

In the work performed in Philadelphia, 10 schizophreinc patients and 10 age and sex matched control individuals from the area were studied. Sera from two of the schizophrenic patients had high titers (1 : 540; 1 : 4860) of BDV-reactive antibodies as determined by Western blot analysis using BDV-antigens prepared from a homogenate of BDV-infected rat brain. One of these patients was bled four times over a 3 month period and showed a consistently high BDV-reactive antibody titer. The RT-PCR assay was conducted using primers specific

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).

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.

[1]Primers specific for the p38 BDV gene were used
[2]Primers 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

[1]Subjects were selected as described in Materials and methods. The psychiatric diagnoses of these individuals are detailed in Table 1
[2]Primers specific for the p24 BDV gene were used

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

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 dedicted 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 schizophenic 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 schizoprehic 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, Berzirkskrankenhaus 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 subjet, 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 RNAzolB (Biotecx, Texas). 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′-GTCACGGCGCGATATGTTTC-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′-AAGGTGAAGGTCGGAGTCAA-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 previously (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 10^7 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 10^6 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).

Acknowledgements

We are grateful to Drs Kay Huebner and Yong Mu Zheng for performing independent RT-PCR analysis and acknowledge the excellent technical assistance of E Gottfried and K Haberzettl. Financial support was provided by Deutsche Forschungsgemeinshaft (SFB 272), Gemeinnützige Hertie-Stiftung and Ewald and Hilde Berge-Stiftung.

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