Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells derived from Japanese patients with chronic fatigue syndrome

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Abstract **CFS, a recently named heterogeneous disorder, is an illness of unknown etiology. The association of CFS with viral infections has been suggested. A common association between CFS and several viruses examined has not been confirmed. Here, we centered on the possible link between CFS and BDV infection. By nested RT-PCR followed by hybridization, BDV RNA was demonstrated as a clear signal in PBMCs in 3 out of 25 CFS patients. The amplified cDNA fragments were cloned and sequenced. A total of 16 clones were studied. Intra-patients divergencies of the p24 were 2–9%, 3–20%, and 3–11% in the deduced amino acids. Inter-patient divergencies among the 16 clones were 3–24%. Antibodies to recombinant BDV p24 protein were detected in 6 CFS patients including one carrying BDV RNA. Overall, these gave the prevalence of 32% (8/25) in Japanese CFS patients, suggesting that Japanese CFS is highly associated with active infection of BDV, or a related agent.**

Key words: Borna disease virus; Chronic fatigue syndrome; Peripheral blood mononuclear cell; RT-PCR

1. Introduction

Viral infections have been widely implicated in the etiology of a variety of neurological and neuropsychiatric syndromes [1]. The majority of the studies have focused on herpesviruses, especially herpes simplex virus type 1, cytomegalovirus and Epstein-Barr virus. However, the studies have yielded varied results in the levels of viral antibodies, viral antigens and viral genomes [1].

CFS is an illness which begins with an acute influenza-like symptoms including feverishness, headache, recurrent tender lymph glands, sore throat, diffuse muscle and joint pain [2]. Debilitating fatigue or easy fatiguability, and neuropsychologic problems such as difficulties with concentration and depression are other symptoms [2]. The Center for Disease Control proposed a working case definition for this syndrome, which relies on clinical and laboratory criteria [3]. CFS, also known as myalgic encephalomyelitis or postviral fatigue syndrome, has been strongly suggested to be sequelae of viral infections. The association with EB virus, human herpesvirus-6, coxsackie B virus, and HTLV-II has been examined in this syndrome [4]. However, a common association between CFS and a certain agent has not been found.

BDV is a neurotropic, yet unclassified, nonsegmented, negative-sense, single-stranded RNA virus which naturally infects horses and sheep [5,6]. In addition, BDV, or a related agent, has been suggested to be closely associated with specific psychiatric disorders, especially major depression, in humans mainly by using conventional serological assays such as immunofluorescence [7,8], although healthy people also have a low prevalence of anti-BDV antibodies [9–11]. Recently, a nested RT-PCR using primers for the BDV p24 gene has been developed to detect the genetic footprints of BDV in PBMCs derived from psychiatric patients [12,13]. As high as 36.7% (22/60) examined showed positive reaction in psychiatric patients in our examination [12], while the same technique showed only (4.7%) 8/172 prevalence in PBMCs derived from healthy blood donors [14]. The phosphorylated p24 protein is encoded in the second open reading frame and it may correspond to the polymerase cofactor found in other nonsegmented, negative-stranded RNA viruses [5]. In this report, we examined a possible link of CFS with BDV infection, because depression is similarly observed in CFS patients as one of the clinical markers for CFS [2].

2. Materials and methods

2.1. Patients

We studied BDV seroprevalence in randomly selected 25 Japanese CFS patients (Table 1), who were diagnosed with CFS in accordance with the guidelines established by the Center for Disease Control [3].

2.2. BDV and cells

MDCK cells uninfected or persistently infected with BDV (MDCK/BDV) [15] were used as controls.

2.3. Detection of BDV RNA in PBMCs

Total RNA fractions were extracted from whole EDTA-treated blood by using an RNA extraction kit (Isogen, Nippon Gene Co., Tokyo, Japan). To detect BDV-specific RNA, the extracted RNA was amplified by nested RT-PCR, under the same conditions as described [12], to obtain a fragment of the p24 coding region which is relatively conserved within the BDV genome [16]. Briefly, one microgram of cellular RNA was amplified by nested RT-PCR using two sets of primers as follows: for the 1st PCR, at nucleotides 1387–1405 and at nucleotides 1865–1847 and for 2nd PCR, at nucleotides 1443–1461 and at nucleotides 1834–1816. RT-PCR consisting of reverse transcription

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Abbreviations: CFS, chronic fatigue syndrome; BDV, Borna disease virus; RT-PCR, reverse transcriptase-polymerase chain reaction; PBMC, peripheral blood mononuclear cell; GST, glutathione *S*-transferase.

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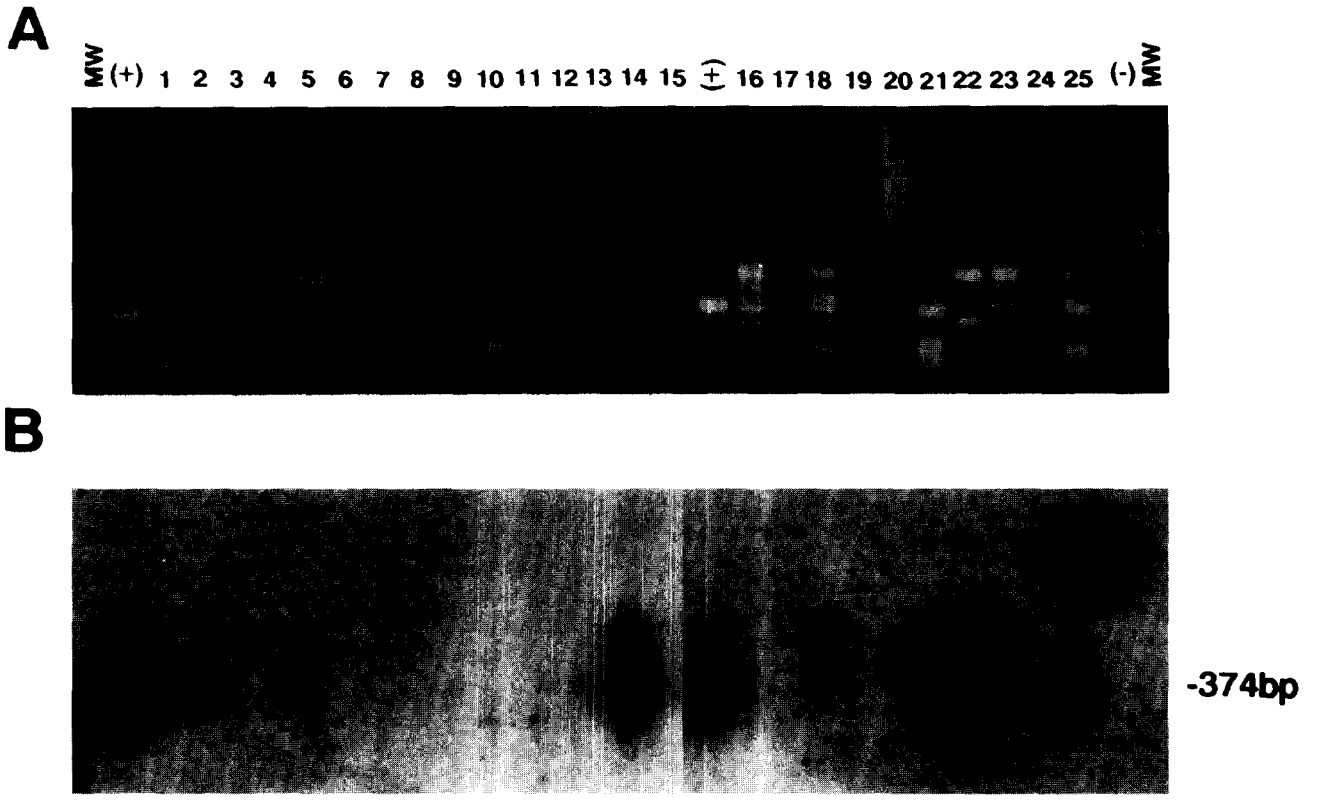

Fig. 1. Nested RT-PCR for the detection of BDV RNA in PBMCs from patients with CFS. A total of 25 samples (lanes 1–25) from CFS patients were subjected to nested RT-PCR. The products of PCR amplification of the BDV p24 region were resolved by agarose gel electrophoresis, then stained with ethidium bromide (A). The results of the Southern blot hybridization using four oligomers as probes are also shown (B). The positive and negative controls were the RNA fractions from MDCK/BDV (+) and MDCK (−), respectively. MW indicates the size markers (Øx174 DNA/*Hae*III fragments).

and amplification of the viral cDNA, was performed according to the protocol described for the EZ rTth RNA PCR kit (Perkin-Elmer Corp., Branchburg, NJ, USA). The final products were separated by 1.5% agarose gel electrophoresis, blotted onto a nylon membrane, then Southern hybridized using four ^{32}P-labeled synthetic oligonucleotides, sense nucleotides 1462–1485, 1485–1507 and 1637–1658, and the antisense nucleotide 1811–1791. Each of the PCR products were cloned into the pCR™II plasmid vector (Invitrogen Corp., San Diego, CA). Each clone was sequenced by use of the Dye Primer Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) in a 373A DNA Sequencer. Nucleotide numbers and the deduced amino acid sequences were those, previously reported for the He/80 strain of BDV [17].

2.4. Detection of anti-BDV antibodies

Anti-BDV antibodies in the plasma obtained from the same EDTA-treated blood as for RNA preparations were examined by immunoblotting, as described [14]. BDV antigen for these assays was horse BDV-derived recombinant full-length p24 fusion protein with GST and a negative control antigen was GST alone. Both GST-p24 and GST proteins were used after purification by glutathione Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) column chromatography. The molecular weight values of the proteins in immunoblotting were calculated by comparing their mobilities with those of marker proteins in a calibration kit (Bio-Rad).

3. Results

3.1. BDV RNA in PBMCs derived from CFS patients

A total of 25 CFS Japanese patients (Table 1) were examined for BDV RNA in their PBMCs. Nested RT-PCR revealed the presence of BDV RNA in CFS patients (Fig. 1). The amplified products derived from patients #14, #21 and #25 showed specific strong signals with 374 bp, which corresponds to the size of the fragment from persistent BDV in MDCK cells, by both ethidium bromide staining and Southern blotting. The amplified products derived from patients #13 and #18 showed only faint signals with 374 bp by Southern blotting. On the other hand, the sample from patient #10 showed a band with a slightly higher mobility which was apparently stained with ethidium bromide but was negative by Southern blotting with synthetic oligonucleotides used. These results are summarized in Table 1.

The amplified cDNA fragments from 3 patients were cloned, sequenced and analyzed. Six or four cDNA clones were derived from each patient, i.e. p14-1, -9, -10, -11, -22 and -25 from

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Fig. 2. Nucleotide and deduced amino acid sequences of BDV p24 region in PBMCs derived from 3 CFS patients. Sequence results of 16 cDNA clones, 6 from patient #14 (p14-1 to -25), 4 from patient #21 (p21-3 to -22), and 6 from patient #25 (p25-3 to -19), at nucleotides 1473–1772 are comparatively shown with those of horse-derived BDV, He/80 [17], strain V [16] and WT-1 [16] (A). Nucleotide sequences identical with those of He/80 were indicated by dots. Dashed lines indicate deleted nucleotides. The deduced amino acids according to these sequences were also similarly shown (B). 'X' indicates amino acid residue which is not determined because of the presence of nucleotide deletion within a corresponding codon.

A

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                1473                                                                                                                1572
He/80           CAGCTGTCGAATGATGAGCTTATCAAGAAGCTAGTGACGGAGCTGGCCGAGAATAGCATGATCGAGGCTGAGGAGGTGCGGGGCACTCTTGGGGACATCT
strain V        ....................A.........T.....................................................................A.......
WT-1            ....................T.....................................A.....................C.....................

P14-1           G.............A.....A.........T.....................................................................A......A
P14-9           G.............A.....A.........T.....................................................................A.......
P14-10          G.............A.....A.........T.....................C...............................................A.......
P14-11          G.............A.....A.......G.T.....................................................................A.......
P14-22          G.............G.....A.........T.....................................................................A.......
P14-25          G.............A.G...A.........T.....................................AC.C.............................A.......

P21-3           A......T..G.........A.........T..........G....T........G...T.......G.....................A.......
P21-4           TTCGA.CTCGG.ACCC....A.C.......T.....................G.....................--..........A.......
P21-11          ..A.................A.C....G..T.....................G.............G.....--..........A.......
P21-22          ....................A.C.......T.....................TG....................--..........A.......

P25-3           ....................G.C.G.....T...................G..G.....................--..................
P25-4           ....................A.C.......T.....G.............G.....................--..........A.......
P25-7           ....................A.C.......T.....................G.....................--..........A.......
P25-13          ......C.............G.C.......T.....................G.....C...............--..........A.G.....
P25-18          .G..................A.C.......T.....................G.....................--..G.......A.......
P25-19          ....................A.C.......T.....................G.....................--..........A.......


                1573                                                                                                                1672
He/80           CGGCTCGCATCGAGGCAGGGTTTGAGTCCCTGTCCGCCCTCCAAGTGGAAACCATCCAGACAGCTCAGCGGTGCGACCACTCCGACAGCATCAGAATCCT
strain V        .......T.....................................................................T.....................G.....
WT-1            .A........T..A.............A..............T.....................................T.........G.....

P14-1           .......T.................A.........T.....................................................T.........G..T..
P14-9           .......T.................A.....................A...............................T.........G..T..
P14-10          .......T.................A.....................A...............................T.........G..T..
P14-11          .......T.................A.....................................................A..T.........G..T..
P14-22          .......T.........G.......A...........G.....................................T.....G..G..T..
P14-25          .......T.................A.....................................................T.........G..T..

P21-3           .......T.................A.........T..............G.....................................G..T..
P21-4           .......T..............C..A.........T.....................................T..............G..T..
P21-11          .......T..............C.....................................................T..............G..T..
P21-22          .......T..............C..A..................................................T..............G..T..

P25-3           .......T.C.............C..A..............T............G.....................T..............G..T.A
P25-4           .......T.C.............C..A.....................................................T..............G..T..
P25-7           .........C.............C..A.....................G.....................T..............G..T..
P25-13          .......T..........A....C..A.....................................................T..............G..T..
P25-18          .......T..............C..A.....................G.G.....................T.........C.....G..T..
P25-19          .......T..............C..A.....................T...TC....C.............T...T..............G..T..


                1673                                                                                                                1772
He/80           TGGCGAGAACATCAAGATACTGGATCGCTCCATGAAGACAATGATGGAGACAATGAAGCTCATGATGGAGAAGGTGGACCTCCTCTACGCATCAACCGCC
strain V        C.....................A.....................................................T.....................
WT-1            C.....................................................................................

P14-1           C..T.....................................................G.............................T...AA.......
P14-9           C..T.....................................................G.....................A.........A....A...
P14-10          C..T....G................................................G.....................................A.......
P14-11          C..T.....................................................G.....................................AA......
P14-22          C..T.....................................A.A..A......A.....G.....................................AA......
P14-25          C..T.....................................................G.....................................A.......

P21-3           C..T.G.................G.......G.....................T.....................T.........TT........
P21-4           C..T.....................G.............G.....................GT...........C.........
P21-11          C..T.....................G.......G.....................T.............T.GG..........G.CGGGGGAT.
P21-22          C..T....G................G.....................................GT...........C.........

P25-3           C..T.....................G.....................................GT...........C.........
P25-4           C..T.....................G.........G...........................C..GT...........C.........
P25-7           C..T.....................G.....................................GT...........C.........
P25-13          C..T.....................G.....................................GT...........C.....A....
P25-18          C..T.....................G.....................C..G...........GT...........C.T......
P25-19          C..T.....T.............G.........G.....G.A.....................G.....GT.........T..C.........
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B

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                68                                                                                                                  167
He/80           QLSNDELIKKLVTELAENSMIEAEEVRGTLGDISARIEAGFESLSALQVETIQTAQRCDHSDSIRILGENIKILDRSMKTMMETMKLMMEKVDLLYASTA
strain V        ....................................................................................................
WT-1            ....................................................................................................

P14-1           E...E.....................T.....................V.....................E...........K..
P14-9           E...E.....................................................V.....................E...........T..
P14-10          E...E.....................................................V.....................E...........T..
P14-11          E...E....R................................................V.....S...............E...........T..
P14-22          E...E.....................................................YV....................E...........K..
P14-25          E...EG..............TP.....................G.....................V.M.............KK....E...........K..
                                                                             V.....................E...........T..

P21-3           K.LS..........V....V...G.....................A.....................G.....G..V.........F.........L...
P21-4           FELGTQ.T.........S........X.....................Y.....................E....A.........G....P..
P21-11          .......T.E.......S....G...X.....................Y.....................G..V.........F.....G...GRGI
P21-22          .......T.........C........X.....................Y.....S...............E.....................G....P..

P25-3           .......TE........GS.......X.........T.............V.......Y......H...........E.....................G....P..
P25-4           .......T....A....S........X.........T.....................Y.....................E..V..........AG....P..
P25-7           .......T.........S........X.........T.....................Y.....................E.....................G....P..
P25-13          ..P....T.........S.T......X....G.......R.....................Y.....................E.....................G....P..
P25-18          R......T.........S........X.....................MR......Y..R.....................E.....................G....PN.
P25-19          .......T.........S........X.....................D.S.P.....Y.....................G...E.V.........R.G...SP..
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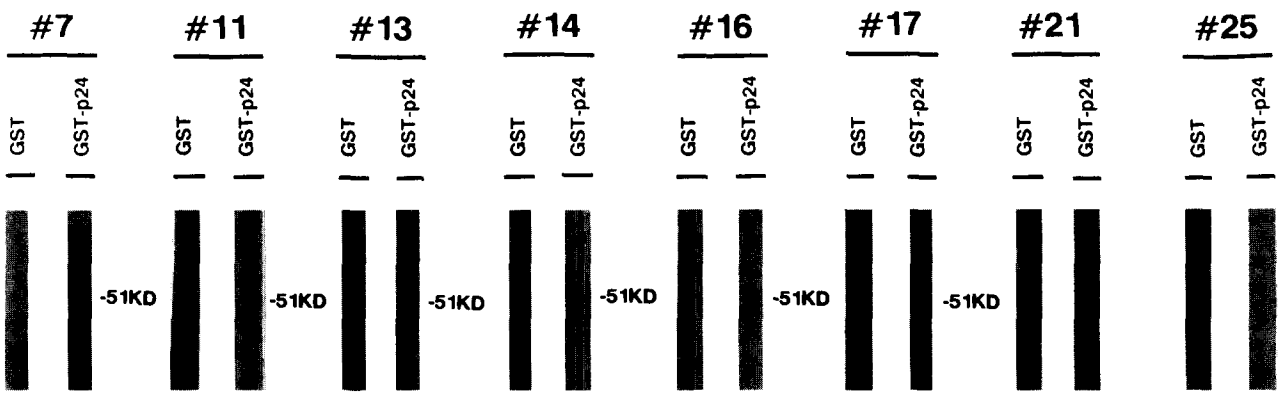

Fig. 3. Anti-BDV antibodies in the plasma samples from patients with CFS. The purified GST-BDV p24 (51 kDa) and GST as a control were similarly resolved by SDS-PAGE followed by its blotting onto a nitrocellulose membrane for immunoblotting. Hundred-fold dilutions of the plasma samples derived from 25 CFS patients were used. The results of 6 patients (#7, #11, #13, #14, #16, and #17) positive for anti-BDV and of 2 patients (#21 and #25) negative for anti-BDV were shown as representatives.

patient #14; p21-3, -4, -11, and -22 from patient #21; and p25-3, -4, -7, -13, -18 and -19 from patient #25. Fig. 2A shows the nucleotide sequence for each of the 16 p24 cDNA clones. Fig. 2B shows the amino acid residues deduced from the nucleotide sequence of each clone. The results showed that the CFS-derived BDV sequences were similar to, but significantly different from, the reported sequences of horse-derived BDV, He/80 [17], strain V [16] and WT-1 [16] which were also shown in Fig. 2 as controls. The two base deletion, GG in place of GGGG at nt 1552–1555, was detected in all cDNA clones from patient #25. Hence, all these cDNA clones were defective for p24. The same deletion was also detected in 3 among 4 clones from patient #21. In addition, there were several amino acid substitutions specific to the patients. Especially, substitutions to the same amino acids at several sites were observed in the clones derived from patients #21 and #25. Computer analyses (GENETYX-MAC, Software Development Co. Ltd., Tokyo, Japan) revealed that intra-patient divergencies in this p24 region at the amino acid level were 2–9% (#14 patient), 4–20% (#21 patient), and 3–11% (#25 patient). Inter-patient divergencies were 9–21% between patients #14 and #21, 13–22% between patients #14 and #25, and 3–24% between patients #21 and #25, which were higher than the divergencies from those of the horse strain V, He/80 and WT-1 by 6–10% (patient #14), 8–12% (patient #21), and 8–14% (patient #25). Thus, in p24, BDV in the PBMCs in CFS patients undergoes mutation at high rates in vivo.

3.2. Detection of anti-BDV antibodies in plasma

Hundred-fold dilutions of 25 plasma samples obtained from the same blood as used for RNA preparations were examined for the presence of anti-BDV antibodies by immunoblotting. The plasma from 6 patients (patients #7, #11, #13, #14, #16 and #17) including patient #14 who showed a positive reaction for BDV RNA were positive by immunoblot analysis using GST-p24, but not GST alone (Fig. 3). In the plasma from patients #21 and #25 showing positive signals for BDV RNA in their PBMCs, no specific reaction by immunoblotting was observed (Fig. 3). These results are summarized in Table 1.

4. Discussion

Our findings suggest that Japanese CFS is associated, at least in part, with active infection of BDV, or BDV-related agent, although no serologic evidence of BDV infection was reported in CFS patients by a German group [18]. This discrepancy seems to be due to the different techniques, namely they used immunofluorescence assay, while we used RT-PCR to detect BDV-related RNA in PBMCs, and immunoblot using purified BDV p24 recombinant protein to detect anti-BDV antibody in plasma.

BDV RNA was not always detected in the PBMCs in the same CFS patients positive for anti-BDV antibodies (Table 1). Essentially similar results were also observed in patients with psychiatric disorders [12,13], blood donors [14], healthy horses [19] and cats [20]. These observations suggest that the group positive for anti-BDV antibodies and negative for BDV RNA in PBMCs had been exposed to BDV antigens. Alternatively, the BDV in this group might be present in the tissue cells other

Table 1
Profiles of 25 CFS patients and their summarized results for BDV

Patient	Age	Sex	Disease onset	BDV RNA	Anti-BDV p24
1	19	M	1990.7	−	−
2	23	M	1991.5	−	−
3	24	M	1994.4	−	−
4	24	M	1988.12	−	−
5	25	M	1991.12	−	−
6	26	M	1994.9	−	−
7	32	M	1990.7	−	+
8	36	M	1994.1	−	−
9	36	M	Around 1988	−	−
10	37	M	1993.10	−	−
11	40	M	Around 1985	−	+
12	40	M	1989.1	−	−
13	43	M	1991.9	−	+
14	44	M	1992.3	+	+
15	57	M	1990.2	−	−
16	21	F	1991.6	−	+
17	21	F	1995.1	−	+
18	22	F	1991.12	−	−
19	25	F	1991.1	−	−
20	25	F	1994.3	−	−
21	28	F	1991.8	+	−
22	30	F	1993.2	−	−
23	35	F	1991.5	−	−
24	36	F	1991.4	−	−
25	52	F	1990.4	+	−

than PBMCs, because we examined BDV RNA only in PBMCs. In contrast, the group positive for BDV RNA in PBMCs and negative for anti-BDV antibodies seems to be latently infected with BDV, as indicated by 2 base deletions in all 6 p24 cDNA clones from patient #25 and 3 among 4 p24 cDNA clones from patient #21 (Table 1, Fig. 2). Alternatively, disruption of viral latency might give rise to high expression of viral proteins which complexed with the circulating anti-BDV antibodies, and interfered with immunoblot analysis. Overall, 8 among 25 (32%) CFS patients examined showed the association of BDV (Table 1). One patient amplified a smaller fragment under the same PCR conditions, but this did not hybridize with the p24-specific probe, suggesting that Southern blotting is a necessary step to avoid false positive results. It is noteworthy that a strong association of BDV was demonstrated with patients with major depression [7,8], because similar depression is also observed in patients with CFS [2]. The association of BDV with 32% CFS patients might be due to the immunocompromized state of their patients which is a clinical marker of CFS [21], or may indicate a relation with the disease at least in part in the Japanese patients. An extensive follow-up study from the onset of the disease on BDV RNA in PBMCs and anti-BDV antibodies, as done in psychiatric patients [13], will determine the prevalence of BDV infection in this syndrome and may clarify whether this agent is related with the disease pathogenesis or the results of reactivation of latent BDV by an unknown stimulation mechanism.

The amplified products of the p24 gene in PBMCs from three patients with CFS were cloned and sequenced. Schneider et al. [16] compared the sequence of the p24 among several BDV strains from horses. They found a 3.1% variation at the nucleotide level and a 1.5% difference at the amino acid level. Binz et al. [22] also found that the p24 in tissues from 4 infected horses had a maximum divergence of 3.3% at the nucleotide level, and 1.5% at the amino acid level. In this study, the intra- and inter-patient sequence variabilities of the p24 were much higher even at the deduced amino acid level (Fig. 2). Our recent analyses of the p24 from 3 psychiatric patients have also revealed slightly more sequence variabilities, i.e. 7.7–14.5%, 10.3–17.1% and 6.0–16.2% in intra-patient divergencies and 12.8–28.2% in inter-patient divergencies at the amino acid level [23]. The reason for the higher variabilities detected in these studies is unknown, but could have resulted from the following reasons, i.e. (1) higher number of clones analyzed in this study; and (2) difference of mutation rates in human and horse cells. In addition, there is also a possibility that the mutations observed here were PCR artifacts. However, misreading or template switching by the *Taq* polymerase would be a random event. Therefore, it would not have caused mutations at a limited number of nucleotide positions in all of 4 or 6 cDNA clones from one patient (Fig. 2). Although the frequency of BDV polymerase error is unknown, it may be as high as rhabdoviruses, i.e. 10^{-4} to 4×10^{-4} substitutions per base incorporated at a single site [24]. These variations suggest that BDV in PBMCs is subjected to higher selective pressure in vivo, especially those immunologic in nature. In addition, high capacity for variability may allow this virus to respond to the influence of host factors, such as the age, immune status or genetic background of the host, which would affect on the pathogenesis of BDV, as already discussed previously [25]. This possibility may be supported by our recent finding of the outbreak (2 developing CFS and another 2 showing related symptoms in the 5 members) in the family of the patient #14, in which 3 of the 5 members had significantly higher antibodies to BDV (not shown). The BDV genome characterization in each family member may reveal the cause of transmission and pathogenicity of the particular BDV in patient #14.

Since we characterized only about 3% of the genome in this study, a comparison of the whole genomes would be required to determine the similarity and dissimilarity of the BDV in CFS patients with those in horse or psychiatric patients.

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