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Lack of Association of Borna Disease Virus and Human T-Cell Leukemia Virus Type 1 Infections with Psychiatric Disorders among Japanese Patients

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Borna disease virus (BDV) infection has been suspected to be a possible etiological factor in human psychiatric disorders and recently in chronic fatigue syndrome. Evidence of the correlation of BDV infection with these disorders remained unclear. Kagoshima is known to be one of the major areas in which human T-cell leukemia virus type 1 (HTLV-1) is endemic; this is the first isolated human retrovirus that causes adult T-cell leukemia with neurological symptoms. The present study aimed to clarify whether BDV and HTLV-1 infections are associated with psychiatric disorders among Japanese patients. Subjects were 346 patients with psychiatric disorders (schizophrenia, 179; mood disorder, 123; and others, 44) and 70 healthy controls. Anti-BDV antibodies from plasma samples were screened by the indirect immunofluorescence (IF) method using BDV-infected MDCK cells. Results revealed that only three samples were found to be weakly positive for BDV in the IF assay and seronegative by Western blot (immunoblot) assay. Furthermore, BDV-p24 related RNA in peripheral blood mononuclear cells from 106 of 346 psychiatric patients and 12 of 70 healthy controls by p24-reverse transcription PCR was examined. Two mood disorder patients were positive for BDV-p24 RNA but seronegative. To detect anti-HTLV-1 antibodies the plasma samples were screened by the particle agglutination method and no significant difference in seropositivity for anti-HTLV-1 antibody was found between the patients and healthy controls. These results also suggested that there is a lack of association between BDV and HTLV-1 infections with psychiatric disorders among Japanese patients.

In order to find a possible etiology of psychiatric disorders, viral infections among psychiatric patients have been examined (8, 16), although evidence that viral infections could be etiological factors in human psychiatric disorders could not be found. Recently, Borna disease virus (BDV) infection as a possible etiological factor in human psychiatric disorders was studied, since BDV causes neurological disorders in horses and sheep in certain areas of central Europe, where it has been endemic for over 150 years (28). BDV was first isolated from a horse with encephalitis (45). The virus is a negative, nonsegmented, single-strand RNA virus (35) that is highly neurotropic (25). Infection with BDV causes an inflammatory response in the brain and can induce degeneration of the nerve cells in the chronic phase of the disease (10, 21). The route of infection to the brain has not yet been determined, although an intranasal or intrapharyngeal pathway is likely to be the natural route of infection and BDV may invade the brain via olfactory or trigeminal nerves (25).

BDV was recently found to cause sporadically occurring progressive enchephalopathies among natural hosts such as sheep (10), cats (22), rabbits (24), and ostriches (23). Furthermore, BDV has been shown to infect experimentally a broad spectrum of animals, ranging from chickens to nonhuman primates (14, 39). Depending on the animal species, the virus infection induces various neurological and behavioral abnormalities, and several seroepidemiological studies on BDV infection in humans have been carried out. The first studies reported that patients with psychiatric disorders, especially unipolar and bipolar depression, showed a higher prevalence of antibodies (4 to 4.5%) specific for BDV antigen than healthy controls (0%) by indirect immunofluorescence (IF) assay (1, 32). Bode et al. (3) reported the presence of BDV-specific antibodies in depressed patients (2.2%), patients infected with human immunodeficiency virus (4.4 to 8.1%), and healthy controls (2.3%) by means of a double-stain IF assay with human serum and BDV-specific monoclonal antibodies to the virus. This study suggested that there was no significant difference in the presence of BDV-specific antibodies between the patients with depression and the healthy controls. Waltrip et al. (42) reported a significantly higher prevalence of anti-BDV antibodies (13.3%) against 14.5-kDa BDV antigen among schizophrenic patients than in healthy controls (0%). In general, these seroepidemiological studies claim an association between BDV infection and human psychiatric disorders. The prevalence of BDV-seropositive patients with psychiatric disorders varies among these reports, performed only in Germany and the United States (1, 3, 9, 32, 42). Recently, Bode et al. (6) reported that infectious human BDV could be first isolated from a patient's blood cells.

Human T-cell leukemia virus type 1 (HTLV-1) is a human retrovirus which causes hematological malignancy and adult T-cell leukemia, as well as a neurological disorder, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (29, 43). In particular, HTLV-1 is found to be especially endemic in Japan (40). Several etiological studies on HTLV-1

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TABLE 1. Positivity of anti-BDV and anti-HTLV-1 antibodies among Japanese psychiatric patients and healthy controls

Diagnosis,[a] sex, and no. of subjects	No. of patients		
	BDV		HTLV-1
	Weakly positive	Negative[b]	Seropositive (%)
Schizophrenia			
M, 111	2[c]	109	7 (6.3)
F, 68	0	68	2 (2.9)
Mood disorder			
M, 42	1[c]	41	2 (4.8)
F, 81	0	81	9 (11.1)
Other disorders			
M, 22	0	22	3 (13.6)
F, 22	0	22	2 (9.1)
Controls			
M, 37	0	37	4 (10.8)
F, 33	0	33	2 (6.1)

[a] The criteria for diagnoses were described in Materials and Methods. "Schizophrenia" includes paranoid, hebephrenic, catatonic, undifferentiated, and residual schizophrenia categorized as F2 with a mean patient age of 48.36 ± 12.17 years (range, 20 to 80 years). "Mood disorders" include manic episodes, bipolar affective disorder, depressive episodes, and recurrent depressive disorder, categorized as F3 with a mean patient age of 52.95 ± 13.24 years (range, 21 to 75 years). "Other disorders" include organic, mental disorders, mental and behavioral disorders due to the use of alcohol, and personal disorders with a mean patient age of 47.33 ± 13.61 years (range, 22 to 82 years). "Controls" had a mean age of 45.43 ± 11.04 years (range, 25 to 74 years).

[b] The samples which showed no fluorescent staining at a dilution of 1:10 were judged negative.

[c] Confirmed as negative for anti-BDV antibody by WB assay (see Fig. 2).

infection among psychiatric patients have also been conducted. Delisi and Sarin (8) and subsequently Robert-Guroff et al. (31) reported that no association of the presence of anti-HTLV-1 antibody was found in serum from schizophrenic patients. These reports suggested that schizophrenia is not associated with HTLV-1 infection. However, there is a little information about HTLV-1 infections among patients with psychiatric disorders in areas in which HTLV-1 is endemic. Furthermore, the correlation of HTLV-1 infection with affective disorders has not been examined.

We undertook a seroepidemiological study of the prevalence of BDV and HTLV-1 seropositivity among Japanese patients with psychiatric disorders, and the results were compared with those for healthy controls, in order to clarify the etiological roles of BDV and HTLV-1 in psychiatric disorders.

MATERIALS AND METHODS

Subjects. Subjects were 346 patients with psychiatric disorders and 70 healthy subjects living in Kagoshima Prefecture, southern Japan. The psychiatric patients included 179 patients with schizophrenia, 123 with mood disorders, and 44 with other disorders. All patients were diagnosed according to the ICD-10 classification of Mental and Behavioural Disorder (44) at the Kagoshima University Hospital and two other affiliated hospitals. All of them were treated with neuroleptics, antidepressants, or antianxiety drugs. The healthy controls were 33 females and 37 males from Kagoshima Prefecture (Table 1).

Heparinized peripheral blood samples (7 to 10 ml) were collected with the informed written consent of each patient. The plasma and the peripheral blood mononuclear cells (PBMCs) were prepared as described previously (15). The plasma was stored at −30°C, and the PBMCs were cryopreserved in liquid nitrogen prior to use.

Cell culture. MDCK cells which are persistently infected with BDV He/80, originally isolated from a horse (11), and uninfected MDCK cells were grown in Dulbecco's Modified Eagle Medium (Nissui Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum at 37°C and 5% CO_2. BDV-infected and uninfected MDCK cells (5×10^4 cells/well) were seeded on Lab-Tek 8 chamber slides (Nunc Inc., Naperville, Ill.). After 48 h of culture, the cells were washed in phosphate-buffered saline (PBS) three times and dried completely, followed by fixation with cold acetone for 30 min. The fixed cells were kept at −30°C prior to use.

IF assay and Western blot (immunoblot) (WB) assay. To detect anti-BDV antibodies, we used the IF method according to a previous description (32) with some modifications as follows. The test plasmas were mixed with 4 volumes of swine serum (Dako, Glostrup, Denmark) diluted 5 times in PBS, and then the plasma samples were absorbed with swine liver powder (100 mg/ml) to eliminate nonspecific background staining. The acetone-fixed BDV-infected and uninfected MDCK cells were incubated with swine serum diluted 5 times in PBS for 10 min at room temperature, washed in PBS containing 0.05% nonionic detergent P-40 (NP-40) (Sigma, St. Louis, Mo.), and dried. Then, 50-μl plasma samples per well were added to BDV-infected and uninfected MDCK cells. The cells were incubated for 30 min at 37°C, and then the cells were washed in PBS containing 0.05% NP-40 three times for 5 min and dried. Anti-BDV rat serum was used at a dilution of 1:250 (provided by J. A. Richt). BDV-positive and -negative known human control samples were obtained from seven German patients with psychiatric disorders (2) and three healthy German subjects. In each IF assay, both the positive and negative control serum were used at a dilution of 1:10. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human immunoglobulin G (IgG) (Dako), and FITC-conjugated goat anti-rat IgG (Organon Teknika Co., Durham, N.C.) were used at dilutions of 1:20 and 1:100 in PBS with 1% fetal bovine serum, respectively. These second antibodies were added to cells (50 μl/well) and incubated for 30 min at 37°C in a moist atmosphere. After cells in PBS containing 0.05% NP-40 were washed three times for 5 min, the cells were dried and covered with 90% glycerin in carbonate buffer. The fluorescently staining cells were examined under a fluorescence microscope, BHS-RFK (Olympus Ltd., Tokyo, Japan).

The samples determined positive or indeterminate by the IF assay were further tested by WB assay using lysate from BDV-infected and uninfected MDCK cells in the Department of Virology in Giessen, Germany, as described previously (33). The lowest dilution tested in WB was 1:10.

RT-PCR assay. Total RNA extraction was carried out by the acid guanidium chloroform method with an RNA extraction kit, Isogen (Nippongene, Tokyo, Japan) (7). The frozen PBMCs from the subjects were quickly thawed, washed in PBS three times, and subjected to RNA extraction. Total RNA extracted from BDV-infected and uninfected MDCK cells was used in reverse transcription-PCR (RT-PCR) as a positive and a negative control. Total RNA (0.5 μg) was used for reverse transcriptase reaction, followed by PCR amplifications using the RNA PCR kit AMV, version 2, according to the manufacturer's instructions (TaKaRa, Tokyo, Japan). The sequences of the primers used for the amplification-encoded part of the open reading frame for the p24 protein of BDV (BDV-p24 primers) were 5′-TGACCCAACCAGTAGACCA-3′ and 5′-GTCC CATTCATCCGTTGTC-3′, as described previously (18). The sequences of β-actin primers were 5′-TACATGGCTGGGGTGTTGAA-3′ and 5′-AAGAGAGG CATCCTCACCCT-3′ (17). The samples were overlaid with 30 μl of mineral oil and amplified through 35 cycles of denaturation at 96°C for 1 min and annealing at 63°C for 1.5 min, with the final extension at 63°C for 10 min, and were cooled to 4°C. An aliquot (10 μl) of PCR products was analyzed in 3% NuSieve and 1% agarose gel electrophoresis (FMC BioProducts, Rockland, Maine) in the presence of 1 μg of ethidium bromide per ml. Gels were visualized and photographed by a UV transilluminator (Funakoshi, Tokyo, Japan). In this study we performed three different RT-PCRs per sample, consisting of BDV-p24 primer with and without RT reaction and β-actin primer with RT reaction.

RT-PCR assay could detect the BDV-related sequence in 10^{-5} μg of total RNA from BDV-infected MDCK cells which corresponded to 1×10 to 2×10 copies of RNA transcript.

Cloning and sequencing of PCR products. PCR products were purified from nonreacted primers, nucleotides, and polymerases by the QIAquick-spin PCR purification kit (Qiagen, Hilden, Germany). The purified PCR products were ligated into the pGEM-T vector (Promega, Madison, Wis.) and transfected to JM109 competent cells (TaKaRa) under conditions recommended by the manufacturer. Positive colonies were selected by a blue-white color screening system on indicator plates. Nucleotide sequence of the recombinant clone was determined by the dideoxy termination sequencing method (34) using the AutoRead sequencing kit (Pharmacia, Uppsala, Sweden). Three independent clones were sequenced to exclude nucleotide sequence changes during the PCR cloning procedure.

Screening of anti-HTLV-1 antibodies. For detection of anti-HTLV-1 antibodies, the plasma samples were screened by the particle agglutination method using the Serodia-atla kit (Fujirebio, Tokyo, Japan) (13). A positive result was recorded if the sample agglutinated the particles at the dilution of over 1:16, and the sample was further titered by using serial twofold dilutions in PBS.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D85768.

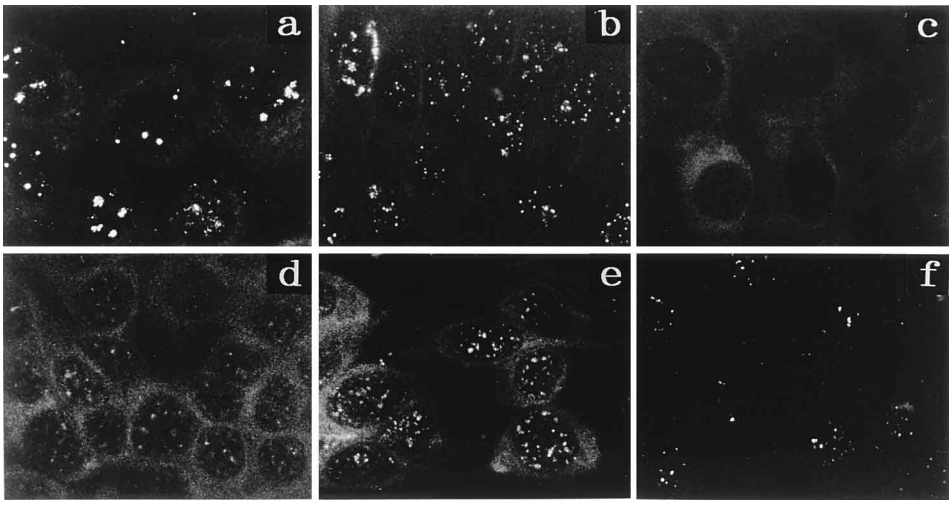

FIG. 1. IF staining pattern of BDV-infected MDCK cells with rat positive control sera (1:250) (a), human positive control sera (1:10) (b), human negative control sera (1:10) (c), patient plasma 12 (1:10) (d), patient plasma 163 (1:10) (e), and patient plasma 303 (1:10) (f).

RESULTS

Subjects. The subjects in this study were admitted to the Kagoshima University Hospital and affiliated hospitals from November 1994 to December 1995, and there was no significant difference in the average age and range among patients with schizophrenia, mood disorders, and other disorders. All of the patients with schizophrenia were inpatients, and the majority of them were in incomplete remission without prominent psychotic symptoms, as in the ICD-10 classification (44). The majority of the patients with mood disorders were outpatients, who were experiencing mild or moderate episodes.

Detection of anti-BDV antibodies by IF assay and WB assay. Anti-BDV rat serum showed a typical staining pattern of granular, focal fluorescence in the nucleus of BDV-infected MDCK cells (Fig. 1a). Positive control serum obtained from a German patient with a psychiatric disorder showed a similar staining pattern (Fig. 1b). Negative control serum obtained from a healthy German subject did not show any nuclear staining (Fig. 1c). Three subjects in the Japanese study showed a similar staining pattern in the nucleus of BDV-infected but not uninfected MDCK cells; however, the intensity of fluorescence with BDV-infected MDCK cells was rather weak compared with that of the positive control (Fig. 1d, e, and f). The three patients were therefore judged weakly positive by the IF assay. Thus, two patients of 179 schizophrenia patients (1.1%) and 1 of 123 mood disorder patients (0.8%) were indefinitely positive for anti-BDV antibody by the IF assay. Neither patients with other disorders or any of the control subjects were positive (Table 1).

Three weakly positive samples were further analyzed by a WB assay. In contrast with the positive human control serum, which reacted clearly with BDV-p38 protein, three IF-weakly positive Japanese samples did not react with BDV-p38 protein or any other proteins, although some nonspecific bands were recognized in BDV-infected and uninfected MDCK cell lysates in all tested samples (Fig. 2). Thus, three Japanese samples were concluded to be negative for BDV-specific antibody in the WB assay.

Detection of BDV-related RNA in PBMCs by p24-RT-PCR and nucleotide sequence of BDV-p24 related RNA. In order to evaluate whether BDV-related RNA is present in the PBMCs of the present subjects, RT-PCR analysis was carried out. First, the effect of annealing temperature on the outcome of amplification was tested (Fig. 3). BDV-p24 RNA was amplified as a 479-bp band from RNA of BDV-infected MDCK cells at a high and a low annealing temperature. At the low annealing temperature, various sizes of DNA bands in human samples were found (Fig. 3a), but these nonspecific amplification products were eliminated at the high annealing temperature (Fig. 3b). Amplification of β-actin mRNA was not affected by the low or the high annealing temperatures. Under this high annealing temperature condition, RNA samples of PBMCs from 106 patients, including three IF weakly positive patients and 12 healthy controls, were screened. Two samples from the mood disorder patients showed the 479-bp bands (Fig. 4), although these patients were negative by BDV-specific IF assay. All samples from schizophrenia or other patients with disorders, as well as healthy controls, did not show the 479-bp amplification products.

The amplified band from a sample from a mood disorder patient was cloned and the nucleotide sequence was determined (Fig. 5). The nucleotide sequence of the isolate from the patient (512) was compared with those from a Japanese patient (p2-9) (19), horse BDV He/80-1, and strain V (30, 36), and a BDV open reading frame-II cDNA clone derived from BDV-

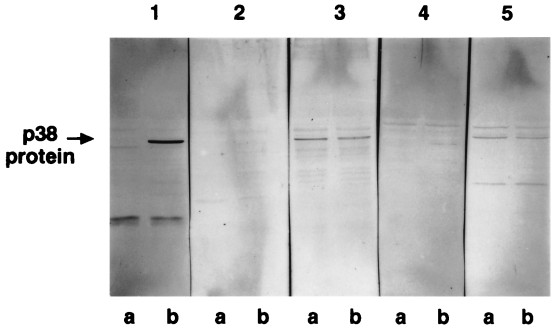

FIG. 2. Detection of anti-BDV antibodies by WB method. WB strips containing extracts from uninfected MDCK cells (a) and BDV-infected MDCK cells (b) were reacted with human positive control sera (1:30) (lane 1), human negative control sera (1:30) (lane 2), patient plasma 12 (1:30) (lane 3), patient plasma 163 (1:30) (lane 4), and patient plasma 303 (1:30) (lane 5).

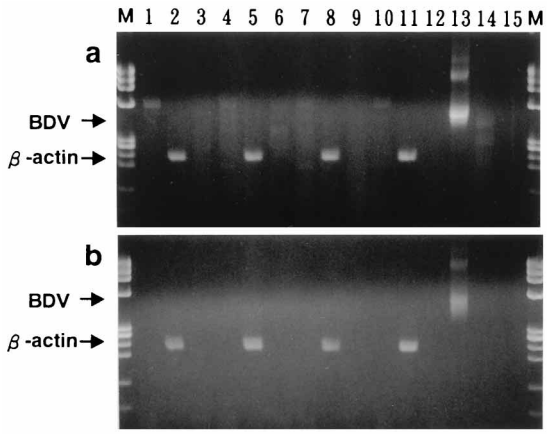

FIG. 3. Effect of annealing temperature on p24 RT-PCR analysis. RNA extracted from PBMCs of psychiatric patients and BDV-infected MDCK cells was subjected to RT-PCR at annealing temperatures of 62°C (a) and 63°C (b). Lanes: 1, 2, and 3, patient 12 p24 primer, β-actin primer, and no RT reaction; 4, 5, and 6, patient 163 p24 primer, β-actin primer, and no RT reaction; 7, 8, and 9, patient 210 p24 primer, β-actin primer, and no RT reaction; 10, 11, and 12, patient 217 p24 primer, β-actin primer, and no RT reaction; 13, BDV-infected MDCK cell p24 primer; 14, uninfected MDCK cell p24 primer; 15, no RNA p24 primer; M, ϕX174 DNA digested with *Hae*III as a size marker.

infected MDCK cells (C-47) (19). The nucleotide sequence of 512 was similar to that of p2-9, although several variations were observed. In the variation cluster region (nucleotides 253 to 271), 512 was identical with p2-9 but not with He/80-1, strain V, or C-47.

Detection of anti-HTLV-1 antibodies. The plasma samples were screened to detect anti-HTLV-1 antibodies by the particle agglutination method, and the prevalence of HTLV-1 infection was evaluated in the present study subjects. The seroprevalence ranged from 2.9 to 13.6% among psychiatric patients, and there was no difference between this and that of the controls (6.1 to 10.8%) (Table 1).

DISCUSSION

IF assay has conventionally been used as the standard method for the detection of BDV-specific antibodies on BDV-infected MDCK cells (3, 33), and the typical staining pattern of granular and/or focal fluorescence in the nucleus is characteristic. Although we found three cases in which a granular, focal fluorescence staining in the nucleus of BDV-infected MDCK cells was shown (Fig. 1), the intensity of fluorescence in these cases was rather weak compared with that of the positive control. These weakly positive samples in the IF assay were also confirmed as seronegative by WB assay (Fig. 2). The results suggested that there was no association of BDV infection with psychiatric disorders.

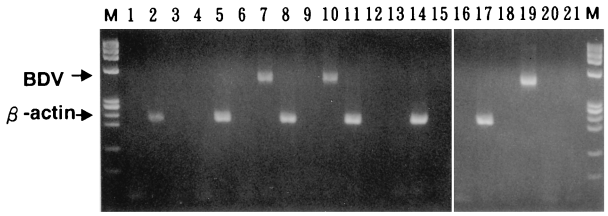

FIG. 4. Detection of BDV-specific genomic transcripts (p24) in PBMCs of psychiatric patients by RT-PCR. Lanes: 1, 2, and 3, patient 12 p24 primer, β-actin primer, and no RT reaction; 4, 5, and 6, patient 163 p24 primer, β-actin primer, and no RT reaction; 7, 8, and 9, patient 512 p24 primer, β-actin primer, and no RT reaction; 10, 11, and 12, patient 515 p24 primer, β-actin primer, and no RT reaction; 13, 14, and 15, patient 506 p24 primer, β-actin primer, and no RT reaction; 16, 17, and 18, patient 525 p24 primer, β-actin primer, and no RT reaction; 19, BDV-infected MDCK cell p24 primer; 20, uninfected MDCK cell p24 primer; 21, no RNA p24 primer; M, ϕX174 DNA digested with *Hae*III as a size marker.

Kishi et al. (18) used the RT-PCR method with human PBMCs to detect BDV-p24-related RNA using specific primers. With the identical primers for amplification of BDV-p24 used, our results demonstrated nonspecific amplification products at a low annealing temperature. The nonspecific amplification products were reduced by the high annealing temperature. These results suggested that some sequences homogenous to the p24 primer existed in the RNA population of PBMCs in humans. We could identify and clone the BDV-p24-related sequence from the RNA of psychiatric disorder patients. Nucleotide sequence analyzed from the isolate (512) was similar to that of other isolates (p2-9) from Japanese patients, as previously reported (19). Nucleotide sequences of the variation cluster region (nucleotides 253 to 271) were identical between the isolates 512 and p2-9. Thus, the BDV-p24-related RNA might exist in some PBMC RNA. Whether these sequences originated from a human strain related to BDV or from endogenous sequences of human genes is unclear.

Whether there is an association of BDV infection with psychiatric disorders has not been resolved yet, and there is no agreement among investigators on this issue. While several seroepidemiological studies suggested the positive association of BDV infection with psychiatric disorders, especially with schizophrenia and mood disorders (6, 9, 42), Sierra-Honigmann et al. (38) reported the failure of BDV-related RNA detection from brain autopsies of schizophrenic subjects. The present study suggested that there is no evidence of BDV infection in Japanese psychiatric patients. Our present results are supported by the epidemiological evidence from psychiatric patients with schizophrenia and mood disorders found in Japan and other countries. There were no significant differences in the estimated rates of schizophrenia and mood disorders in Japan and other areas of the world, while there was some variation in the estimated rates (26, 27, 41). However, the prevalence of BDV-specific antibodies and BDV-related RNA among patients with psychiatric disorders has varied in several reports (3, 9, 42). The highest seropositive rate among psychiatric patients was reported as 36 to 38% (4), while the lowest seropositive rate was reported as 3.7% (3) by the IF method. Furthermore, a marked discrepancy between RT-PCR and immunoassay has been reported (5, 18). The discrepancy between the worldwide prevalence of psychiatric disorders and BDV infection suggests that BDV might not be a common etiological factor in human psychiatric disorders. Further studies are needed to clarify the pathogenic significance of BDV infections in humans with psychiatric and neurological disorders, and a reliable and specific detection method for anti-BDV antibodies needs to be developed.

The subjects in this study were from Kagoshima Prefecture, southern Japan, where HTLV-1 and the human retrovirus that causes leukemia as well as a neurological disorder, HAM/TSP, are known to be prevalent (40). Table 1 demonstrated seroprevalence of HTLV-1 among the subjects, and patients showed a seroprevalence rate similar to that for the controls. Since the prevalence of psychiatric disorders in Kagoshima Prefecture was similar to those of other areas of Japan where HTLV-1 is not endemic (27), these results supported the idea that HTLV-1 infection appeared to have no correlation with psychiatric disorders (8).

Furthermore, cytomegalovirus (CMV) infection was com-

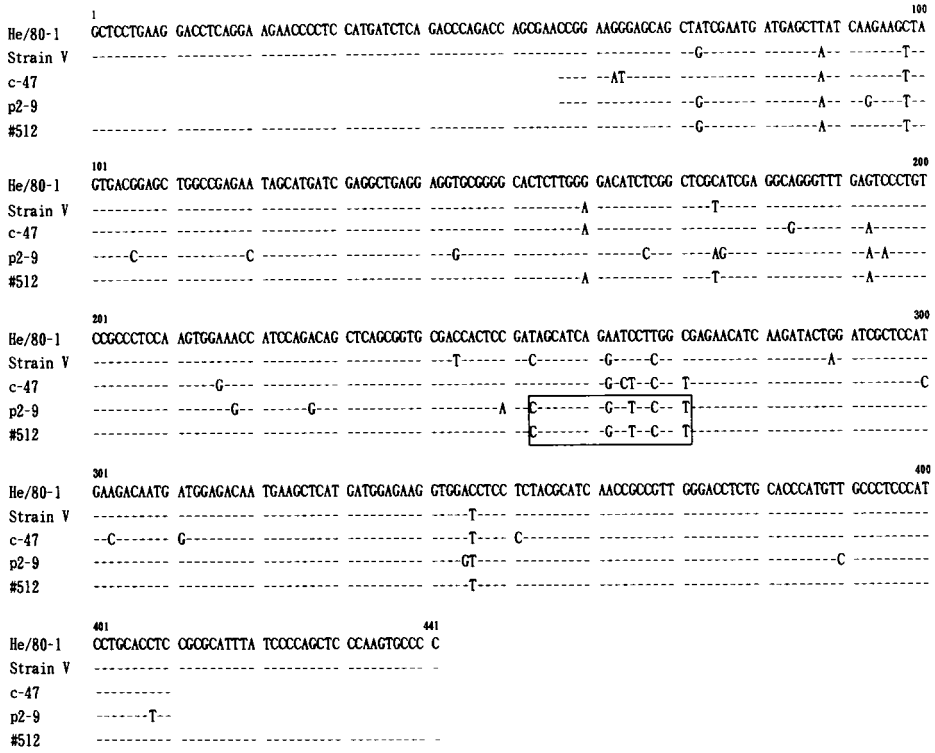

FIG. 5. Nucleotide sequence of BDV cDNA clone isolated from the PBMCs of a psychiatric patient. cDNA fragments (479 bases long) of the BDV p24 region, amplified from total RNA extracted from the PBMCs of the psychiatric patient by RT-PCR, were sequenced. The sequences presented are in antigenomic polarity (positive sense). Isolate 512 was derived from patient 512. Sequences for the horse-derived He/80-1, strain V, C-47, and p2-9 had been previously reported (19, 30, 36). Nucleotide sequences 1 to 56 and 411 to 441 of p2-9 and C-47 had not been reported. Bars show nucleotide sequences identical to those of He/80-1. The box indicates the variation cluster region.

monly found in Japanese populations, as well as other Asian populations (12), in contrast to a low prevalence in Caucasian populations (37). The seropositive rate of CMV infection was as high as 93.5 to 96.1% among the subjects in this study. The titers of both antibodies in patients were as high as those of the controls (data not shown). Although it has been reported that psychotropic drugs may have antiviral activities (20), titers of anti-CMV antibodies as well as anti-HTLV-1 antibodies among the subjects in this study were not affected by treatment with neuroleptics. This suggested that the results of the present study may not be affected by administration of chronic psychotropic medication.

In conclusion, there was a lack of association of BDV and HTLV-1 infections with psychiatric disorders in the present study, and further studies are needed to elucidate the possible etiology of psychiatric disorders in humans.

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