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High prevalence of Borna disease virus in domestic cats with neurological disorders in Japan

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Abstract

A total of 15 (T-1–T-15) domestic cats with neurological disorders in Tokyo area were examined for association with Borna disease virus (BDV). None had detectable antibodies to feline immunodeficiency virus (FIV), feline leukemia virus, feline infectious peritonitis virus and *Toxoplasma gondii*, and only cat T-8 had detectable antibody to FIV. Serological and molecular epidemiological studies revealed a significantly high prevalence of BDV infection in these cats: antibodies against BDV p24 and/or p40 proteins in 10/15 (66.7%) and p24 and/or p40 RNA in peripheral blood mononuclear cells in 8/15 (53.3%). Further, in situ hybridization and immunohistochemistry analyses of the autopsied brain samples derived from one of the cats (T-15) revealed BDV RNA predominantly in neuronal cells in restricted regions, such as olfactory bulb and medulla of cerebrum. Thus, BDV is present in Japanese domestic cats with neurological disorders at a high prevalence.

Keywords: Borna disease virus; Cat; Neurologic disorder; Staggering disease

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1. Introduction

Borna disease virus (BDV) is a neurotropic, nonsegmented, single-, negative-stranded RNA virus (Briese et al., 1994; Cubitt and de la Torre, 1994; Cubitt et al., 1994; de la Torre, 1994; Schneemann et al., 1995) which causes encephalitis in horses and sheep, mainly in endemic areas in Central Europe (Zwick, 1939). Natural infection with BDV, in both the same and other areas, also occurs in healthy and diseased cattle (Bode et al., 1994; Caplazi et al., 1994; Hagiwara et al., 1996), ostriches with neurological signs (Malkinson et al., 1993), and cats with (Lundgren and Ludwig, 1993; Lundgren et al., 1993, 1995) or without neurological signs (Nakamura et al., 1996). In addition, it was reported that BDV is correlated with human psychiatric disorders because such patients showed significantly higher prevalence of BDV infection as determined by screening for anti-BDV antibodies (Rott et al., 1985; Bode et al., 1988) and BDV RNA in peripheral blood mononuclear cells (PBMCs) than healthy controls (Bode et al., 1995; Kishi et al., 1995a,b; Sauder et al., 1996).

Epidemiological studies of Swedish cats revealed a significantly higher BDV association with cats showing unknown neurological disorders characterized by behavioral and motor disturbances, referred to as ‘staggering disease’ (Kronevi et al., 1974; Lundgren, 1992), than in cats without neurological symptoms (Lundgren and Ludwig, 1993; Lundgren et al., 1993). This disease was first reported in Sweden as having no specific correlation with several known microbial agents (Lundgren, 1992). Histopathological and immunohistochemical analyses of the diseased cats showed that the inflammatory reaction of the central nervous system was most pronounced in the gray matter of the brain stem, basal ganglia and hippocampus (Lundgren, 1992). High seroprevalence of BDV (44%) was identified in cats with ‘staggering disease’ in Sweden (Lundgren and Ludwig, 1993), while 13% seroprevalence was reported in randomly selected cats in Germany (Lundgren et al., 1993). Similar results were also reported in cats with ‘staggering disease’ from Austria (Weissenböck et al., 1994). In addition, BDV antigen and nucleic acid were demonstrated in the feline brain tissues from cats with ‘staggering disease’ (Lundgren et al., 1995), although other attempts to detect BDV RNA or antigens in the brains from diseased cats failed (Nowotny and Weissenböck, 1995). Feline BDV in the spinal cord from a diseased cat grew in embryonic mink brain cells during three passages, but thereafter at the next passage of the cells the virus disappeared (Lundgren et al., 1995). However, it was shown that injection of BDV derived from diseased cats into healthy animals induced neurological symptoms and non-suppurative encephalitis (Lundgren et al., 1997).

Recently, we tested normal domestic cats in Japan for detectable specific antibody to BDV and for BDV RNA in PBMCs and found that 13.3% of the cats were positive for specific anti-BDV antibodies in plasma and 8.3% for BDV RNA in PBMCs (Nakamura et al., 1996). There have been no reports of the occurrence of ‘staggering disease’ in cats in Japan. In this study, we performed serological and molecular epidemiological studies to determine the prevalence of BDV in domestic cats with neurological disorders in Tokyo, Japan. Further, examination of the brain from one of the diseased cats confirmed BDV expression and severe lesions in restricted regions of the brain.

Table 1
Clinical symptoms observed in domestic cats examined for BDV infection

Cat	Breed	Sex	Age (years)	Major symptoms
T-1	Persian	Female	0.8	Head tilt, Ataxia
T-2	Persian	Male	2	Epileptic seizure
T-3	Mixed	Male	14	Epileptic seizure
T-4	Mixed	Male	1	Tonic convulsion, Circling
T-5	JDC[a]	Female	13	Posterior paralysis
T-6	JDC	Male	1	Depression
T-7	JDC	Female	3	Epileptic seizure
T-8	JDC	Male	7.5	Tremor
T-9	JDC	Female	9	Posterior paralysis, Head tilt
T-10	Russian blue	Female	2	Circling, Epileptic seizure
T-11	JDC	Female	1.6	Agitation
T-12	JDC	Female	5	Epileptic seizure
T-13	JDC	Female	1	Circling, Epileptic seizure
T-14	JDC	Female	17	Head tilt, Circling
T-15	Persian	Female	13	Ataxia, Unconsciousness

[a] Japanese domestic cat.

2. Materials and methods

2.1. Cats with neurological symptoms

A total of 15 domestic cats with various neurological symptoms (Table 1) were studied. Cats T-1–T-14 and a cat T-15 were referred to the Veterinary Hospital of the University of Tokyo and Machida Animal Hospital, respectively. These cats were 0.8–17 years old (average age, 6.06 years old). The cats showed neurological signs including epileptic seizure, circling, head tilt, ataxia and tremor. Infection of these cats with feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline infectious peritonitis virus (FIPV) and *Toxoplasma gondii* was examined as follows. Serum samples were tested for anti-FIV p24 antibody and FeLV p26 antigen using a commercial test kit (Sanp^{TM}; IDEXX, Portland, ME, USA). Antibodies to FIPV and *Toxoplasma* were tested with enzyme-linked immunosorbent assay and latex agglutination test, respectively (MARUPI LIFETECH, Osaka, Japan). Because known infectious diseases, hydrocephalus and brain tumors were not found in these cats with neurological signs, the cause of disease for them had not been identified.

2.2. Preparation of PBMCs from cats

About 5 ml of EDTA-treated blood were obtained from each cat. After removing plasma by centrifugation, the PBMCs were isolated by centrifugation on Ficoll-Paque (density, 1.077 g/ml).

2.3. Detection of anti-BDV antibodies

Anti-BDV antibodies in plasma obtained from EDTA-treated blood were examined by Western blotting using the recombinant full-length p24 and p40 fusion proteins with glutathione *S*-transferase (GST) expressed in *Escherichia coli* as BDV antigens (Bahmani et al., 1996). GST alone was used as a negative control. All these proteins were used after purification by glutathione Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) column chromatography. The purified proteins were separated by SDS-PAGE with a 15% polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). The membrane strips were reacted with a 50-fold dilution of plasma pre-absorbed with GST protein (10 μg/ml) at 37°C for 1 h, then immunostained using an HRP-1000 kit (Konica, Tokyo, Japan). As positive and negative control antibodies, rabbit antiserum to BDV p40 or p24 and rabbit antiserum to GST or preimmune rabbit serum, respectively, were used. The molecular weight values of the proteins on Western blots were calculated by comparing their mobilities with those of marker proteins in a calibration kit (Bio-Rad Laboratories).

2.4. Detection of BDV RNA in PBMCs

To detect BDV-specific RNA in PBMCs, total RNA was extracted from the PBMCs by an RNA isolation kit (ISOGEN, Nippon Gene Co., Tokyo, Japan), as described previously (Nakamura et al., 1996). The RNA samples (2 μg) were reverse-transcribed before amplification with 200 units of SuperScript II, RNase H-negative reverse transcriptase (RT) (GIBCO BRL) and random hexamers (100 pmol/ml) at 42°C for 1 h, then treated at 92°C for 5 min to stop the reaction. The resulting cDNA products were amplified by nested polymerase chain reaction (PCR) using two sets of primer pairs corresponding to sequences in the p24 region; i.e. for the 1st PCR, nucleotides 1387–1405 and 1865–1847, and for the 2nd PCR, nucleotides 1443–1461 and 1834–1816 (Kishi et al., 1995b). The cDNA products were also subjected to nested PCR using primer pairs corresponding to the sequences to the p40 region; i.e. for the 1st PCR, nucleotides 242–261 and 989–969, and for the 2nd PCR, nucleotides 277–296 and 717–698 (Bode et al., 1995). Nested RT-PCR of the p40 region in cat brain samples was performed using different primer sets; i.e. for the 1st PCR, nucleotides 242–261 and 511–492 (Sierra-Honigmann et al., 1993); and for the 2nd PCR, nucleotides 259–278 (Sierra-Honigmann et al., 1993) and 483–464 (Sauder et al., 1996). The predicted lengths of these nested amplified products were 392 bp (base pairs) using the p24 primers, 441 bp using the former p40 primers, and 225 bp using the latter p40 primers. All RNA samples were also subjected directly to nested PCR without the RT step to confirm RT-dependent amplification of the products. As a positive BDV RNA, we used the RNA extracted from MDCK cells persistently infected with horse brain-derived BDV (MDCK/BDV) (Herzog and Rott, 1980). As negative controls, the RNA extracted from uninfected MDCK or distilled water (absence of nucleic acid template) were used.

These final nested PCR products were separated by 1.5% agarose gel electrophoresis, blotted onto nylon membranes (Amersham), then Southern-hybridized using ^{32}P-labeled probe corresponding to internal sequences of BDV; i.e. nucleotides 1462–1484, 1485–

1507, 1637–1658 and 1811–1791 of the p24 region (Kishi et al., 1995b), and nucleotides 407–427 and 611–590 for 441 bp and nucleotides 407–427 for 225 bp PCR products of the p40 region (Bode et al., 1995).

Nucleotide numbers corresponded to those previously reported for the He/80 strain of BDV (Cubitt et al., 1994).

2.5. Histopathological study of cat brain sections

Thin sections (4 μm) of cat brains in paraffin were stained with hematoxylin-eosin (HE) for histological evaluation by light microscopy.

2.6. In situ hybridization of cat brain sections for BDV RNA

The thin sections (4 μm) of cat brain samples were deparaffinized, then processed for in situ hybridization. Digoxigenin (DIG)-labeled sense and antisense riboprobes corresponding to full-length BDV p40 (1113 nucleotides) were generated by in vitro transcription using T7 polymerase (Boehringer Mannheim). The RNA probe was partially hydrolyzed by incubation in buffer containing 40 mM $NaHCO_3$ and 60 mM Na_2CO_3 for 30 min at 60°C, then neutralized by 300 mM CH_3COONa containing 1% CH_3COOH. The hybridization mixture consisted of 50% formamide, 3 × SSC, 50 mM HEPES (pH 7.0), 2 × Denhardt's solution, and 2.5 mg/ml salmon sperm DNA. Hybridization was performed for 16 h at 50°C. After hybridization, the sections were rinsed with 2 × SSC and washed with 0.2 × SSC at 57°C for 15 min. Sections were then reacted with alkaline phosphatase-labeled anti-DIG polyclonal antibody (Boehringer Mannheim) and finally visualized using nitroblue tetrazolium and X-phosphate (Boehringer Mannheim). After in situ hybridization, individual slides were counterstained with methyl green.

2.7. Preparation of rabbit polyclonal antibodies to BDV p40

The GST-p40 protein purified as described above was cleaved by PreScission™ protease (Pharmacia Biotech AB). The p40 protein again purified by glutathione Sepharose 4B to remove the free GST protein, was used as an immunogen. Rabbits (4 weeks old, female) purchased from SLC were intramuscularly immunized with the p40 protein with Freund's complete adjuvant. The polyclonal anti-p40 antibody fraction was obtained from the resultant rabbit anti-p40 sera by affinity chromatography using Sepharose 4B coupled with purified p40 protein.

2.8. Immunohistochemistry of cat brain sections for BDV antigen expression

The thin sections (4 μm) of cat brain samples were deparaffinized, then processed for detection of BDV antigen expression using polyclonal rabbit anti-BDV p40 purified as described above, followed by the labeled streptavidin-biotin (LSAB) immunohistochemistry method. After deparaffinization, the specimens in 10 mM citric acid at pH 6.0 were heated in a microwave for 10 min. After washing with distilled water, the specimens were further treated with 0.3% H_2O_2 in methanol for 10 min to suppress endogenous

peroxidase activity, followed by incubation with 10% normal goat serum for 30 min. The first antibody was reacted at 4°C overnight. The sections treated with the first antibody were reacted with biotinylated goat anti-rabbit IgG. The LSAB reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride, then counterstained with hematoxylin.

3. Results

3.1. Detection of anti-BDV antibodies in domestic cats with neurological symptoms

A total of 15 domestic cats with neurological symptoms, admitted to two animal hospitals, were examined for BDV infection. All except one (T-8 positive for FIV antibody) of the cats were negative for serum antibodies against FIV, FIPV and *T. gondii* and FeLV antigen (not shown). About 5 ml of EDTA-treated blood was obtained from each cat. After removing plasma, the PBMCs were prepared by centrifugation through Ficoll-Paque.

Anti-BDV antibodies in the plasma were examined by Western blotting using recombinant GST-p24 and GST-p40. GST alone was used as a negative control. Several cat sera specifically reacted with GST-p24 and/or GST-p40, similarly as with control rabbit anti-p24 or -p40 antiserum (Figs. 1a and b). The results showed BDV seroprevalences of 46.7% (7/15) for anti-p24 antibodies and of 53.3% (8/15) for anti-p40 antibodies. None of these samples reacted with GST alone (Fig. 1c). The results are summarized in Table 2. Consequently, 66.7% (10/15) of the cats examined were seropositive for antibodies against p24 and/or p40. Among the seropositive cats, 50% (5/10) were positive for antibodies against both p24 and p40.

Table 2
Summarized results of antibodies against BDV and BDV RNA in PBMCs from diseased cats

Cat	BDV antibodies		BDV RNA in PBMCs	
	p24	p40	p24	p40
T-1	+	−	+	−
T-2	−	−	−	−
T-3	−	+	−	+
T-4	−	−	−	−
T-5	−	−	−	−
T-6	+	+	+	+
T-7	+	+	−	+
T-8	+	+	−	+
T-9	−	−	+	−
T-10	−	+	−	−
T-11	−	−	−	−
T-12	+	+	−	−
T-13	+	+	−	−
T-14	+	−	+	−
T-15	−	+	−	+

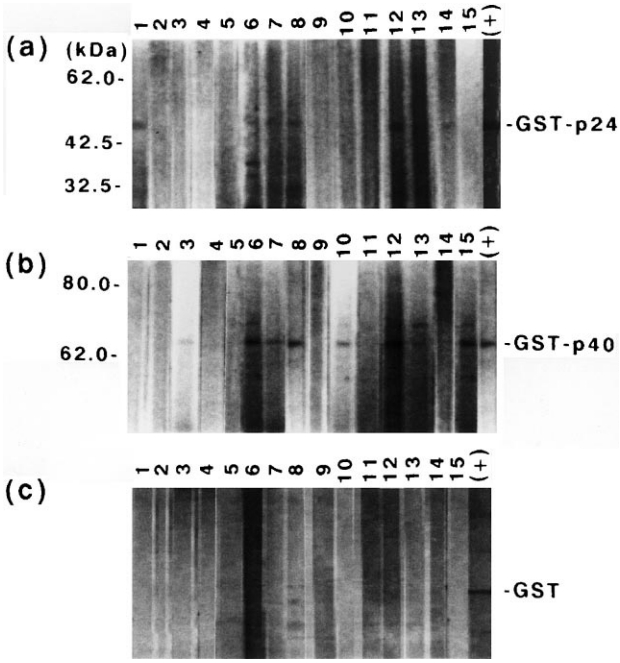

Fig. 1. Western blotting for BDV antibodies in plasma samples from 15 cats with neurological symptoms. The purified GST-BDV p24 (51 kDa) (a), GST-BDV p40 (67 kDa) (b), and GST alone as a negative control (c) were similarly resolved by SDS-PAGE, followed by blotting onto PVDF membranes. A 50-fold dilution of each plasma sample derived from diseased cats (T-1–T-15, lanes 1–15, respectively) was reacted with the blotted membrane. As positive control antibodies (+), polyclonal antibodies to BDV p24, p40 and GST were used. The molecular weights of the proteins were calculated by comparing their mobilities with those of marker proteins (MW) in a calibration kit.

3.2. *Detection of BDV RNA in PBMCs from domestic cats with unknown neurological symptoms*

To detect BDV-specific RNA in PBMCs, total RNA was extracted from the PBMCs. The RNA samples (2 μg) were reverse-transcribed, then the resulting cDNA products were amplified by nested PCR using two sets of primer pairs for p24 as well as p40 regions. The specificity of the final products of the nested PCR were confirmed by Southern blot hybridization using ^{32}P-labeled synthetic oligonucleotide probes. All PCR products showed discrete bands for the p24 fragment of 392 bp and p40 fragment of 441 bp, corresponding to the sizes of the fragments from MDCK/BDV cells. BDV RNA in PBMCs was demonstrated in 26.7% (4/15) using the probe for the p24 region and 33.3% (5/15) using that for the p40 region (Fig. 2a). No positive signals were detected when the nested PCR was performed directly without the RT step (not shown), indicating that these BDV signals were derived from viral RNA. The RNA from uninfected MDCK cells or distilled water, in place of RNA, did not produce bands around the sizes corresponding to the p24 or the p40 products (Fig. 2a). The results are summarized in Table 2.

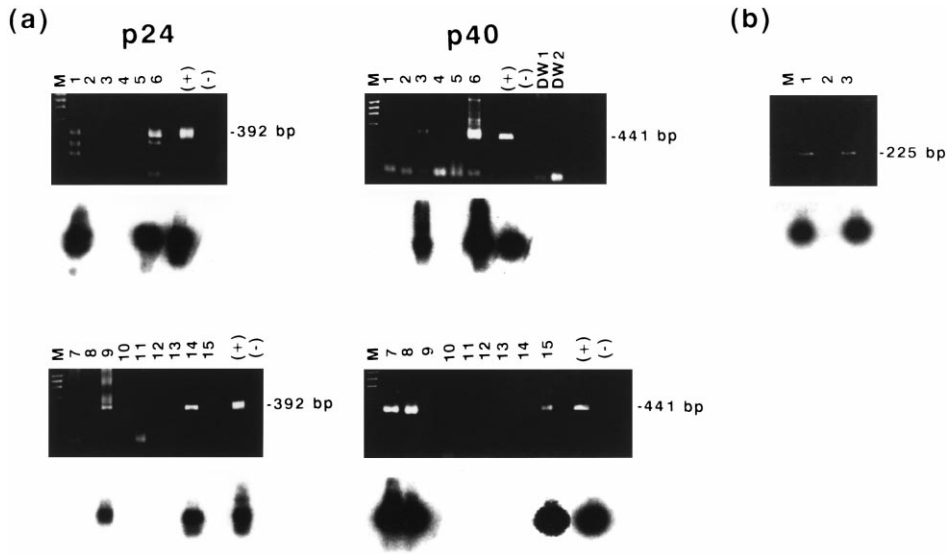

Fig. 2. PCR detection of BDV RNA in PBMCs from cats with Borna-like disease using primers corresponding to p24 and p40 regions. The PBMCs from the diseased cats (T-1–T-15, lanes 1–15, respectively) were subjected to PCR for BDV RNA with primers corresponding to p24 (392 bp) and p40 (441 bp) (a). In addition, RNA samples from several brain regions (olfactory bulb, cerebral cortex, and medulla of cerebrum in lanes 1–3, respectively) were also subjected to nested RT-PCR to amplify BDV RNA using primers corresponding to the region of p40 (225 bp) (b). In addition to the RNA extracted from the PBMCs and brain regions, that from uninfected MDCK cells (−) and from persistently BDV-infected MDCK cells (+) as negative and positive controls, respectively, were reverse-transcribed, then the resulting cDNA was subjected to nested PCR. Further, distilled water (DW1 and DW2) in place of RNA was reverse-transcribed, then subjected to the first round PCR and to nested PCR, respectively. The final PCR products were separated by agarose gel electrophoresis. The results of ethidium bromide-staining (upper panel) and Southern blotting (lower panel) are shown. M, size markers (øx174 DNA/*Hae*III fragments).

All these BDV p24 and/or p40 RNA-positive cats, with the exception of T-9 which was positive for BDV p24 RNA but negative for anti-p24 antibody, were also seropositive for p24 and/or p40, respectively. T-6 was the only animal positive for p24 and p40 RNA and antibodies among the total of 15 cats examined here.

3.3. Demonstration of BDV expression in brain regions from T-15 cat

To confirm the presence of BDV in the brain of Japanese cats with neurological disease, we analyzed BDV RNA in several regions of the brain from one cat (T-15), which was the only case available for autopsy.

T-15 was positive for anti-p40 antibodies and p40 RNA in PBMCs, but negative for anti-p24 antibodies and p24 RNA in PBMCs (Table 2). Therefore, we next examined the possible amplification of BDV in the brain samples with primer pair corresponding to the p40 region to amplify a 225 bp product. The results revealed the presence

Table 3
Summarized results of BDV infection in brain regions from T-15 cat

Brain region	Lesion[a]	RT-PCR	ISH[b]		IHC[c]
			Sense	Antisense	
Olfactory bulb	−	+	++	+	+
Cerebrum Cortex	−	−	−	−	−
Cerebrum Medulla	+[d]	+	++	+	+
Hippocampus	−	−	−	−	−
Pons	++[e]	−	−	−	−
Lateral ventricle	−	−	−	−	−
Medulla oblongata	++	ND[f]	+	+	−
Amygdaloid body	−	−	ND	ND	−
Thalamus	−	−	ND	ND	−
Cerebellum	−	−	+	+	−
Spinal cord	++	−	−	−	−

[a] The presence of lesion was determined by histological examinations of brain regions by HE staining.

[b] In situ hybridization with BDV p40-specific sense or antisense riboprobe.

[c] Immunohistochemistry with polyclonal rabbit anti-BDV p40 antibodies.

[d] +Mild or weak.

[e] ++ Severe or strong.

[f] Not determined.

of BDV RNA only in the olfactory bulb and the medulla of the cerebrum, but not in the cerebral cortex, hippocampus, pons, lateral ventricle (medial wall), amygdaloid body, thalamus, cerebellum or spinal cord (Table 3). The results in representative regions (olfactory bulb, cerebral cortex, and medulla of the cerebrum) are shown in Fig. 2b.

Surprisingly, HE staining of brain sections from T-15 revealed severe ventrolateral axonal degeneration and loss of myelin at all levels of the spinal cord (Fig. 3f). In the medulla oblongata (Fig. 3e) and pons (not shown), both of which were negative for BDV RNA by the nested RT-PCR (Table 3), mild axonal degeneration and loss of myelin were observed. In addition, neuronophagia and a slight mononuclear cell infiltration in addition to mild axonal degeneration and loss of myelin were found in some areas in the medulla of cerebrum (Fig. 3c), but no apparent abnormalities were seen in the olfactory bulb (Fig. 3a), both of which were the only regions positive for BDV RNA by the nested RT-PCR (Fig. 2b). The other brain regions examined were all negative for such lesions (Table 3).

Since no correlation between BDV infection and lesions in the T-15 cat brain regions examined, except for the medulla of the cerebrum, was observed, we next performed detailed examination of BDV expression on sections prepared from brain materials. In situ hybridization with BDV p40-specific riboprobes revealed strong signals in the olfactory bulb and the medulla of the cerebrum. More positive cells were identified in these regions by the sense than the antisense riboprobe. Faint signals were also identified in the medulla oblongata and cerebellum, similarly in more number of the

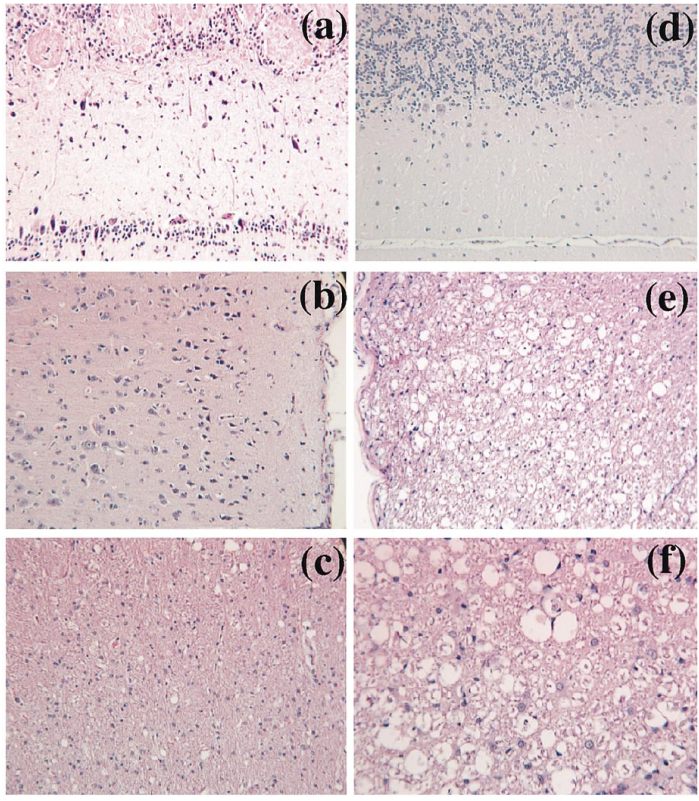

Fig. 3. Histopathology of the brain from T-15. The olfactory bulb (a), cerebral cortex (b), medulla of cerebrum (c), cerebellum (d), medulla oblongata (e) and spinal cord (f) were examined. HE staining revealed axonal degeneration and loss of myelin in c, e and f.

positive cells by the sense than the antisense riboprobe. However, the signal intensity of the positive cells was not markedly different between the antisense and sense riboprobes. The positive cells seemed to be predominantly neurons due to their morphology. In contrast, the other regions, including the spinal cord and pons, were negative by in situ hybridization (Table 3). The representative in situ hybridization profiles in the olfactory bulb, medulla of cerebrum, medulla oblongata and spinal cord are shown in Figs. 4a–d, respectively.

Next, we examined the expression of BDV p40 antigen by immunohistochemistry using polyclonal rabbit anti-p40 antibodies purified by affinity chromatography as described in Section 2. Clear positive signals were identified only in the olfactory bulb and cerebral medulla, but not in the other regions including the spinal cord, cerebellum, medulla oblongata and pons (Table 3). Again, the positive cells in the olfactory bulb and the medulla of the cerebrum seemed to be predominantly neurons due to their morphology. The representative profiles in the olfactory bulb, cerebral medulla, medulla oblongata and spinal cord are shown in Figs. 5a–d, respectively.

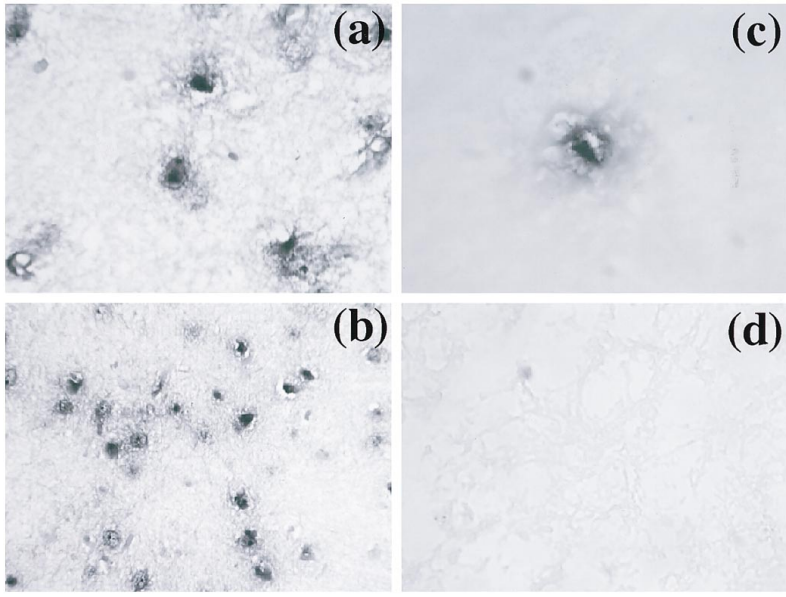

Fig. 4. In situ hybridization of BDV RNA in the brain sections from T-15. Brain sections from T-15 were hybridized with a DIG-labeled BDV p40-specific sense riboprobe, then counterstained with methyl-green: olfactory bulb (a), medulla of cerebrum (b), medulla oblongata (c) and spinal cord (d).

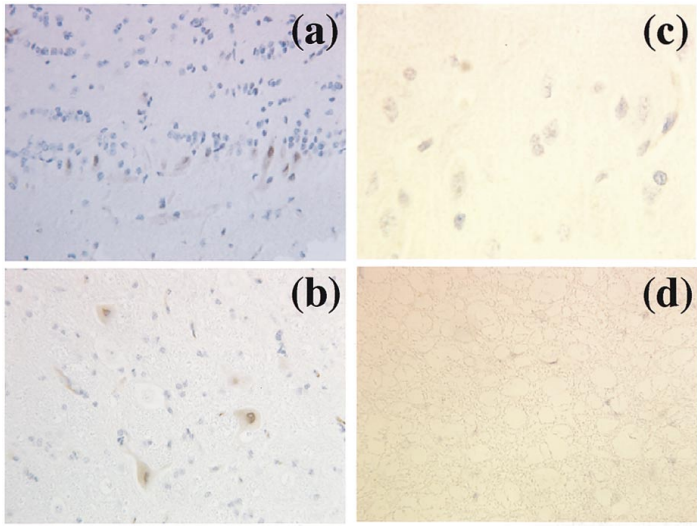

Fig. 5. Immunohistochemistry to detect BDV protein expression in the sections from several regions of T-15. Brain sections were subjected to immunohistochemistry using rabbit polyclonal anti-BDV p40 antibody: olfactory bulb (a), medulla of cerebrum (b), medulla oblongata (c) and spinal cord (d).

4. Discussion

Cats with 'staggering disease' show various neurological symptoms, such as ataxia, depression, and also other symptoms, such as fever, reduced appetite and dehydration (Lundgren, 1992). Similar encephalitis in cats has also been reported not only in Sweden (Lundgren, 1992) and Austria (Weissenböck et al., 1994), but also from the other parts of the world, including Australia (Borland and McDonald, 1965) and the United States (Vandevelde and Braund, 1979). However, there have been no reports on cats with 'staggering disease' in Japan.

In this study, we examined BDV prevalence in domestic cats with various neurological signs in Japan (Table 1); 11/15 (73.3%) animals were shown to be positive for BDV by screening for anti-p24 and-p40 antibodies (Fig. 1), as well as BDV RNA using primers corresponding to p24 and p40 regions in PBMCs (Fig. 2), as summarized in Table 2. The same results were reproducibly obtained by triplicate assays in both Western blotting and nested RT-PCR. This prevalence was significantly higher than 8.4% seroprevalence and 13.3% prevalence of BDV RNA in PBMCs reported in randomly selected 83 domestic cats with no apparent neurological signs in Japan, although this figure was obtained by screening only for anti-p24 antibodies and p24 RNA in PBMCs (Nakamura et al., 1996). Similarly, a BDV serosurvey in 111 cats in the UK recently revealed that the incidence of antibody to BDV in cats with neurological disease was higher than in cats with other types of disease (Reeves et al., 1998)

The 'staggering disease' in Swedish cats showed no specific correlation with FIPV, FeLV, FIV, pseudorabies virus, tick-borne encephalitis virus, canine distemper virus, *Borrelia burgdorferi* or *T. gondii* by serological and immunohistochemical screening (Lundgren, 1992). Similarly, most of the cats examined in the present study, with the exception of T-8, were also negative for FeLV, FIV, FIPV and *T. gondii*.

Many cats examined in this study were positive for both BDV RNA and anti-BDV antibodies: all of p40 RNA-positive cats were positive for anti-p40 antibodies and three of four p24 RNA-positive cats were positive for anti-p24 antibodies (Table 2). This was in marked contrast to our previous results in healthy or neurological disorder-unrelated cats in Japan, all of which were positive only for RNA or antibodies (Nakamura et al., 1996). The reason for this difference is not known at present. However, one possible explanation is the difference in the BDV load in the blood. More active replication of BDV may allow us to easily detect viral signals. Nevertheless, only one (T-6) of the 15 cats examined here was positive for both p24 and p40 antibodies and RNA (Table 2). Thus, the viral load in the blood even in diseased cats seemed to be still lower than in horse or sheep as suggested previously (Lundgren et al., 1995; Nowotny and Weissenböck, 1995).

Examination of BDV RNA and antigen in several regions of the brain from one cat (T-15) by nested RT-PCR (Fig. 2b), in situ hybridization (Fig. 4) and immunohistochemistry (Fig. 5) showed strong positive signals in the olfactory bulb and the medulla of cerebrum, and faint signals in the medulla oblongata and cerebellum, with no apparent signals in other regions (Table 3). In contrast, HE staining (Fig. 3) revealed axonal degeneration and loss of myelin in the medulla oblongata, pons and spinal cord, while only mild lesions were seen in the medulla of the cerebrum with no apparent abnormalities in the other

regions including the olfactory bulb (Table 3). In this animal, we could not detect any lesions in the hippocampus (Table 3), although this site is believed to be one of the major regions for inflammatory reactions in cats with 'staggering disease' (Lundgren, 1992). Thus, the regions positive for BDV antigens or RNA were partially different from those positive for histopathological abnormalities.

Two groups examined for BDV antigens and RNA in the brains from cats with 'staggering disease'. One group demonstrated BDV RNA in the brain samples from diseased cats by RT-PCR, although BDV antigen was not convincingly demonstrated by immunohistochemistry in the brain from diseased cats (Lundgren et al., 1995, 1997). In contrast, all attempts by the other group to demonstrate BDV RNA and antigen in the cat brains failed (Nowotny and Weissenböck, 1995). Nevertheless, the latter group observed that rabbits intracerebrally inoculated with brain homogenates from affected cats developed BDV-specific antibodies. Thus, the results by two groups were different. Recently, a case of feline Borna disease with muscle fasciculation and proprioceptive defects in the absence of encephalitis was shown to be positive for BDV in numerous neurons by in situ hybridization (Berg and Berg, 1998). Another report showed that five of the six brains found to be infected with BDV were from cats with signs of neurological disease (Reeves et al., 1998). Histologically, four of the five showed encephalitis, while the fifth had a normal brain. The sixth BDV-positive cats had no evidence of neurological disease and its brain was histologically normal. These results together with our observations from the brain of T-15 suggest that BDV persists only in restricted brain regions in cats and the levels of such BDV seem to be extremely low compared with those of BDV in the brains from horse and sheep, as described above. Alternatively, the levels of BDV in the brains may become low by the time that neurological signs are clinically manifested. This may be the major reason for the partial discrepancy in the data concerning the feline brain regions positive for BDV and showing lesions. Similar examinations with more diseased cats are needed to clarify this possibility.

Following experimental inoculation of adult rats, intracerebrally or intranasally, with horse brain-derived BDV, infectious BDV and antigens were detected in the brain, retina, cerebrospinal fluid, peripheral nerves and adrenal gland (Narayan et al., 1983a,b; Carbone et al., 1987, 1989; Deschl et al., 1990). Especially, although the BDV antigens can be detected in the eye in all retinal layers during the early stages of the infection, the virus disappears at later stages from the retina and the animals become blind, with persistent, productive infection maintained in the other tissues (Narayan et al., 1983a). Generally, the pathogenesis in experimentally BDV-infected adult rats is closely related to the host immune responses (Rott et al., 1988; Stitz et al., 1993, 1995; Planz et al., 1995) as well as cytokine production (Shankar et al., 1992; Morimoto et al., 1996) and inducible nitric oxide (Koprowski et al., 1993; Zheng et al., 1993; Akaike et al., 1995) in the brain. In addition, intracerebral clearance of several viruses by immune T cells as well as cytokines such as interferons and tumour necrosis factor-α during viral encephalitis and meningitis have been demonstrated in patients and animal models (Oldstone et al., 1986; Frei et al., 1988; Sussman et al., 1989; Lebon et al., 1989; Schijns et al., 1991). Thus, it is possible that the major regions with lesions such as the medulla oblongata, pons and spinal cord in T-15 might have been triggered by BDV infection in the earlier

phase of the disease and the regions which were positive for BDV RNA and antigen such as the olfactory bulb and the medulla of the cerebrum in this study could have been the major sites of BDV replication at the time of the examination. Another possibility for the lesions in the medulla oblongata, pons and spinal cord is the presence of an unknown agent.

The reason we could detect BDV RNA in these brain regions by in situ hybridization more strongly with the sense than the antisense BDV-specific riboprobe is also unknown, since the viral RNA was more strongly detectable in MDCK/BDV cells with the same antisense than the sense riboprobe (not shown). However, the results by in situ hybridization and immunohistochemistry indicate the presence of minus-stranded genomic RNA and a major virion structural protein p40, but no or very low levels of plus-stranded viral mRNAs, suggesting that the BDV replication rate in the brain might be extremely low. Further, it is also suggested that this abnormal viral life cycle may reflect a latent state of BDV in nondividing neuronal cells in the brain.

In conclusion, we showed a significantly higher prevalence of BDV in cats with undefined neurological symptoms in Japan, compared with that in our previous paper in which healthy or neurological disease-unrelated Japanese cats were examined for BDV (Nakamura et al., 1996). The BDV signals were identified in restricted regions of the brain from one of the diseased cats. The significance of BDV infection in cats, close human companions, should be determined to clarify the possible risk to humans.

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