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Lack of association between Borna disease virus infection and neurological disorders among HIV-infected individuals

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Viruses have been proposed as etiologic cofactors in the pathogenesis of HIV-related neurological disease. To investigate the etiologic potential of Borna disease virus (BDV) in these disorders, two populations were studied: (1) 27 prospectively identified patients with various neurological disorders were evaluated with BDV RT–PCR (CSF, PBMC), and BDV serology, and (2) a separate group of 25 retrospectively studied patients with AIDS dementia complex was evaluated using BDV serology only. A novel, BDV p40 gene RT–PCR assay was developed: conserved primers were used in a non-nested amplification, detecting less than 100 BDV RNA copies and all of nine wild-type, confirmed animal BDV infections. BDV seroprevalences were 12.5% and 8.0%, respectively, which are similar to the general HIV-infected population. None of the prospectively studied patients had detectable BDV RNA in their CSF or PBMC. Our findings do not support the hypothesis that BDV infections are responsible for HIV-related neurological disorders.

Keywords: BDV; AIDS dementia complex; human immunodeficiency virus; PCR; serology; CSF

Introduction

Two lines of evidence suggested that BDV may cause disease in HIV-infected patients. First, reports that BDV seroprevalence is higher among HIV-infected persons than among the general population (Auwanit *et al*, 1996; Bode *et al*, 1988) suggest that there may also be a higher prevalence of BDV infection in HIV-infected groups. Second, idiopathic neurologic disorders are frequently found among HIV-infected patients with advanced HIV disease. Other viruses have been proposed as possible cofactors in the etiology of HIV-related neurological disease and, in particular, AIDS dementia complex (ADC) (Lipton and Gendelman, 1995).

The presence of viral antigens, BDV RNA and histopathological changes have been demonstrated in human brain tissues (De La Torre *et al*, 1996; Haga *et al*, 1997; Salvatore *et al*, 1997). Although some reports describe the presence of BDV RNA in PBMCs from psychiatric patients (Bode *et al*, 1995; Kishi *et al*, 1995b), others have not detected BDV RNA in similar groups (Lieb *et al*, 1997; Richt *et al*, 1997; Sierra Honigmann *et al*, 1995).

In order to characterise the possible role of BDV as a viral cofactor in HIV-associated neurological disorders, we analysed cerebrospinal fluid (CSF) and peripheral blood mononuclear cells (PBMC) with a newly developed RT–PCR, and plasma or sera were evaluated with a previously published BDV serologic test (Caplazi *et al*, 1994). This is the first report to our knowledge that uses RT–PCR to evaluate BDV infections among HIV-infected persons.

Results

Development of new BDV PCR primers
Novel BDV primers and probes were developed for this study. To identify conserved primer-binding sites, all BDV sequences published in the GenBank and EMBL sequence databases as of November 15, 1996, were identified. The BDV p40 gene was the most often reported BDV sequence, with six independent sequence strings (BDVPT1P40, BDVPT2P40, BDVPT3P40 (Bode *et al*, 1995), TOGBDVSEQ (Cubitt *et al*, 1994), BD04608 (Briese

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et al, 1994) and S62821 (Pyper *et al*, 1993). An alignment of all six available p40-sequences revealed an interrupted consensus sequence in which all six sequence strings had homology (Figure 1).

The consensus sequence was evaluated for suitable primers and probes that had the best sequence conservation, minimum intra- and interstrand duplex formation, minimum false priming sites, and the lowest melting temperature difference between the primers. The resulting BDV PCR primer pair (upper: BD139U, Lower: BD139L) and probe (BD139P) produce a product of 139 base pairs (Figure 1). BD139U and BD139P had no mismatches compared to the consensus sequence. The lower primer, BD139L, contained two mismatches relative to the consensus sequence at positions four and seven from the 5′ end (Figure 1). To avoid potential amplification problems due to these mismatches, BD139L was synthesised with inosine substitutions at positions four and seven because inosine can reportedly hybridise to any of the four normal nucleotides with approximately equal affinity (Cassol *et al*, 1991).

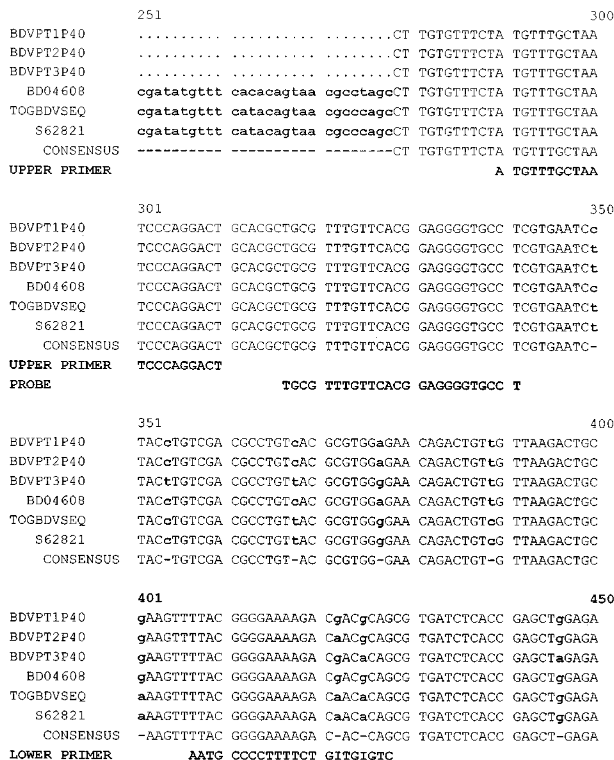

Figure 1 Alignment of six p40 gene sequences and their consensus sequence. Consensus at each position was only accepted when all sequences were identical. Lower case bold indicates absence of consensus at that position for one or more of the six sequences. The BDV139 primers and probe are indicated in bold upper case. The sequence numbering corresponds to the complete genomic sequence BD04608 (Briese *et al*, 1994).

The PCR reaction was optimised with respect to $MgCl_2$ concentration (1, 1.5, 2 and 3 mM), glycerol concentration (0, 10 and 20%), TaqStart Antibody (0 or 0.5 μl per reaction), annealing temperature (50, 52, 54, 56, 58, 60 and 62°C), annealing time (20 s, 20 s plus 1 s cycle, 45 s) and denaturation temperature (95, 94 and 93°C). In the RT step, reactions primed with the specific BDV primer (BD139U) produced more PCR product than reactions primed with random hexamers.

The sensitivity of the BD139 RT–PCR assay was tested in three ways. Conservation of the primer sequences was evaluated by testing brain tissues from nine animals with documented BDV infections (classical clinical syndrome and positive brain tissues by immunohistochemistry), including six horses, two sheep and one donkey. All nine cases were positive in the BD139 RT–PCR assay. The lower limit of detection was evaluated first by amplifying dilutions of RNA extracted from BDV-positive, cultured cells. This demonstrated BDV RT–PCR positivity in RNA from 0.1 infected cell equivalents per reaction. The lower limit was further evaluated by amplifying dilutions of *in vitro* generated, quantitated p40 gene RNA, demonstrating detection of fewer than 100 BDV RNA copies per amplification reaction.

Prospectively studied cohort with various neurological disorders

Clinical and demographic data from the prospectively studied cohort are summarised in Table 1. The median age of the 27 subjects (seven women) was 34 years (range, 28–71). The median CD4 count at the time of spinal tap was 60 CD4 cells/μl (range 0 to 440). The median duration of followup was 175 days (range, 4–433 days).

BDV RNA was not detected in any of the 27 PBMC or CSF specimens from HIV-infected patients with neurological diseases. The average amount of total RNA recovered from 26 of 27 PBMC specimens was 2.44 μg per specimen; the RNA content of one specimen could not be assessed for technical reasons. Since many patients in this cohort had lymphopenia secondary to advanced HIV disease, RNA yields varied from 0.12–6.66 μg per PBMC specimen. Suitability of the extracted RNA for amplification was confirmed by semiquantitative beta-actin RT–PCR, which was positive in all 26 RNA specimens tested. Since detectable RNA was not expected from 0.14 ml CSF, RNA levels in these specimens were not measured.

RNAs extracted from all 27 PBMC and CSF specimens were tested by BDV RT–PCR in duplicate, and several positive and negative controls were included in every run, assuring consistent performance of the assay. In addition to the PCR data, BDV serologies performed on simultaneously collected sera from 16 of the 27 subjects revealed 2 (12.5%) that were positive (titers, 1:40 and 1:80).

Table 1

Subject	Age[a]	Gender	Stage[b]	CD4/μl	Reason for tap[c]	Final neurological diagnosis[c]
1	47	M	C3	0	R/O PML (apraxia, brain atrophy, paralysis)	ADC
2	38	F	C3	90	Confirm ADC	ADC
3	28	M	A3	110	R/O Neurosyphilis	None
4	34	M	C3	0	R/O PML (ataxia, memory loss)	Presumptive PML
5	45	M	C3	60	Confirm cerebral toxoplasmosis	Cerebral toxoplasmosis
6	71	M	B3	60	Progressive paralysis	Idiopathic hemorrhagic spinal cord necrosis
7	34	M	C3	30	Polyneuropathy	d4T-associated polyneuropathy
8	41	M	C3	10	Left hemiparesis	Suspected CNS lymphoma
9	32	F	C3	80	R/O PML (hemiparesis)	Presumptive PML
10	30	F	C3	274	R/O PML	PML
11	34	M	C3	0	Confirm ADC (word-finding abnormality)	None
12	33	M	B3	0	R/O toxoplasmosis, lymphoma (aphasia, cerebral hyperdensity)	Suspected TIA, microangiopathy
13	39	F	C3	10	R/O cerebral cryptococcosis	Cryptococcal meningitis
14	40	M	C2	250	R/O residual cerebral borreliosis	Status post borreliosis
15	37	M	A3	200	Radiculitis	Lumboradicular syndrome
16	33	M	C3	20	Confirm cerebral toxoplasmosis	Cerebral toxoplasmosis
17	31	M	B3	80	R/O PML	Presumptive PML
18	32	M	A2	440	R/O neurosphyilis	Syphilis ruled out
19	28	F	C3	30	Paraparesis	ADC
20	29	F	C3	10	R/O PML	PML
21	34	M	C3	0	Left hemiparesis	Polyradiculitis, CMV retinitis
22	42	M	C3	70	Confirm ADC (general brain atrophy)	None
23	49	M	A2	318	Confirm neurosyphilis	Neurosyphilis
24	33	M	C3	40	R/O PML	Presumptive PML, status post toxoplasmosis
25	35	F	C3	150	Confirm cryptococcal meningitis	Cryptococcal meningitis
26	36	M	B2	284	R/O residual neurosyphilis	Status post neurovascular syphilis
27	36	M	C3	10	Romberg's sign	CMV encephalitis/retinitis

[a]Age in years. [b]CDC stage of HIV infection. [c]R/O, rule out; ADC, AIDS dementia complex; PML, progressive multifocal leukoencephalopathy; TIA, transient ischemic attack.

Retrospectively studied cohort with ADC
Sera from 25 retrospectively studied HIV-infected subjects with a diagnosis of AIDS dementia complex (ADC) were evaluated by BDV serology: 2 of 25 (8.0%) subjects were antibody positive (both titers, 1 : 40).

Discussion

This study suggests that BDV infections do not play an important role in the pathogenesis of neurological illnesses among the two cohorts of HIV-infected subjects reported here. The prospectively studied cohort of 27 HIV-infected subjects with various neurological problems had no detectable BDV RNA in their CSF or PBMCs. The BDV seroprevalence in this group was 12.5% (2/16). The retrospectively studied cohort of 25 HIV-infected patients with AIDS dementia complex (ADC) was evaluated with BDV serologies only, resulting in a seroprevalence of 8.0% (2/25). These seroprevalences are similar to those previously reported for the general HIV-infected population (Auwanit *et al*, 1996; Bode *et al*, 1988), suggesting no increased seroprevalence among those with neurological disorders.

The use of PBMC specimens for BDV detection was based on other reports that assessed BDV infections in humans (Bode *et al*, 1995; Kishi *et al*, 1995b; Lieb *et al*, 1997; Nakaya *et al*, 1996; Richt *et al*, 1997). The topic of BDV RNA in PBMC specimens has stirred controversy. Sauder *et al* (1996) reported BDV RNA positivity by p24 and/or p40 RT–PCR in 50% of PBMC specimens from 26 patients with psychiatric disorders. The PCR results were also confirmed in a separate laboratory. Interestingly, most of the BDV RNA-positive patients were BDV seronegative. In contrast, Lieb *et al* (1997) found no BDV RNA in blood from a different group of psychiatric patients attending the same hospital as used by Sauder *et al* (1996). The negative PCR results from Lieb *et al* (1997) were also confirmed in two separate laboratories. In view of this ongoing debate, we do not rule out the presence of BDV RNA in brain tissue on the basis of negative PBMC BDV RNA data.

The newly developed BDV RT–PCR assay reported here has theoretical advantages over some existing BDV RT–PCR methods. The single round of amplification used here can provide better quantitation than the nested PCR protocols previously reported (Bode *et al*, 1995; Kishi *et al*,

1995a, b; Sierra Honigmann *et al*, 1993; Sorg and Metzler, 1995; Zimmermann *et al*, 1994), while reducing the risk of contamination inherent in nested methods. Although single round PCR can be less sensitive than nested PCR, sensitivity of less than 100 BDV RNA copies was achieved by detecting the PCR products with radioactive probes. Furthermore, the primers were based on conserved BDV sequences, improving the chances of detecting wild-type variants that may differ from characterised strains. Success in this respect was demonstrated by detecting BDV RNA in all of nine BDV-infected animal brain tissues.

Patients meeting our case definition do not typically undergo brain biopsies, so brain tissues from these subjects were not available. Instead, we studied CSF specimens to assess CNS infection. However, the RT–PCR-negative results from CSF specimens in this study must be interpreted with caution. First, we are not aware of evidence that CSF contains BDV RNA in documented infections. Since BDV is cell associated, the failure to detect BDV-RNA in CSF could reflect the inadequacy of this specimen type. Second, we used small volumes of CSF for PCR and did not document RNA recovery from these specimens. The use of BDV RT–PCR in CSF specimens was previously assessed in a cohort of schizophrenic patients in which no viral RNA was detected (Sierra Honigmann *et al*, 1995). Attempts to culture BDV from CSF of three BDV-seropositive patients suggested the presence of BDV, but the results were inconclusive because the cultures failed to propagate virus after passage (Richt *et al*, 1993).

BDV seroprevalence is reportedly higher in HIV-infected populations than in the general population, raising the possibility that HIV-infected patients are more frequently infected with BDV. Bode *et al* previously reported that HIV-infected individuals in the US and England have a higher BDV seroprevalence (7.8%) than the general population (2.0%) (Bode *et al*, 1988). A study performed on HIV-1-infected subjects in Thailand reported BDV seroprevalences of 8.3% among prostitutes, 0% among IV drug users, and 2% among HIV-negative blood donors (Auwanit *et al*, 1996). Surprisingly, a subgroup of HIV-infected patients with sexually transmitted diseases had a very high BDV seroprevalence (38%). Our study of HIV-infected individuals with neurological disorders revealed an overall BDV seropositivity of 9.8%, which is similar to the seroprevalence in unselected HIV-infected individuals from England and the US. The BDV PCR data reported here are the first, to our knowledge, addressing actual viral replication among HIV-infected patients.

In summary, our RT–PCR and serological data did not confirm the presence of BDV in these cohorts of HIV-infected patients with neurological disorders. Although these negative results do not rule out BDV infections, they suggest that BDV infections are not a prominent etiology of neurological disorders in this immunocompromised population.

Materials and methods

Subjects
Two groups of subjects were studied. In the first group, 27 HIV-infected patients with various neurological disorders were prospectively enrolled between May, 1995 and April, 1996 at the Zurich University Hospital. The enrollment criteria were (1) documented HIV infection and (2) spinal tap for any reason. At the time of spinal tap, at least 1 ml CSF and 16 ml anticoagulated blood (CPT Vacutainer, Becton Dickinson, Meylan, France) were collected. Within 4 h of collection, aliquots of peripheral blood mononuclear cells (PBMC), plasma and CSF were stored at −70°C. Clinical data from each patient were recorded on case report forms, including neuropsychological, cognitive, social, and motor disorders, history of animal contact, primary diagnosis, reason for spinal tap, results of brain imaging and followup information including definitive diagnosis (Table 1). This research on human subjects was approved by the Ethical Committee (Human Subjects Review Board) of the University Hospital.

The second group of 25 patients was retrospectively identified by searching the Swiss HIV Cohort Database (Ledergerber *et al*, 1994) for patients with (1) AIDS dementia complex (ADC) and (2) stored serum obtained within 1 month of ADC diagnosis. The 25 cases of ADC were diagnosed between November, 1988 and July, 1994. The tested sera were closely matched to the date of ADC diagnosis, with a median difference between diagnosis and serum collection of 1 day.

BDV RT–PCR
Searches of the GenBank and EMBL sequence databases (November 15, 1995) and sequence alignments were performed with GCG-8 software (Wisconsin Package, Version 8 for Silicon Graphics computers, Genetics Computer Group, Madison, WI, USA). Identification of primers and probes in the BDV p40 consensus sequence was performed with Oligo software (OLIGO primer analysis software, Version 5.0 for Windows, National Biosciences, Inc., Annapolis Lane, Plymouth, MN, USA).

BDV RT–PCR was performed as follows. RNA (final volume, 50 μl) was extracted from 5×10^6 patient PBMCs, animal brain tissue, or control cell lines (BDV He/80-infected and -uninfected C6 cells) using the RNeasy Kit (Qiagen AG, Basel, Switzerland) or from 140 μl CSF using the QIAamp HCV Kit (Qiagen) according to the manufacturer's instructions. For PBMC extractions, RNA yields were

determined by measuring fluorescence with RiboGreen RNA quantitation reagent (Molecular Probes Europe BV, Leiden, The Netherlands) according to the manufacturer's instructions. Before reverse transcription, a mixture containing 5 μl RNA (10% of total extracted RNA) and 6 μl primer BD139U (5′-ATG TTT GCT AAT CCC AGG ACT-3′) (1.3×10^{12} molecules, or 22 pmoles, primer) was denatured at 70°C for 10 min. The mixture was then reverse transcribed using the Expand Reverse Transcriptase kit (Boehringer Mannheim AG, Rotkreuz, Switzerland) according to the manufacturer's instructions, except that the incubation proceeded for 10 min at 30°C followed by 42°C for an additional 45 min. Amplification reactions (100 μl) containing 5 μl (25%) of the RT reaction and 50 μl 2× master mix [2.5 U AmpliTaq polymerase, 2× PCR buffer (both Perkin Elmer AG, Rotkreuz, Switzerland), 400 μM each dNTP, and 0.55 μg TaqStart antibody (Clonetech Laboratories Inc., Palo Alto, CA, USA), 5×10^{13} molecules (83 pmoles) of each primer, BD139U and BD139L (5′-CTG IGT IGT CTT TTC CCC GTA A-3′)] were denatured (2 min at 94°C), amplified with 35 cycles (30 s at 94°C, 45 s at 60°C and 30 s at 72°C) and extended for 5 min at 72°C. Amplified products were detected using ethidium bromide-stained gels and by liquid hybridisation. For hybridisation, 7 μl of PCR-product and 18 μl hybridisation solution [per hybridisation, 12.5 μl (50%) deionised formamide (Fluka, Buchs, Switzerland), 10^6 c.p.m.^{32}P-labelled probe (BD139P: 5′-TGC GTT TGT TCA CGG AGG GGT GCC T-3′), 1 μl 5 M NaCl, 0.5 μl 5 mM each dNTP (dNTP Set, Pharmacia, Piscataway, NJ, USA) and water to 18 μl] were denatured at 97°C for 5 min and hybridised by linear cooling to 30°C over 15 min in a thermal cycler. Ten μl of hybridised mixture were electrophoresed in agarose gels (2.25% NuSieve/0.75% MP agarose (FMC, Rockland, ME, USA, and Boehringer-Mannheim, respectively). The gels were dried and autoradiographed. Radioactive hybridisation improved the sensitivity of detection by at least two orders of magnitude.

Beta-actin RT–PCR
Recovery of amplifiable PBMC RNA was documented by semi-quantitative beta-actin RT–PCR. Primers designed in house correspond to beta-actin mRNA exons 3 (35up; 5′-GTCACCAACTGGGACGACATGGAGAA) and 4 (36low; 5′-CATGGCTGGGGTGTTGAAGGTCTCA), resulting in a 171 bp PCR product. Amplifications were performed with the EZ r*Tth* RNA PCR kit (Perkin-Elmer, Foster City, CA, USA) according to the suppliers suggestions. Briefly, 25 μl master mix (0.2 mM dNTP, 1× EZ buffer, 1.5 mM $Mn(OAc)_2$, 0.5 μM each primer and 0.15 U/μl r*Tth* DNA polymerase) and 1 μl template RNA were amplified in a T3 thermocycler (Biometra, Göttingen, Germany) as follows: 94°C for 45 s, 62°C for 20 min, 3 cycles of 95°C for 15 s, 62°C for 10 s and 72°C for 30 s, then 22 cycles of 90°C for 15 s, 62°C for 10 s and 72°C for 30 s, followed by 72°C for 15 min and cooling to 4°C. PCR products were electrophoresed in agarose gels and visualised with SYBR Gold nucleic acid stain (Molecular probes, Eugene, Oregon, USA). Digital images of the gels were created by scanning Polaroid photographs, and band intensities were determined using image analysis software (Intelligent Quantifier, BioImage, Ann Arbor, MI, USA). Copy numbers were calculated from band intensities by comparison to actin RT–PCR products from PBMC RNA standards.

BDV serology and immunohistochemistry
BDV serologies were performed as previously described (Caplazi *et al*, 1994). Briefly, BDV strain He/80-infected MDCK or C6 cells were fixed in acetone, incubated with threefold serum dilutions (37°C, 1 h, washed three times, incubated with anti-human FITC conjugate (37°C, 1 h), washed three times, counterstained with Evans blue, washed and mounted. Serum dilutions were scored as positive or negative based on the intensity of intranuclear signal under fluorescent microscopy.

BDV infection in the brain tissue samples used as positive control material for PCR was established by immunohistochemistry as described (Caplazi *et al*, 1994).

Paraffin sections of brain tissues from nine animals suffering from clinically manifest Borna encephalitis were deparaffinised and treated with 0.1% (w/v) protease (type XXVII, Sigma, Buchs, Switzerland). The monoclonal anti-BDV antibody 38/17 C1, recognising BDV p38, was used in a peroxidase streptavidin-biotin method (LSAB, DAKO, Glostrup, Denmark) with AEC as a chromogen. Specific signals were detected microscopically. A positive tissue sample and mock reactions without primary antibody were included in each assay to monitor the performance of the protocol.

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