Demonstration of human Borna disease virus RNA in human peripheral blood mononuclear cells

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Abstract BDV naturally infects horses and sheep, and causes sporadic neurological disease. Serological evidence suggests an association of BDV, or a related virus, with specific psychiatric diseases in humans. Here, by using a nested RT-PCR technique, we demonstrate that human BDV RNA is present in the PBMC of psychiatric patients. In an examination of a total of 60 patients from 5 wards of a hospital in Japan, the detection rate differed within each ward, ranging from 8% to >50% (37% on the average). Of particular note was the finding that the human derived BDV sequences, which included deleted forms in about 23% of the positive samples, were slightly different from those derived from horse BDV. These results suggest urgent consideration of the measures to be taken to cope with the effects of blood transfusion. In addition, the detection of a high level of BDV in the PBMC of patients will help our understanding of the pathogenesis in the disease.

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

1. Introduction

BDV is a neurotropic, yet unclassified, nonsegmented, negative-sense, single-stranded RNA virus which naturally infects horses, sheep, cats, cattle, and ostriches [1]. Serological data suggest an association of BDV, or a related agent, with specific psychiatric diseases in humans [2,3], although healthy people also contain a low prevalence of anti-BDV antibodies [4,5]. Experimental BDV infection of animals such as rats induces behavioural changes that resemble some types of affective neuropsychiatric disorders in humans [1]. However, whether BDV is a natural pathogen of humans remains to be determined as infectious BDV, or related virus, has yet to be isolated from human tissue or body fluids.

Borna disease has been most extensively characterized in experimentally infected rats, in which the agent is highly neurovirulent invading the brain from peripheral sites by axonal transport [6]. By an RT-PCR technique, the BDV-specific p40 gene was identified in the brain and many peripheral organs of

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

intranasally infected rats although it was not found in blood [7]. In contrast, the presence of the gene was also demonstrated by a similar technique in PBMC of neonatally, intracranially infected rats [8]. In naturally BDV-infected horses however, the presence of BDV-specific RNA has been demonstrated by a similar technique in brain tissue [9], and in conjunctival fluid, nasal secretions and saliva [10]. In addition, many seropositive samples from horses were negative by RT-PCR [10], indicating either that BDV is shed irregularly and in intervals or that the BDV genome is sufficiently variable in a number of seropositive animals to evade detection by this technique.

Here, we examined the possible presence of BDV-specific RNA in PBMC from humans with psychiatric disorders. For this, we developed a more sensitive technique, nested RT-PCR, for the amplification of a fragment of p24, a region which is relatively well conserved within BDV genomes [11].

2. Materials and methods

2.1. Patients
We studied PBMC from 35 males aged 34–69 from three wards and 25 females aged 37–73 from two further wards in a local psychiatric hospital in Hokkaido (Table 1). PBMC was isolated from EDTA-treated blood by centrifugation on Ficoll–Paque (density 1.077).

2.2. BDV and cells
MDCK cells uninfected or persistently infected with BDV (MDCK/BDV; kindly provided by Dr. R. Rott, Justus-Liebig-Universität Giessen, Giessen, Germany) [12] were used.

2.3. Preparation of cellular RNA
Total RNA fractions were prepared by using an RNA extraction kit (RNAzol B; Cinna/Biotecx Laboratories International, Inc., Friendswood, TX, USA) as described [13].

2.4. Detection of BDV RNA
For the detection of BDV-specific RNA genome, nested RT-PCR was used. One microgram of total cellular RNA was used in a reverse transcription reaction, followed by an amplification reaction of cDNA using the EZ rTth RNA PCR kit (Perkin-Elmer Corporation, Branchburg, NJ, USA) with a primer pair 5'-TGACCCAACCAGTAGAC-CA-3' (Bo-p24.D2) at nucleotides 1,387 to 1,405 and 5'-GTCCCAT-TCATCCGTTGTC-3' (Bo-p24.A1) at nucleotides 1,865 to 1,847 of the BDV genome. The reverse transcription step was performed in a total volume of 50 microliters in a thermal cycler (Perkin-Elmer Corporation) at 60°C for 30 min, followed by 93°C for 2 min. The reverse transcription products were subsequently subjected to the amplification for 35 cycles of denaturation at 94°C for 1 min and annealing and polymerization at 60°C for 1.5 min, followed by a final polymerization step for 10 min at 60°C. Five microliters of the RT-PCR product were used in a second PCR with a nested set of primers, 5'-TCAGACCC-AGACCAGCGAA-3' (Bo-p24.D3) at nucleotides 1,443 to 1,461 and

5'-AGCTGGGGATAAATGCGCG-3' (Bo-p24.A2) at nucleotides 1,834 to 1,816. The 2nd PCR consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and polymerization at 72°C for 1.5 min, followed by a final polymerization step for 7 min at 72°C. The PCR products were separated by 1.5% agarose gel electrophoresis, blotted onto a nylon membrane, then hybridized with ^{32}P-labeled synthetic oligonucleotides, sense stranded nucleotides (1,462 to 1,484 [Bo-seq.D1], 1,485 to 1,507 [Bo-seq.D2] and 1,637 to 1,658 [Bo-seq.D3] and an antisense stranded nucleotide (1,791 to 1,811 [Bo-seq.A1]). In addition, the PCR products were cloned in a pCRII vector using a TA cloning kit (Invitrogen Corporation, San Diego, CA, USA) and several representative clones were sequenced according to the protocol described for the CircumVent Thermal Cycle Dideoxy Sequencing Kit (New England Biolabs Ltd., Beverly, MA, USA) using ^{32}P-labeled primers, Bo-p24.D3 and Bo-p24.A2. The PCR products were also directly sequenced by the method of Engelke et al. [14] using ^{32}P-labeled primers, Bo-Seq.D1 and Bo-Seq.A1. All the numbers for BDV nucleotide sequences described here correspond to the previously reported BDV numbering scheme [15].

2.5. Detection of anti-BDV antibodies

For detection of anti-BDV antibodies in plasma from the same blood samples as those used for the PBMC preparations, full-length p24 protein prepared as a fusion protein with GST in E. coli [16] was used as an antigen for Western blot. GST was used as a negative control protein. Both GST-p24 and GST proteins were used after purification by glutathione Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) column chromatography.

3. Results

3.1. Detection of BDV RNA in PBMC

When the nested RT-PCR technique was applied to the detection of BDV-related RNA in the PBMC from 60 psychiatric inpatients, clear positive signals were detected in 36.7% (22/60) of the samples by both ethidium bromide staining and Southern blotting (Table 1). Most of the PCR products showed a discrete band corresponding to the size (374 bp) of the fragment from persistent BDV in MDCK cells. However, patient numbers 9, 27 and 57 showed a band with slightly increased mobility, while patient number 26 showed a band with much faster mobility. Patient number 42 showed two bands, one with normal and one with faster mobility. The results of the staining and Southern blots of patient numbers 25 to 36 in Table 1 are shown in Fig. 1 as representative of the total analysis obtained. Sequence results from the random sampling of the PCR products showed some differences from the reported sequences (He/80-1, Strain V and WT-1) of horse-derived BDV [11,15] (Fig. 2). In addition, one (pHBDV-9-27) of two DNA clones derived from the products with normal size (patient numbers 8 and 9 in Table 1) contained a one base deletion which results in the appearance of stop codon just after the deletion site. Direct sequencing of two forms (HBDV-26 and -27) of RT-PCR products with smaller sizes revealed misalignment mutations which generate large deletions of 230 bases from nucleotides 1,489 to 1,718 and 115 bases from nucleotides 1,503 to 1,617, respectively. For this deletion mechanism, repeat sequences AATGATG and AAG, respectively, seem to play a role, as has been similarly reported in retroviruses [17]. Thus, most of the BDV in PBMC from certain patients was homogeneous and contained only the same mutant form.

3.2. Detection of anti-BDV antibodies in plasma

The plasma from the same blood samples used for the BDV genome-positive PBMC preparations were also examined for the presence of anti-BDV antibodies (Table 1). Western blot analysis was done using purified GST-BDV p24 fusion protein as the test antigen. The results showed a 30% seroprevalence (18/60) when 1:50 dilutions of plasma were used (Table 1). Only

Fig. 1. Nested RT-PCR for the detection of BDV RNA in PBMC from psychiatric inpatients. Representative results of 12 samples (the same samples shown as patient numbers 25 to 36 in Table 1) analyzed by nested RT-PCR are shown here. The products of PCR amplification of the BDV p24 region were subjected to agarose gel electrophoresis, then stained with ethidium bromide (A). The results of the Southern blot hybridization using four oligomers as a probe are also shown (B). As positive and negative controls, the RNA fractions from MDCK/BDV (+) and MDCK (−) were similarly subjected to nested RT-PCR. DW means distilled water in place of the RNA. MW indicates the size markers (Φx DNA/HaeIII fragments).

9 of the 60 patients (15%) showed positive signals by both RT-PCR and Western blot analyses (Table 1).

4. Discussion

Our study has demonstrated the presence of BDV genomic

sequences in PBMC fractions from psychiatric inpatients by a nested RT-PCR technique (Fig. 1). A recent report [18] that some paraformaldehyde-fixed monocytes in the PBMC from psychiatric inpatients were positive for BDV antigens by flow cytometry would be in agreement with our results. More recently, the same group also demonstrated the presence of BDV-

Table 1
Profiles of 60 patients in 5 wards and their summarized results for BDV

Ward	Patient	Sex	Age	BDV genome in PBMC	Size of PCR product	Serum antibody[a]
A	1	M	52	+	normal	+
A	2	M	61	+	normal	−
A	3	M	52	−		−
A	4	M	65	−		−
A	5	M	63	−		−
A	6	M	38	+	normal	+
A	7	M	47	−		+
A	8	M	63	+	normal	+
A	9	M	61	+	small deletion	+
A	10	M	43	−		−
A	11	M	66	−		−
B	12	M	56	−		−
B	13	M	49	−		−
B	14	M	62	−		−
B	15	M	69	−		−
B	16	M	42	−		+
B	17	M	42	−		+
B	18	M	41	+	normal	+
B	19	M	51	−		−
B	20	M	34	−		−
B	21	M	52	−		−
B	22	M	53	−		−
B	23	M	60	−		+
C	24	M	44	−		−
C	25	M	56	−		−
C	26	M	58	+	large deletion	−
C	27	M	64	+	small deletion	−
C	28	M	58	+	normal	+
C	29	M	62	−		+
C	30	M	43	−		−
C	31	M	62	−		−
C	32	M	62	+	normal	+
C	33	M	56	+	normal	−
C	34	M	53	−		+
C	35	M	65	+	normal	+
D	36	F	70	−		−
D	37	F	67	−		−
D	38	F	37	−		+
D	39	F	60	−		+
D	40	F	73	+	normal	−
D	41	F	54	−		−
D	42	F	42	+	normal & small deletion	−
D	43	F	40	−		−
D	44	F	52	−		−
D	45	F	63	−		−
D	46	F	62	+	normal	−
D	47	F	70	−		−
E	48	F	53	−		−
E	49	F	62	+	normal	−
E	50	F	62	+	normal	+
E	51	F	54	−		−
E	52	F	69	−		−
E	53	F	63	+	normal	−
E	54	F	64	−		−
E	55	F	55	+	normal	−
E	56	F	47	+	normal	−
E	57	F	52	+	small deletion	−
E	58	F	42	+	normal	−
E	59	F	61	−		+
E	60	F	62	−		−

[a]Plasma fraction (1:50 dilution in PBS) was applied to Western blotting using purified GST-BDV p24 fusion protein.

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A        1671          1681          1691          1701          1711          1721
         |             |             |             |             |             |
He/80-1  CTT GGC GAG AAC ATC AAG ATA CTG GAT CGC TCC ATG AAG ACA ATG ATG GAG ACA ATG AAG
Strain V --C --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- --- ---
WT-1     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
pHBDV-8-22 --C --T --- --- -C- --A G-- --- --- --- C-- --- --- --- --- --- T-- --- G-- G-- ---
pHBDV-9-27 --C --T --- --- G-- --- --- T-- --- --- -x- -TG A-- --- --- -C- --- --- --- ---

         1731          1741          1751          1761          1771          1781
         |             |             |             |             |             |
He/80-1  CTC ATG ATG GAG AAG GTG GAC CTC CTC TAC GCA TCA ACC GCC GTT GGG ACC
Strain V --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- ---
WT-1     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
pHBDV-8-22 --- --- --- -G- --- --C --T --- --- --- -G- --- -A- --- --- --- ---
pHBDV-9-27 --- --- --- --- --- --- -TT --- --- --- --- --- -A- --- --A --- ---


B        1464                        1489                          1718          1730
         |                           |                             |             |
He/80-1  CCG AAG GGA CAG CTA TCG AAT GAT GAG----- // -----ACA ATG ATG GAG ACA ATG AAG
HBDV-26  CCG AAG GGA CAG CTG TCG AAT GAT GAG---- (Δ 230 bases) ---ACA ATG ATG GAG ACA ATG AAG

         1482                        1503                          1617          1634
         |                           |                             |             |
He/80-1  AAT GAT GAG CTT ATC AAG AAG CTA----- // -----CAA GTG GAA ACC ATC CAG ACA
HBDV-27  AAT GAT GAG CTA ATC AAG AAG CTA--- (Δ 115 bases) ---CAA GTG GAA ACC ATC CAG ACA
```
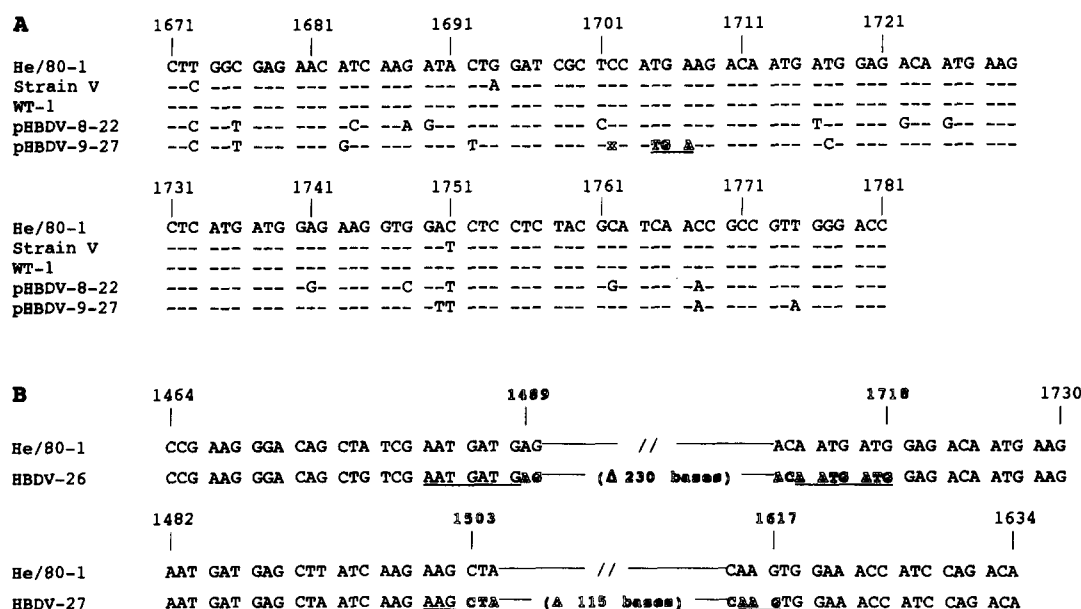
Fig. 2. Comparison of p24 sequence derived from human PBMC BDV and previously reported horse-derived BDV. The sequence results of p24 at nucleotides 1,671 to 1,781 in two cDNA clones (pHBDV-8-22 and pHBDV-9-27) corresponding to patients 8 and 9 in Table 1 are shown in section A. The sequences (He/80-1, Strain V and WT-1) of horse-derived BDV which were reported previously [11,15] are also shown in section A. x, a deleted nucleotide. TGA, a stop codon appeared by one-base deletion at nucleotide 1,702. The PCR products with increased mobility in Fig. 1 (HBDV-26 and -27 derived from patients 26 and 27, respectively, in Table 1) were sequenced directly and the results are shown in section B. As a control, horse-derived sequence of He/80-1 [15] is shown. Δ, deletion. Direct repeats observed at deletion junctions in HBDV-26 and -27 are underlined and the sequences and region deleted are shown in outline.

specific RNA in humans by similar technique as us [19]. Essentially, their result seems to be almost similar with our result. However, sequence analysis of the PCR products in this report revealed mutant forms of the BDV genome in PBMC from a significant proportion of the patients examined, irrespective of the presence or absence of anti-BDV antibodies (Table 1). In addition, the sequences obtained were slightly different from those of horse-derived BDV genome (Fig. 2). Thus, further studies are necessary in order to confirm the pathogenicity of BDV and to clarify the relationship between the human BDV-related agent and horse BDV.

The hypothesis that there is an association of BDV with psychiatric disorders in humans is due to the high prevalence of anti-BDV antibodies in those patients [2–5]. However, before the pathogenesis of BDV in human psychiatric disorders can be fully understood, screening for the BDV genome in healthy donors and horses or sheep is clearly urgent for a thorough understanding of the epidemiology of BDV. The PBMC samples examined here were derived from inpatients of Hokkaido, the northernmost island of Japan. Many imported horses and sheep are bred and maintained in Hokkaido and one possibility is that BDV has been transmitted from horses to humans. In fact, our examination using RT-PCR as used here has demonstrated that BDV is endemic in healthy horses in Hokkaido (about 30% prevalence in 57) [20]. In addition, BDV RNA was also demonstrated in PBMC from healthy peoples (about 6.5% prevalence in 77; M. Kishi, unpublished). In conclusion, our results strongly support the hypothesis that the BDV-related agent or agents we have identified is a hematopoietic cell-related virus that presumably develops disease in the brain only after a long-term persistent incubation period. To confirm these findings, more widespread screening for BDV is urgently required. In particular, measures to cope with the possibility of virus transmission by blood transfusion would seem to be especially important.

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