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SHORT PAPER

A Variant Form of Feline Borna Disease

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Summary

Borna disease virus (BDV) is a neurotropic agent with capacity to infect and cause encephalomyelitis in a wide range of animals, including horses, sheep, cattle and cats. Recent interest in BDV as a potential human pathogen has been stimulated by reports of BDV-specific antibodies and nucleic acid in patients with neuropsychiatric diseases. The pathogenesis of Borna disease (BD) in naturally infected animals is believed to be immune-mediated, mainly through the action of cytotoxic T cells. In this paper, a case of feline BD with atypical clinical and histopathological features is reported. Clinically, the cat showed muscle fasciculation and proprioceptive defects. Despite absence of encephalitis, numerous neurons were infected with BDV as shown by in-situ hybridization. This indicates that BDV infection may lead to various disease patterns, depending on differences in viral pathogenicity, or on as yet unidentified host-specific factors.

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Introduction

Borna disease virus (BDV) is a neurotropic, negative-strand RNA virus with worldwide distribution, but affecting animals in northern Europe in particular (Briese *et al.*, 1994: Cubitt *et al.*, 1994). The host spectrum of BDV is broad, including horses, sheep, cattle, cats, rabbits and ostriches (Malkinson *et al.*, 1993; Bode *et al.*, 1994; Lundgren *et al.*, 1995; Rott and Becht, 1995). Recent reports of BDV-specific antibodies, as well as BDV proteins and nucleic acid, in peripheral blood mononuclear cells (PBMCs) of human patients with affective disorders and schizophrenia suggest a link between BDV and certain neuropsychiatric diseases (Rott *et al.*, 1985; Bode 1995; Bode *et al.*, 1995; Sauder *et al.*, 1996). Whether BDV plays a contributory or a full aetiological role in such diseases remains to be elucidated.

In the majority of naturally infected species, BDV causes a severe neurological syndrome called Borna disease (BD), morphologically manifested as a non-suppurative encephalomyelitis with a predilection for the limbic system, basal ganglia and brain stem (Gosztonyi and Ludwig, 1995). Experimental studies in rats indicate that the pathogenesis of BD is immune-mediated, mainly through the action of $CD8^+$ cytotoxic T cells (Stitz *et al.*, 1995). The

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absence of a cytopathogenic effect of BDV in tissue culture would seem to support this notion. However, experimental infection of newborn rats with high passages of BDV may lead to acute BD, accompanied by severe cytopathogenic changes in neurons, with little or no sign of inflammation (Gosztonyi and Ludwig, 1995). Therefore, it cannot be ruled out that some strains of BDV are capable of inducing neurological disease *per se*.

Here we report a case of feline BD in which both the clinical and morphological manifestations differed from what has been previously described in naturally infected hosts. This may be of importance in understanding the pathogenesis of BDV infection in animals and man.

Materials and Methods

Histopathology

Tissue specimens, from a cat whose clinical history is described under Results, were fixed in buffered 10% formalin for light microscopy. After fixation, the brain was cut in coronal sections, which included the frontal, parietal and temporal lobes, basal ganglia, hippocampus, thalamus, mesencephalon and cerebellum. From the spinal cord, sections were taken from cervical, thoracic and lumbar segments. Samples were also taken from the kidney, spleen and sartorius muscles. Paraffin wax sections were cut 4 μm thick and stained with haematoxylin and eosin (HE).

Molecular Biology

Tissue samples were taken from the frontal cortex and basal ganglia, snap frozen in liquid nitrogen and stored at −70°C. Total RNA was extracted from the tissue samples with TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). One microgram of total RNA was reverse-transcribed to cDNA by Superscript II (Life Technologies), with oligo $d(T)_{12-18}$ (Pharmacia Biotech, Uppsala, Sweden) as a primer. The cDNA was amplified by a nested polymerase chain reaction (PCR), with two sets of primers specific for a fragment of the BDV p23 gene: for the first round of PCR, 5′-TGA CCC AAC CAG TAG ACC A-3′ at nucleotides 1387–1405 and 5′-GTC CCA TTC ATC CGT TGT C-3′ at nucleotides 1865–1847; for the second round of PCR, 5′-TCA GAC CCA GAC CAG CGA A-3′ at nucleotides 1443–1461 and 5′-AGC TGG GGA TAA ATG CGC G-3′ at nucleotides 1834–1816. Ten microlitres of each reaction mixture were analysed by electrophoresis in a 2% agarose gel in the presence of ethidium bromide 0·5 μg/ml. The nested PCR reaction from the basal ganglia was purified (Wizard PCR Preparations; Promega, Madison, WI, USA), cloned into the pGEM-T vector (Promega) and sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) with the T7 Sequencing kit (Pharmacia Biotech) and the primers SP6 and T7. The nucleic acid sequence was compared and aligned with previously reported BDV sequences from German and Swedish horses, Swedish and Austrian cats ("cat B" and "cat C", respectively) with BD and the two laboratory reference strains C6BDV (Cubitt *et al.*, 1994) and BDV strain V (Briese *et al.*, 1994); the MegAlign program of the DNA Star software package (DNA Star Inc.) was used. Phylogenetic analysis was performed with the Phylip software package (Felsenstein, 1993). A distance matrix was constructed and an "unrooted tree" was fitted, by means of the Kitsch program.

In-situ hybridization was performed on paraffin wax sections from the brain, with a digoxigenin-labelled anti-sense cRNA probe prepared by in-vitro transcription from the BDV p23 cDNA clone PAB4 (Lipkin *et al.*, 1990). After dewaxing, sections were "permeabilized" with Proteinase K (Boehringer Mannheim, Mannheim, Germany) at a concentration of 100 μg/ml at 37°C for 15 min. The sections were then washed in phosphate-buffered saline (PBS) and post-fixed for 10 min at room temperature

in PBS containing paraformaldehyde 2%. After washing in PBS, sections were prehybridized with a solution containing 50% formamide, 5 × saline sodium citrate (SSC), salmon sperm DNA 500 μg/ml, 5 × Denhardt's solution and yeast transfer RNA 250 μg/ml, at room temperature for 4 h. The probe was diluted with the same solution to a concentration of 1 μg/ml and heated at 85°C for 5 min. Hybridization was performed in a humid chamber at 65°C overnight. Thorough washing was carried out twice in 2 × SSC at 57°C (5 min each wash) and once in 0·2 × SSC at 57°C (10 min). Between the first two washes, the sections were treated with RNAse A (Boehringer Mannheim) at a concentration of 20 μg/ml at 37°C for 30 min. Detection of the digoxigenin-labelled probe was performed according to the Boehringer–Mannheim manual for non-radioactive in-situ hybridization, with an alkaline phosphatase-conjugated anti-digoxigenin antibody and the two colour substrates NBT and BCIP. Colour development was allowed to proceed overnight and the sections were mounted in glycerol–gelatin, without counterstaining.

Results

Clinical Observations

A 3·5-year-old female domestic shorthair cat ("cat A") with muscle fasciculation (a phenomenon in which individual muscle fasciculi contract spontaneously) was presented to a veterinary clinic for examination. The fasciculations, which had been observed for 3 days, had been preceded by apathy and loss of appetite of several days' duration. On examination, muscle fasciculations were evident throughout the whole body, giving the impression of a wave passing through the skin. The muscular tone of the front legs was increased. Body temperature and proprioceptive reflexes were normal. Treatment with an antibiotic (enrofloxacin) was prescribed.

During the following weeks, the condition of the cat deteriorated. The muscle fasciculations worsened and were accompanied by markedly decreased proprioception in the front legs. The cat seemed mentally alert, but was reluctant to move around. Three weeks after the onset of clinical signs, the animal was killed with an intraperitoneal overdose of pentobarbital. The carcass was frozen at −20°C for 2 days before being sent to the Department of Pathology, Swedish University of Agricultural Sciences, for necropsy.

Histopathology

No macroscopical abnormalities were observed. Histopathological examination revealed necrosis of fibres in the sartorius muscles. Some fibres were undergoing regenerative repair, as demonstrated by the presence of macrophages within the sarcolemma (Fig. 1). In the brain and spinal cord, no inflammatory lesions were found. Due to the artefactual effects of freezing, it was not possible to evaluate the neuronal and glial morphology. However, positive hybridization signals for BDV p23 were detected both in the nucleus and in the cytoplasm of numerous neurons (Fig. 2).

Molecular Biology

RT-PCR (reverse transcription followed by PCR), carried out on a sample from the basal ganglia, revealed distinct fragments of the expected sizes (478 bp

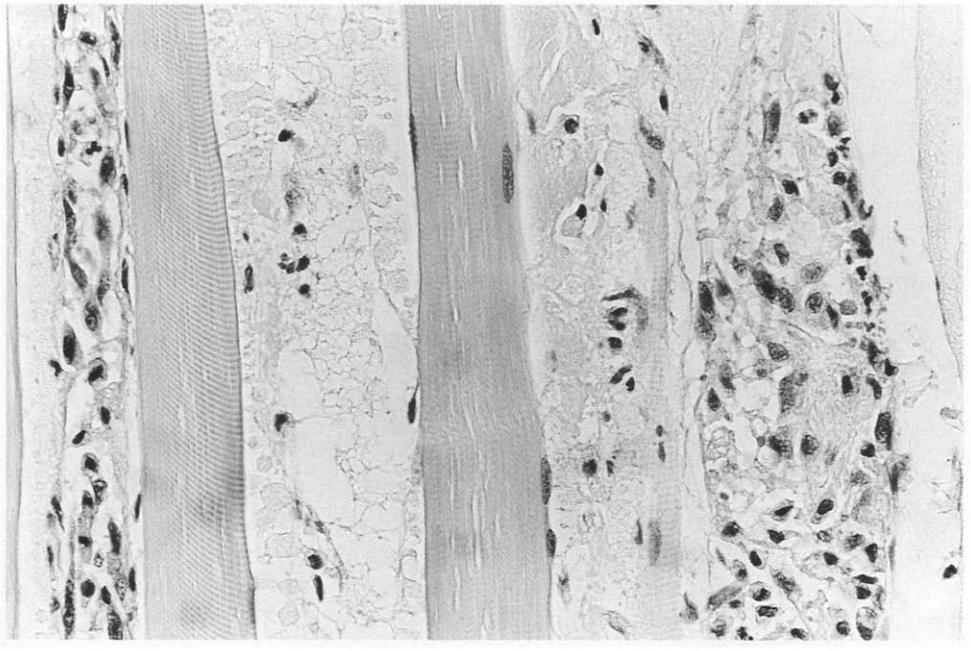

Fig. 1. Necrotic muscle fibres, side by side with normal fibres, in the sartorius muscle. Regenerative repair is demonstrated by the presence of macrophages within the sarcolemma of some fibres. HE. × 360.

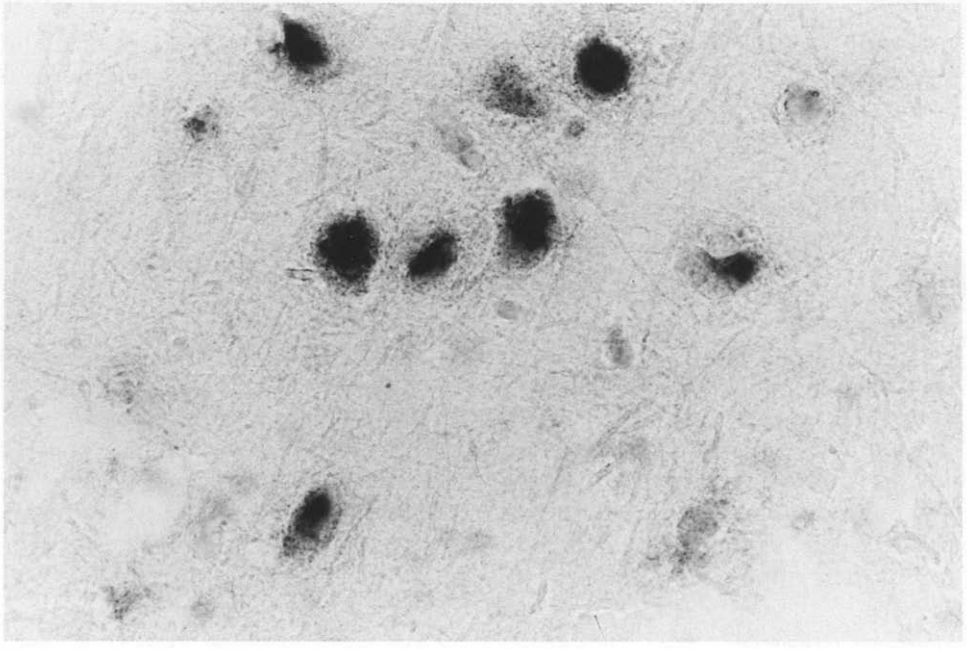

Fig. 2. Neurons in the cerebral cortex showing positive hybridization signals for BDV p23 (dark staining of nucleus and cytoplasm). In-situ hybridization with a digoxigenin-labelled cRNA probe (no counterstain). × 576.

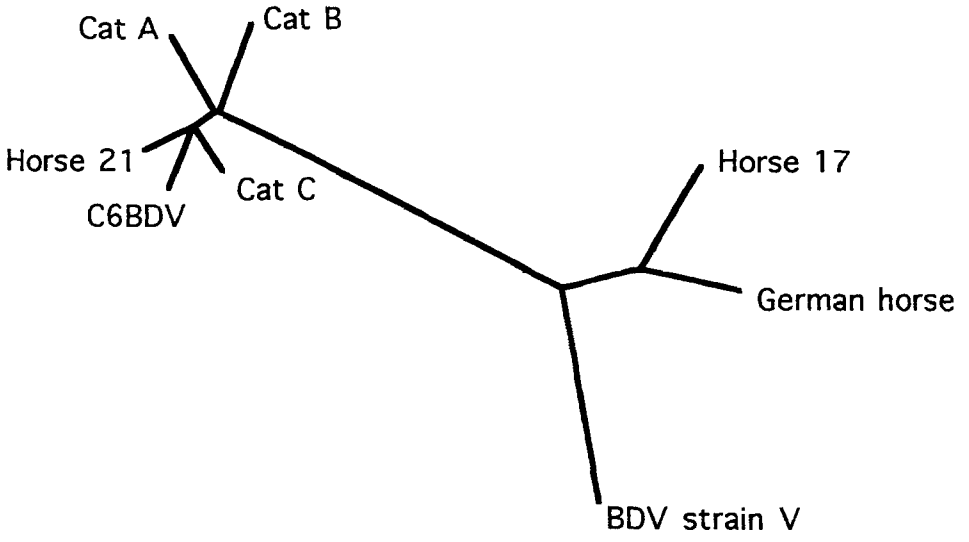

Fig. 3. Proposed phylogenetic tree for the p23 gene of BDV derived from cats and horses in Europe. The cat A isolate was obtained from the cat described in this paper. The cat B isolate was obtained from the PBMCs of a Swedish cat with BD and the cat C isolate from the brain of an Austrian cat with BD. For details of the Swedish horses 17 and 21, see Berg and Berg (1998b). The GenBank accession number for the German horse is U94868. The phylogenetic analysis was performed with the PHYLIP software package (Felsenstein, 1993).

and 389 bp) after both rounds of PCR. The sequence of the nested PCR product (Fig. 3; "cat A") was found to be closely related to other feline BDV sequences (cats B and C) as well as to a sequence obtained from the PBMCs of a Swedish horse ("horse 21") and to the BDV reference strain C6BDV. In contrast, a more distant relationship was found with BDV sequences from two other horses, one German and one Swedish ("horse 17"), and the BDV reference strain V (Fig. 3). However, the divergence at the nucleotide level between cat A and BDV strain V was only 3·6% and at the amino-acid level 2·3%. An alignment of the amino-acid sequences showed three substitutions in cat A as compared with C6BDV (Fig. 4). The first one was from asparagine [N] to serine [S] at position 40 (corresponding to position 71 in the full-length protein); the second was from glutamic acid [E] to glycine [G] at position 46 (position 73 in the full-length protein); and the third was from arginine [R] to histidine [H] at position 136 (position 103 in the full-length protein).

Discussion

It may be argued that BDV, although present in the central nervous system (CNS), was not the direct cause of disease in this case. However, neither the clinical picture nor the histopathology was consistent with any known feline CNS disorder, except possibly BD. In addition, the in-situ hybridization results showed that BDV nucleic acid was not only detectable in the CNS but also present in numerous neurons. This massive infection of neurons supports the

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BDV strain V . . . . . 118
German horse . . . . . 118
Horse 17 . . . . . 118
Cat A . . . . . S . G . . . . . 118
Cat B . . . . . 118
Cat C . . . . . 118
Horse 21 . . . . . 118
C6BDV S D P D Q R T G R E Q L S N D E L I K K L V T E L A E N S M I E A E E V R G T L 118

BDV strain V . . . . . 238
German horse . . . . . 238
Horse 17 . . . . . V . . . . . K . 238
Cat A . . . . . H . . . . . 238
Cat B . . . . . H . . . . . 238
Cat C . G . . . H . . . . . 238
Horse 21 . . . . . H . . . . . 238
C6BDV G D I S A R I E A G F E S L S A L Q V E T I Q T A Q R C D H S D S I R I L G E N 238

BDV strain V . . . . . 358
German horse . . . . . 358
Horse 17 . . . . . L . . . . 358
Cat A . . . . . 358
Cat B . . . . . V . . . 358
Cat C . . . . . 358
Horse 21 . . . . . 358
C6BDV I K I L D R S M K T M M E T M K L M M E K V D L L Y A S T A V G T S A P M L P S 358

BDV strain V . . . . . 391
German horse . . . . . 391
Horse 17 . . . . . 391
Cat A . . . . . 391
Cat B . . . . . 391
Cat C . . . . . 391
Horse 21 . L . . . . . 391
C6BDV H P A P P R I Y P Q L 391
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Fig. 4. Alignment of the amino-acid sequences for the p23 gene of BDV derived from cats and horses in Europe. BDV strain V and C6BDV are laboratory reference strains. The alignment was constructed with the MegAlign program of the DNA Star software package (DNA Star Inc.). Residues that are identical with the reference strain C6BDV are hidden. The numbers in the margin do not correspond to the positions of amino-acids in the full-length protein.

argument that BDV was the aetiological agent, or at least a factor of major importance in the pathogenesis of the disease.

The clinical picture differed from that previously described in cats and other animal species naturally infected with BDV. The most common clinical manifestations in cases of feline BD are ataxia of the hindlegs, proceeding to paresis and finally paralysis (Lundgren, 1992). In addition to these motor disturbances, many cats develop mental changes, such as increased affection or aggressive behaviour. No such clinical signs were observed in the present cat. Instead, the predominant clinical feature was muscle fasciculation. In man, the latter is commonly associated with lower motor-neuron disease, and to some extent with polyneuropathy and other lesions of the peripheral nerves (Walton, 1985). Lesions of lower motor neurons cause atrophy and loss of tone in muscles. In this case, the muscular tone was increased and no atrophy was observed. Therefore, it is more likely that the fasciculations were related to lesions of the peripheral nerves.

In addition to muscle fasciculation, the cat showed markedly decreased proprioception in the front legs. This has previously been noted in cases of

feline BD (Lundgren, 1995). Loss of proprioceptive positioning is a sensitive test for the assessment of CNS disease in small animals, and lesions of the sensory cortex will often cause severely abnormal reactions (Oliver and Mayhew, 1987). However, it is also possible that peripheral nerve disease or lesions of the spinal cord or higher ascending pathways cause the proprioceptive reaction to be delayed or absent (Braund, 1994).

Experimental studies in rats have shown that about 2–6 weeks after viral antigen first appears in the brain, BDV spreads centrifugally to the spinal cord, cranial and spinal roots, and nerves. Late in infection, BDV antigen appears in peripheral nerve fibres of several different tissues and organs (Gosztonyi and Ludwig, 1995). Therefore, it is entirely possible that BDV interferes with the function of peripheral nerves. Whether this occurred in the present cat remains unknown, since samples from the peripheral nerves were not available for molecular biological investigation.

The most interesting aspect of this case of feline BD was the presence of severe neurological signs despite lack of inflammatory brain lesions. Due to the effects of freezing on tissue morphology, it was not possible to determine whether cytopathogenic changes in neurons or glial cells were present. However, the finding of BDV nucleic acid in many neurons points to a direct, virus-induced dysfunction of the CNS. An atypical disease pattern such as this might be due to a variation of the viral genome, or to host-specific factors such as age, breed, sex and genetic makeup.

The phylogenetic analysis showed the BDV isolate from cat A to be closely related to other feline BDV sequences as well as to the reference strain C6BDV. This may seem to argue against the notion of a variant BDV with "new" pathogenic properties. However, it should be kept in mind that this analysis was confined to a small part of the viral genome. Since the molecular basis for the pathogenicity of BDV is presently unknown, the possibility that the isolate from cat A represents a variant BDV with unusual pathogenic properties remains open. In fact, two of the amino-acid substitutions in the p23 sequence of cat A (from asparagine to serine at position 71 and from glutamic acid to glycine at position 73) could be regarded as major changes with potential functional implications for the p23 protein.

A previous epidemiological study of feline BD showed that age and breed are of no significance as risk factors, but that males are at greater risk than females (Berg *et al.*, 1998a). In this case, the animal was a female. There were no indications of immunodeficiency, the cat having been completely healthy previously and the lymphoid organs showing no histopathological abnormalities. The most likely explanation for the lack of inflammation in the CNS is that the cat was infected with BDV either *in utero* or during the neonatal period, when the immune system was too immature to respond. Experiments with rats have shown that newborn animals infected with BDV fail to develop neurological disease and encephalitis, although the virus is replicating to high titres in the brain; the rats later develop subtle behavioural changes, such as deficiency in memory and learning (Dittrich *et al.*, 1989; Bautista *et al.*, 1994).

We speculate that the present cat was persistently infected with BDV after exposure to the agent at an early age, clinical signs appearing when viral

replication became sufficient to interfere with vital neuronal functions. Recently, we reported a similar absence of encephalitis despite neurological signs in a horse with BD (Berg *et al.*, 1998b). These observations indicate that BDV infection may lead to various disease patterns, depending on differences in viral pathogenicity or on as yet unidentified host-specific factors. Most important, they show that the virus itself is capable of causing severe neurological disturbances by mechanisms that remain to be determined.

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