Evidence of Parvovirus Replication in Cerebral Neurons of Cats

Angelika Url,1* Uwe Truyen,2 Barbara Rebel-Bauder,1 Herbert Weisseneck,1 and Peter Schmidt4

Institute of Pathology and Forensic Veterinary Medicine, Veterinary University of Vienna, A-1210 Vienna, Austria,1 and Zentralinstitut, Tiergesundheitsdienst Bayern e.V., D-85586 Poing, Germany2

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The correlation between parvovirus infections and lesions in the central nervous system other than cerebellar hypoplasia was studied in 100 cats. The animals were necropsied with a history of various diseases, one third showing typical clinical and pathomorphological signs of panleukopenia. In 18 cats polyclonal antisera against canine parvovirus consistently labeled neurons mainly in diencephalic regions, whereas the cerebellar cortex remained negative in all cases. In situ hybridization with digoxigenin-labeled minus-sense RNA probes, hybridizing with monomer-replicative form DNA or mRNA, revealed positive signals in nuclei of several neurons of the brain, again excluding the cerebellum. PCR applied to formalin-fixed and paraffin-embedded brain tissue and intestinal tissues of the diseased cats and subsequent DNA sequence analysis yielded canine parvovirus type 2 (CPV-2)-like sequences in the central nervous system. Two aspects of these findings are intriguing: (i) parvoviruses appear to be capable of replicating in neurons, cells that are considered to be terminally differentiated and (ii) CPV-like viruses of the old antigenic type CPV-2 appear to be able to infect cats.

Canine parvovirus (CPV) and feline panleukopenia virus (FPV) are considered to be host range variants among the feline parvovirus subgroup within the genus Parvovirus (12). Whereas FPV is known to have infected cats for many decades, CPV emerged suddenly in the mid-1970s and spread throughout the world in 1978. It was a new pathogen for the dog, and retrospective studies revealed the first signs of its appearance in 1976. The original virus from 1978, designated CPV type 2 (CPV-2) to separate it from a nonrelated parvovirus isolated in 1973 from skin tissue of a dog (2), was replaced throughout the world between 1979 and 1985 by two different but closely related antigenic variants: CPV type 2a (CPV-2a) and CPV-2b (10). Besides the antigenic differences between CPV-2 and the antigenic types CPV-2a and -2b, they show distinct biological properties. Whereas CPV-2 is only known to infect and replicate in dogs, CPV-2a and -2b can infect, replicate, and cause disease in cats (14). Experimental infections of cats with CPV-2 consistently failed to demonstrate virus replication (14, 15).

Parvovirus replication is restricted to the nucleus and is dependent on certain helper functions from the host cell. This is due to the single-stranded DNA genome of the virus that needs to be completed to a double-stranded intermediate to start transcription and translation of the viral genome and proteins, respectively. The DNA polymerase responsible for the synthesis of the complementary strand is a cellular polymerase that is only expressed in mammalian cells during the S phase of the cell cycle (1).

Replication of FPVs in dogs and cats is predominantly seen in some highly mitotically active tissues, such as the lymphoid tissue, including lymph nodes, spleen, and thymus, as well as bone marrow and the epithelium of the gastrointestinal tract. Infection of the central nervous system (CNS) has been observed in cats after FPV infection during the first days of life. The developing and then dividing Purkinje cells of the cerebellum are lytically infected, leading to cerebellar hypoplasia and the development of the cerebellar ataxia syndrome (3, 5, 11).

In our study we provide strong evidence for parvovirus infection of neurons other than cerebellar Purkinje cells in cats.

MATERIALS AND METHODS

One hundred cats were sent for necropsy after unsuccessful therapy of various clinical symptoms and were examined pathologically. In 39% of the necropsied cats, gross pathology and histopathology revealed lesions of the gut considered characteristic for panleukopenia. Thirty-three percent suffered from infectious diseases other than parvovirus infections, such as feline leukemia, feline infectious peritonitis, or bacterial infections, and 28% of the examined cats died from noninfectious diseases, like cardiopathy or metabolic diseases. Only three cats showed neurological symptoms.

A wide range of tissues, including brain, was fixed in 7% buffered formalin, embedded in paraffin wax, and routinely stained with hematoxylin and eosin (H&E).

Immunohistochemistry (IHC) with the avidin-biotin complex technique was applied to formalin-fixed and paraffin wax-embedded brain sections and in some cases to sections of the small intestine. Briefly, deparaffinized and rehydrated sections were incubated with 1.5% H2O2 in methanol for blocking of endogenous peroxidase activity. To reduce background staining, the sections were incubated with 10% normal goat serum for 1 h at room temperature in a humidified chamber. Subsequently, the sections were incubated with the primary antibody (polyclonal antibodies against nonadenatured CPV [kindly provided by Colin Parrish, Ithaca, N.Y.]; dilution, 1:2,000; monoclonal antibodies against CPV-1-2A1 [kindly provided by Ti-Ho Hannover (Custom Monoclonals International, Sacramento, Calif.)]; dilution, 1:700; monoclonal antibodies against feline herpes virus type 1 [FHV-1]; [FVR 4A1 R; kindly provided by Ludwig Haas, Hannover, Germany]; dilution, 1:500; and polyclonal antisera against feline leukemia virus [bovine anti-FeLV precipitating antibody; Antibodies Inc., Davis, Calif.]; dilution, 1:2,000), overnight at 4°C in a humidified chamber. After extensive washing with phosphate-buffered saline, the sections were incubated with a biotinylated secondary antibody for 30 min at room temperature in a
Humidified chamber. Consecutive steps for avidin-biotin complex binding and visualization of positive reaction products were performed according to the manufacturer’s instructions (Vectorstain ABC kit and peroxidase substrate kit DAB: Vector Laboratories, Burlingame, Calif.). Gut samples from a cat, histopathologically characteristic for panleukopenia, samples from a cat with malignant FeLV-induced lymphoma, and skin from a cat that was positive by histopathology, IHC, and molecular pathology positive for FHV-1 served as positive controls.

For electron microscopy, cubes of formalin-fixed brain of cat 1 were fixed in glutaraldehyde and osmium tetroxide and were embedded in agar 100 resin (Agar Scientific Ltd., Essex, United Kingdom). Ultrathin sections were stained with uranyl acetate and lead citrate according to standard techniques and were examined with a Zeiss EM 900 transmission electron microscope.

For RNA in situ hybridization, 2-μm sections of paraffin-embedded brain were placed on Superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). For all steps RNAse-free glassware and water treated with diethyl pyrocarbonate were used. The sections were deparaffinized with Neo-Clear (Merk, Vienna, Austria) and rehydrated with a declining ethanol series. Before proteolysis, the sections were denatured with 0.2 M HCl and were incubated in 2× SSC (standard saline citrate buffer) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Proteolytic digestion with proteinase K (Roche, Vienna, Austria) at a concentration of 5 μg/ml was performed for 15 min at 37°C. Digestion was stopped by postfixation in 4% paraformaldehyde and washing in 2× SSC. For prehybridization the sections were covered with a mixture of 2.5 ml of formalin, 1 ml of 20× SSC, 200 μl of Denhardt’s solution (Sigma, Vienna, Austria), 130 μl of tRNA (baker’s yeast tRNA type X; Sigma), and 1.3 ml of diethyl pyrocarbonate treated 50% dextran sulfate (Calbiochem, La Jolla, Calif.), 61 μl of tRNA, and 1 μl of the digoxigenin-labeled probe (final concentration of 100 ng/ml) per ml of prehybridization mixture. Posthybridization washes were performed in 2× SSC followed by treatment with 10 μl of RNase A (10 mg/ml; Sigma) in a solution containing 8.3 ml NaCl (3 M), 500 μl of Tris-HCl (1 M, pH 8.0), 100 μl of EDTA (0.5 M, pH 8.0), and 41.1 ml of water. After additional washing steps and incubation in 5 ml of 10% blocking reagent (Roche, Vienna, Austria) with 1 ml of Tween 20 and 44 ml of maleate-buffer (pH 7.5), the hybridized probes were visualized immunohistochemically by incubation with an antidigoxigenin, alkaline phosphatase-conjugated antibody (dilution, 1:100 in blocking reagent) and by subsequent staining with X-phosphate–nitroblue tetrazolium chloride according to the manufacturer’s recommendations (Roche). After the color reaction was stopped in 10 ml of Tris-HCl (1 M, pH 8.0), 2 ml of EDTA (0.5 M), and 1,000 ml of water, counterstaining was performed with hematoxylin; finally the sections were mounted with Aquatex (Merk, Vienna, Austria).

The probe was generated from a plasmid based on pCR2.1 (Invitrogen, Carlsbad, Calif.) containing an 800-bp insert of the FPV VP1 gene. A digoxigenin-labeled, minus-sense, single-stranded RNA probe was generated by in vitro transcription of the SceI-linearized plasmid with T7 polymerase. Since the viral genome of FPV and CPV represents a single-stranded DNA of negative polarity, the minus-sense probe hybridizes with both mRNA and positive-sense DNA. Both are synthesized only in infected cells, and a hybridization signal with the minus-sense probe therefore indicates DNA replication and/or transcription (15).

PCR was performed as described previously (13). In brief, formalin-fixed and paraffin wax-embedded tissue was deparaffinized by xylene and consecutive alcohol washings. Template DNA was extracted and purified by using standard procedures (17); parvovirus-specific sequences were amplified with Td polymerase. The primers used and their position in the genome are summarized in Table 1. The amplics were purified by using a HighPure PCR kit (Boehringer Mannheim, Mannheim, Germany) and were fivefold concentrated by vacuum centrifugation. The amplics were cloned into the plasmid pCR2.1, and custom cycle sequencing (Seqlab, Göttingen, Germany) was performed by using plasmid DNA purified with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany).

### RESULTS

In 45% of the cats’ brains, histopathology revealed morphological changes suspicious for neurodegeneration, mainly in the lateral geniculate nucleus (LGN). Bilaterally, the neuropil of this area was more or less vacuolated and the neurons underwent peculiar shrinkage. The shrinking process was either accompanied with pallor and fading of the perikarya or with pyknosis of the nuclei and darkening of the cell (Fig. 1A). In some cases, isocortex, hippocampus, and pons, as well as diencephalic nuclei other than LGN, were suspicious for similar lesions. Besides two cases with involvement of the vestibular nuclei, the cerebellum did not show any abnormalities. In two cats, demyelinated areas were found in the neocortex and the pons. IHC of the brain was performed for all 100 cats by using polyclonal antibodies against parvovirus (monoclonal antibodies were tested in one case and revealed identical signals). Eighteen of the 100 tested cats revealed positive immune signals in different areas of the brain, like diencephalon, especially the LGN, neocortex, hippocampus, gray matter nuclei of...
pons, cerebellar medulla, and medulla oblongata. In 15 cats, the brownish signals were localized either in the neuronal nuclei or in the entire neurons, including their processes (Fig. 1C). In three cats, immune signals were found only in glial cells and/or in endothelial cells in neocortex and in LGN. The cerebellar cortex remained negative in all cases, as did IHC with antibodies against FHV-1 and FeLV in the parvovirus-positive cats.

Electron microscopy of brain tissue of cat 1 revealed particles suspicious for parvovirus within the cytoplasm of neurons, but unfortunately, due to fixation artifacts, a definite characterization was impossible. In situ hybridization applied to the brains from 14 of the 18 immunohistochemically parvovirus-positive cats yielded dark blue signals in some nuclei of neurons of the LGN (Fig. 1B) and of neurons adjacent to the lateral ventricles in the allocortex and in the hippocampal region. Neither the intensity nor localization of ISH signals corresponded well with the IHC signals.

PCR revealed parvovirus-specific amplicons in five tissue samples of three cats. In general, only weak signals were obtained from brain material (Fig. 2) Amplicons were generally cloned into the plasmid pCR2.1 and were sequenced by using standard primers. The amplicon obtained from the gut material of cat 3 was sequenced directly by using the primer 19 (Table 1). Sequence analysis revealed CPV-2-like sequences in the brains of the cats examined; in one cat a FPV-like sequence could be amplified from the corresponding gut sample (Table 2). A paraffin-embedded, formalin-fixed tissue sample from a horse that was processed in parallel with the cat tissues always remained negative.

**DISCUSSION**

FPV infection in cats is known to cause cerebellar hypoplasia if infection occurs mid- to late gestation and perinatally due to destruction of the cerebellar Purkinje cells and is known to cause panleukopenia after postnatal infection. The characteristic tissue tropism of parvoviruses is confined to cells of high mitotic activity, such as the intestinal epithelium, lymphoid tissue, and bone marrow stem cells (11). Within the CNS, parvovirus infection and replication are restricted to the cerebellar Purkinje cells related with cerebellar hypoplasia. To our knowledge, parvovirus infections of the CNS other than Purkinje cell infections have never been reported, although in one report, demyelination of the spinal cord was associated with high FPV titers (4), and in another report, FPV was isolated from brain homogenates of cats (16). However, the infection of neural tissue or neurons other than Purkinje cells by parvoviruses has never been shown in situ (by in situ hybridization or IHC).

In our study, we demonstrated in independent approaches the presence of parvovirus proteins and nucleic acid in neurons of cats other than cerebellar Purkinje cells. In 18 cats, most of them parvovirus infected and diseased (Table 3), immunohistochemical analysis revealed marked staining of neuronal cells in various regions of the brain, predominantly in the LGN of the diencephalon, in cerebral cortex, and in hippocampus. As the number of cats with suspicious neuronal degenerations in H&E staining, mainly within the LGN (n = 45), differs widely

<table>
<thead>
<tr>
<th>Material</th>
<th>Nucleotide position</th>
<th>Virus type</th>
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<tbody>
<tr>
<td>CPV-d reference strain</td>
<td>3629 3647 3685 (aa305)</td>
<td>CPV-2</td>
</tr>
<tr>
<td>FPV reference strain</td>
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<td>CPV-2</td>
</tr>
<tr>
<td>Cat 3 brain</td>
<td>3629 3647 3685 (aa305)</td>
<td>CPV-2</td>
</tr>
<tr>
<td>Cat 2 brain</td>
<td>3629 3647 3685 (aa305)</td>
<td>CPV-2</td>
</tr>
<tr>
<td>Cat 1 brain</td>
<td>3629 3647 3685 (aa305)</td>
<td>CPV-2</td>
</tr>
<tr>
<td>Cat 1 gut</td>
<td>3629 3647 3685 (aa305)</td>
<td>CPV-2</td>
</tr>
<tr>
<td>Cat 3 gut</td>
<td>3629 3647 3685 (aa305)</td>
<td>CPV-2</td>
</tr>
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* a CPV-specific nucleotides, differing between FPV and all CPV types.
* b CPV-2a- and 2b-specific nucleotides, differing between CPV-2 and CPV-2a and 2b.
* c aa, amino acid.
from the number of IHC-positive cats \((n = 18)\), autolytic processes may also be responsible for the peculiar morphology of those neurons. Thus, in contrast to IHC, H&E staining seems not to be an appropriate tool for identifying neuronal degeneration due to parvovirus infection.

In situ hybridization confirmed the immunohistochemical findings, although only rarely was a colocalization of antigen and nucleic acid observed. Most likely, the different amounts of proteins and nucleic acid occurring within the neurons combined with the different sensitivity of the methods used may be responsible for the lacking colocalization of IHC and in situ hybridization signals. As minus-sense RNA probes were used for in situ hybridization, a positive signal not only revealed the presence of parvovirus DNA but also demonstrated virus replication in the affected cells. The amplification of parvovirus-specific sequences by PCR further confirmed the presence of parvovirus in the CNS of those cats. These data clearly indicate that parvovirus can infect and replicate in the CNS of cats, apart from perinatal FPV infections of the cerebellar Purkinje cells.

However, as parvoviruses are known to be S phase dependent \((1)\) and as neurons, unlike what was stated in earlier dogmas, are now believed to be capable of reentering the cell division cycle but incapable of completing the division cycle, as the cycle is arrested in the G1 phase \((8)\), our findings are intriguing.

Furthermore, DNA sequence analyses of parvovirus DNA amplified from brain sections revealed results that are interesting in two regards: first, the demonstration of CPV-2-like sequences in neuronal tissues was very surprising, as this virus type was believed to be extinct and replaced in the dog populations by the antigenic types CPV-2a and -2b \((10)\). Experimental infections of cats with CPV-2 consistently failed to show virus replication, and the cat was therefore considered not to be susceptible to this virus. However, to our knowledge virus replication in neuronal cells has never been examined and a possible infection of these cells may have been overlooked. Second, some cats appeared to be infected with more than one parvovirus, i.e., cat 3, from which CPV-2- and FPV-like sequences were amplified.

Although true persistent infections of cats or dogs with FPVs have never been demonstrated, recent findings of CPV-2a and CPV-2b in clinically asymptomatic cats in Japan and Taiwan indicate the possibility of a coexistence of these viruses and their hosts, at least their heterologous hosts (CPV in cats) \((6, 7)\). Our findings suggest that this may also be true for the CPV-2 infection of cats and that infection of one animal with several parvoviruses may also occur.

CPV-2 does not circulate naturally in dog populations anymore but is widely used in modified live virus vaccines for dogs. Whether these are the source of the cat infections is not known but warrants further investigation.

The virus typing was based exclusively on analysis of PCR-amplified DNA sequences and therefore has to be carefully interpreted. Cross-contamination with viral nucleic acid leading to false-positive results is an inherent risk of PCR. For all PCR assays CPV-2 was used as a positive control (CPV isolate CPV-d) \((9)\). In one cat the amplified sequences showed a complete homology to this virus and a contamination cannot be excluded, although the negative controls were valid. However, in two cats a parvovirus sequence that was very different from the control virus was amplified and a cross-contamination in these cases is highly unlikely. Nevertheless, the virus typing based on analysis of PCR-generated DNA has to be considered preliminary and needs to be confirmed by examination of virus isolated from the cases in question. This needs to be addressed to further studies, as for the cases described, only formalin-fixed material was available.

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**REFERENCES**