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Absence of evidence of Borna disease virus infection in Swedish patients with Chronic Fatigue Syndrome

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Chronic Fatigue Syndrome (CFS) is characterized by debilitating fatigue, somatic symptoms and cognitive impairment. An infectious basis has been proposed; candidate agents include enteroviruses, herpesviruses, retroviruses and Borna disease virus (BDV), a novel neurotropic virus associated with neuropsychiatric disorders. Sera and peripheral blood mononuclear cells (PBMC) from Swedish CFS patients were assayed for evidence of infection using ELISA and Western immunoblot for detection of antibodies to BDV proteins N, P and gp18; and using nested reverse transcriptase polymerase chain reaction (RT–PCR) for detection of BDV N- and P-gene transcripts. No specific immunoreactivity to BDV proteins was found in sera from 169 patients or 62 controls. No BDV N- or P-gene transcripts were found through RT–PCR analysis of PBMC from 18 patients with severe CFS. These results do not support a role for BDV in pathogenesis of CFS.

Keywords: CFS; Borna disease virus; serum; PBMC; RT–PCR

Introduction

The pathogenesis of Chronic Fatigue Syndrome (CFS) is unknown. Criteria for diagnosis of CFS include debilitating fatigue lasting more than 6 months, impairment of short-term memory and concentration, pharyngeal pain, tender lymphadenopathy, myalgias, arthralgias, headache, sleep disturbance, and post-exertional malaise (Fukuda *et al*, 1994). Although CFS is usually sporadic, outbreaks have been reported. The observation of immunologic abnormalities, particularly in severely affected patients, is consistent with viral infection and has led to the speculation that CFS is the result of virus-induced immune activation (Landay *et al*, 1991; Cannon *et al*, 1997; Bennett *et al*, 1997; Gupta *et al*, 1997).

A wide spectrum of viruses has been proposed as factors or cofactors in pathogenesis of CFS including enteroviruses, Epstein-Barr virus, cytomegalovirus, human herpesvirus type 6, varicella zoster virus, retroviruses and Borna disease virus (Bode *et al*, 1992; Buchwald *et al*, 1996; Kitani *et al*, 1996; Nakaya *et al*, 1996). Bode and coworkers examined sera from 50 North American CFS patients and found no evidence of BDV infection (Bode *et al*, 1992). In contrast, two studies in Japan reported increased prevalence of Borna disease virus (BDV) infection in CFS patients: Kitani and colleagues found serum antibodies to the viral phosphoprotein (P) in 30 of 89 patients (34%), and P-gene sequences in peripheral blood mononuclear cells (PBMC) in seven of 57 patients (12%) (Kitani *et al*, 1996); Nakaya *et al* (1996) reported immunoreactivity to P-protein in six of 25 patients (24%) and P-gene sequences in PBMC of three of 25 patients (12%).

Borna disease virus is a neurotropic, negative-stranded RNA virus. Natural infection is most commonly reported in ungulates (horses, sheep and cattle); however, all warmblood species are likely to be susceptible. Infection may result in profound motor and cognitive dysfunction or mild disturbances in behavior dependent on the integrity and intensity of the host immune response (Hatalski *et al*, 1997). Infection of ungulates has not been reported in Sweden; however, an epidemic of encephalitis in domesticated cats (staggering disease) has been attributed to BDV suggesting a potential reservoir and vector for transmission to humans (Lundgren *et al*, 1993). The geographic region in which this epidemic was observed

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represents the cachement area of a well-characterized cohort of patients with CFS; thus, we examined these patients for evidence of BDV infection.

Results

Serology

A total of 169 sera samples from CFS patients were analyzed by enzyme-linked immunosorbent assay (ELISA) for reactivity to recombinant BDV proteins N, P, gp18, and an irrelevant control protein, β-galactosidase. Patients were divided into subgroups according to severity of illness – CFS-Severe and CFS-Mild. Sera from 62 normal subjects, including 30 asymptomatic individuals recruited by the CFS patients and 32 random blood donors, served as controls. Immunoreactivity to two or more BDV proteins was observed in nine out of 22 cases in the CSF-Severe group (40.9%) and in 18 out of 147 cases in the CFS-Mild group (12.2%, $P<0.003$) (Table 1). Immunoreactivity among controls was seen in two out of 30 cases in the recruited control group (7%) and in none of the 32 cases in the random blood donor group. Average immunoreactivity readings to BDV antigens were higher in CFS patients than in normal controls (Figure 1). However, immunoreactivity was not restricted to BDV proteins but was also observed with the irrelevant control protein β-galactosidase. Endpoint titration of 'reactive' sera with N, P, gp18 and β-galactosidase indicated that binding to BDV antigens was not specific (data not shown).

Western immunoblotting (WIB) was used as a second serologic system to assess samples from all 22 subjects in the CFS-Severe group and 31 subjects in the CFS-Mild group including those immunoreactive in ELISA with at least two BDV proteins. None of the CFS sera were immunoreactive with any of the BDV proteins in WIB.

Sera from experimentally infected rats and rhesus macaques were specifically reactive with BDV proteins in ELISA and WIB. Sera from normal rats and rhesus macaques did not react with BDV proteins in either ELISA or WIB (data not shown).

Nested RT–PCR

PBMC samples were obtained from 22 patients in the CFS-Severe group, coded, and shipped to the

Table 1 Serum immunoreactivity to Borna disease virus and β-galactosidase in Swedish CFS patients.

	ELISA	*Western blot*
Group A CFS-Severe	9/22 (9/22)	0/22
Group B CFS-Mild	18/147 (18/14)	0/31
Group C Recruited Controls	2/30	N/D
Group D Random Blood Donors	0/33	N/D

Serum immunoreactivity was defined as binding to at least two viral proteins (N, P, gp18). Parentheses indicate the number of patients immunoreactive in ELISA to irrelevant control protein, β-galactosidase.

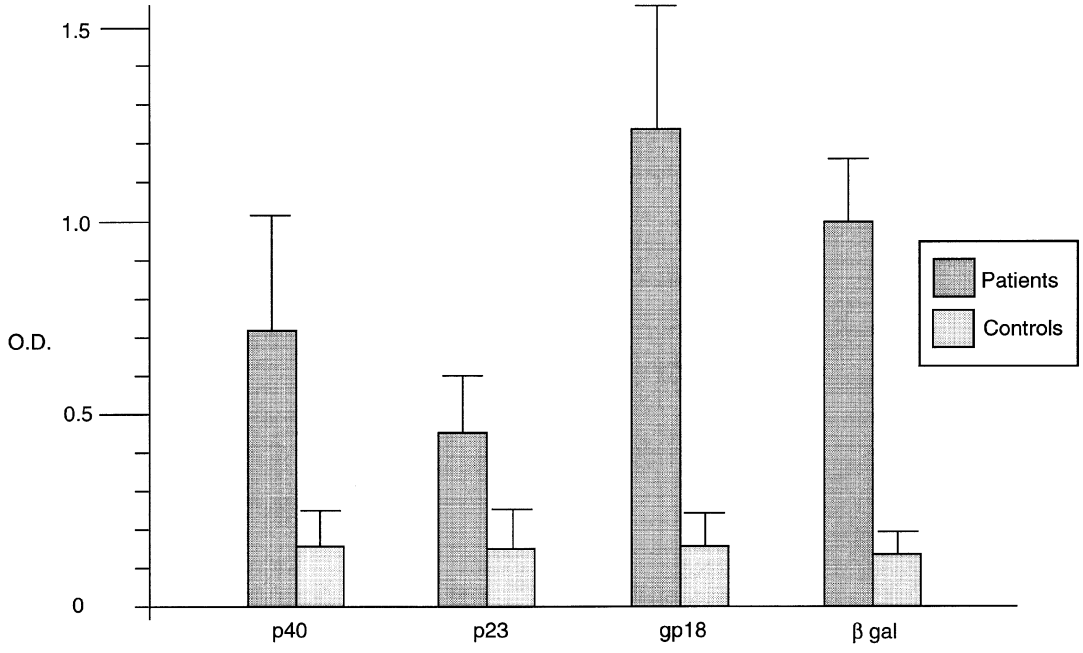

Figure 1 Mean reactivity of CFS patient and control sera to BDV N, P, gp18 and β-galactosidase control in ELISA.

University of California-Irvine for blinded RT–PCR analysis. RNA was extracted from PBMC, reverse transcribed with oligo d(T) and random hexamer primers, and initially analyzed by PCR with porphobilinogen deaminase (PD) primers to assess the quality and quantity of cDNA template. Thereafter, first strand cDNA templates from 28 samples representing 18 patients were subjected to nested RT–PCR for amplification of BDV N-gene (random hexamer primed cDNA) and P-gene (oligo d(T) primed cDNA) transcripts. Neither N-gene nor P-gene sequences were detected in any of the CFS samples. The threshold for detection of synthetic N-gene or P-gene RNA transcripts was in the range of 10–100 copies/reaction.

Discussion

The potential association between CFS and BDV in Japan (Kitani *et al*, 1996; Nakaya *et al*, 1996), together with data indicating an endemic focus of feline BDV infection (Lungren *et al*, 1993) within a cachement area for CFS in Sweden, led us to investigate the prevalence of BDV infection in a well-characterized cohort of Swedish CFS patients and normal subjects living in this cachement area.

ELISA analysis of sera from 169 CFS patients and 62 normal subjects revealed elevated immunoreactivity with N, P and gp18 in CFS patients, particularly in patients with severe disease. However, CFS sera also bound β-galactosidase, suggesting that immunoreactivity to BDV proteins was not specific. Furthermore, immunoreactivity observed in ELISA was not confirmed by WIB. The failure of ELISA-reactive CFS sera to bind BDV proteins in WIB may be due to lower sensitivity of the WIB assay or the presence of conformational determinants on BDV proteins that are accessible in ELISA but not WIB format. However, sera from experimentally infected rats and rhesus macaques readily detected BDV proteins in ELISA and WIB and did not bind β-galactosidase.

Nested RT–PCR was employed to investigate whether PBMC of CFS patients contained BDV transcripts. Although previous reports of BDV nucleic acids in CFS subjects have demonstrated only P-gene transcripts, N-gene transcripts have been described in PBMC of human subjects with other neuropsychiatric disorders (Bode *et al*, 1995; Kishi *et al*, 1995; Sauder *et al*, 1996). Thus, we assayed for the presence of both N-gene and P-gene transcripts. It has been suggested that levels of BDV nucleic acids in PBMC may fluctuate over the course of disease. Therefore, where possible, we investigated multiple samples from individual CFS subjects over a period of several months. Neither P-gene transcripts nor N-gene transcripts were detected by nested RT–PCR in PBMC of CFS patients with severe disease, using methods sensitive to detection of $\sim$100 copies of RNA template.

Our findings in Swedish CFS patients differ from reports of BDV infection in Japanese CFS patients (Nakaya *et al*, 1996; Kitani *et al*, 1996) where similar methods were employed for serology and RT–PCR detection of P-gene transcripts in PBMC. This could be due to geographic distinctions between the two groups with respect to diagnosis, clinical course, or differential exposure to infectious agents. Although serum immunoreactivity to BDV proteins observed in Swedish CFS patients by ELISA may reflect infection with related microbial agents that induce cross-reactivity with conformational determinants on BDV proteins (Kliche *et al*, 1996) and β-galactosidase, the serologic findings are also consistent with nonspecific polyclonal B-cell activation. Indeed, increased levels of antibodies against different microbial agents and other viruses, such as EBV, have previously been shown in sera from CFS patients (Jones *et al*, 1985; Straus *et al*, 1985) and interpreted as evidence of polyclonal activation.

In summary, we find no serologic or molecular evidence for BDV infection in Swedish CFS patients. Absence of evidence is not equivalent to evidence of absence. Nonetheless, our findings do not support the hypothesis that BDV can be implicated in the pathogenesis of CFS in Swedish patients.

Materials and methods

Patients and controls

One hundred and sixty-nine patients from an area northeast of Stockholm, Sweden, diagnosed with Chronic Fatigue Syndrome (CFS) according to CDC criteria (Fukuda *et al*, 1994) were recruited for this study. Disease was judged to be severe in 22 patients (CFS-Severe group: 11 female, 11 male; average age 31.4 (24–41) years and 40.1 (29–53) years, respectively) and mild in 147 patients (CFS-Mild group: 106 female, 41 male; average age 43.7 (14–65) years and 43.6 (23–60) years, respectively). Normal controls were asymptomatic non-blood relations recruited by CFS study patients (n=30; 26 female, 4 male; average age 42 (22–61) years and 43.8 (27–57) years, respectively), and random blood donors from Uppsala, a region 100 kilometers west of Stockholm (n=32; 9 female, 23 male; average age 43.6 (35–55) years and 46.3 (21–57) years, respectively).

Forty ml blood samples were collected from patients and controls: 5 ml were removed for serology; 35 ml were heparinized and subjected to Ficoll gradient centrifugation for isolation of PBMC. Multiple samples were collected in 3 month intervals over a period of up to 1 year from nine patients in the CSF-Severe group (four patients-four

samples; two patients-three samples; four patients-two samples). All samples were coded to protect patient privacy and ensure blinded analysis.

Recombinant BDV proteins
Recombinant BDV proteins N, P and gp18 as well as a negative control antigen, β-galactosidase, were expressed in *Escherichia coli* for use as antigens in enzyme-linked immunosorbent assay (ELISA) and Western immunoblot (WIB) serology experiments. Details of constructs, methods for protein expression and purification have been described elsewhere (Briese *et al*, 1995). All proteins were confirmed to be immunoreactive in ELISA and WIB with sera from experimentally infected rodents (rats) and primates (rhesus macaques) as well as naturally infected ungulates (horses and sheep) before use in assays with human materials.

ELISA
96-well Immulon I microtiter plates with lids (Dynatech) were coated overnight at 37°C with 50 ng of recombinant protein (N, P, gp18, β-gal) per well in 100 μl of borate buffer (100 mM boric acid, 50 mM sodium borate, 75 mM sodium chloride, pH 8.4). Plates were washed three times with wash buffer (0.05% Tween 20 in phosphate buffered saline [PBS]) and incubated for 1 h at 37°C with ELISA diluent (0.5% bovine serum albumin fraction V [BSA] in wash buffer). After three washes, 100 μl of 1 : 25 diluted sample sera in ELISA diluent were applied to each well (duplicate wells for each serum) and incubated for 2 h at 37°C. Plates were washed three times and 100 μl per well of horseradish peroxidase-conjugated goat anti-human IgG (Sigma, goat anti-rat IgG in case of rat controls) diluted 1 : 2500 in ELISA diluent were added for another 2 h at 37°C. After five washes, 100 μl 3,3′5,5′-tetramethylbenzidine substrate solution (Sigma) were added to each well and left for 30 min in the dark at room temperature before stopping the reaction with 50 μl/well of 25% sulfuric acid. The OD at 450 nm was determined for each well using a microplate reader (Thermo max; Molecular Devices). Sera from normal human subjects and normal rhesus macaques and rats served as negative controls. Sera from BDV infected rhesus macaques and BDV infected rats were used as positive controls. Samples were considered immunoreactive in ELISA if they bound at least two of three BDV proteins at reciprocal dilution titers at least 2 SD above the mean of an established population of healthy controls.

Western Immunoblot (WIB)
One-hundred ng of each recombinant protein per 0.5 cm gel width were size-fractionated by discontinuous sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE, 13%) and transferred to nitrocellulose membranes (Schleicher & Schuell). After blocking with WIB diluent (0.5% nonfat dry milk (Carnation)/0.05% Tween 20/PBS) for 1 h, the membranes were incubated with sample sera at a 1 : 40 dilution in WIB diluent for 2 h at RT. Membranes were washed three times with wash buffer (0.05% Tween 20 in PBS) and incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma; goat anti-rat IgG in case of rat controls) diluted 1 : 1000 in WIB diluent for 2 h at RT. After five washes, immunoreactive bands were visualized using Western Blue reagent (Promega). Sera from normal human subjects and normal rhesus macaques and rats served as negative controls. Sera from BDV infected rhesus macaques and BDV infected rats were used as positive controls.

Analysis of PBMC for the presence of BDV transcripts
PBMC were isolated from heparinized blood samples within 12 h after collection using Ficoll-Paque centrifugation (Pharmacia). RNA from 1–2×10^6 PBMC was extracted using Tri-Reagent (MRC). Two microgram RNA aliquots from each sample served as template for reverse transcription in a 20 μl volume using either an oligo d(T) primer (BDV P-gene transcript amplification; porphobilinogen deaminase (PD) transcript amplification) or random hexamer primer (BDV N-gene transcript amplification; porphobilinogen deaminase (PD) transcript amplification) and 200 U of Superscript Plus (Gibco BRL). After incubation with 1 unit of RNase H (Amersham), 2.5 μl of the reaction mixture were removed for 40 cycles of PCR in a DNA thermal cycler (Ericomp, Power Block II) using gene-specific primers (BDV-P gene, BDV-N gene, or PD) and 2.5 U of Taq DNA polymerase (Promega). One microliter of the initial PCR reaction was then removed for an additional 40 cycles of PCR using gene-specific primers nested with respect to the primers used for the initial PCR. For detection of BDV N sequences we adopted the RT–PCR protocol described by Sauder *et al* (1996, 1998) for analysis of human samples using primers based on BDV strain HE/80 sequence. RT–PCR conditions for detection of BDV P mRNA were identical to those used by Salvatore *et al* (1997) to detect BDV sequences in human brain material employing primers 5′-TGACCCAACCAGTAGACCA-3′ (nt 1387–1405) and 5′-GTCCCATTCATCCGTTGTC-3′ (nt 1865–1847) for initial PCR (40 cycles of 1 min 94°C denaturation; 1 min 58°C annealing and 1 min 72°C extension) and primers 5′-TCAGACCCAGAC-CAGCGAA-3′ (nt 1443–1461) and 5′-AGCTGGG-GATAAATGCGCG-3′ (nt 1834–1816) for nested PCR (same cycle conditions as above). Nucleotide positions for primers refer to BDV strain V (Briese *et al*, 1994, GenBank accession number U040608). Primers used for PD gene amplification were PD10 5′-ATTCGGGGAAACCTCAACAC-3′ and PD11 5′-

CCCACAGCATACATGCATT-3′. PD primers were selected for use as an index to the integrity of mRNA and presence of chromosomal DNA contaminants in RNA preparations. Amplification from human chromosomal DNA yields a 750 bp fragment; amplification from spliced mRNA yields a 152 bp fragment (Shimizu *et al*, 1994).

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