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Experimental Chronic Wasting Disease (CWD) in the Ferret

C. J. Sigurdson[*,†,¶], C. K. Mathiason[*], M. R. Perrott[*], G. A. Eliason[*], T. R. Spraker[*], M. Glatzel[†,#], G. Manco[†], J. C. Bartz[‡], M. W. Miller[§] and E. A. Hoover[*]

[*] *Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1619, USA,* [†] *Institute of Neuropathology, University Hospital of Zürich, Schmelzbergstrasse 12, CH-8091, Zürich, Switzerland,* [‡] *Department of Medical Microbiology and Immunology, Creighton University, Omaha, NE 68178, USA and* [§] *Colorado Division of Wildlife, Wildlife Research Center, 317 West Prospect Road, Fort Collins, CO 80526-2097, USA*

Summary

Chronic wasting disease (CWD), a prion disease of North American deer, elk and moose, affects both free-ranging and captive cervids. The potential host range for CWD remains uncertain. The susceptibility of the ferret to CWD was examined experimentally by administering infectious brain material by the intracerebral (IC) or oral (PO) route. Between 15 and 20 months after IC inoculation, ferrets developed neurological signs consistent with prion disease, including polyphagia, somnolence, piloerection, lordosis and ataxia. Upon first sub-passage of ferret-adapted CWD, the incubation period decreased to 5 months. Spongiform change in the neuropil was most marked in the basal ganglia, thalamus, midbrain and pons. The deposition of PrP^{CWD} was granular and was occasionally closely associated with, or localized within, neurons. There were no plaque-like or perivascular PrP aggregates as seen in CWD-infected cervids. In western blots, the PrP^{CWD} glycoform profile resembled that of CWD in deer, typified by a dominant diglycosylated glycoform. CWD disease in ferrets followed IC but not PO inoculation, even after 31 months of observation. These findings indicate that CWD-infected ferrets share microscopical and biochemical features of CWD in cervids, but appear to be relatively resistant to oral infection by primary CWD inoculum of deer origin.

Keywords: chronic wasting disease; prion; species barrier; transmissible spongiform encephalopathy

Introduction

Chronic wasting disease (CWD) is a prion disease of North American mule deer, white-tailed deer, Rocky Mountain elk and moose (family Cervidae), and is the only prion disease known to naturally affect wildlife. CWD has been recognized for at least three decades in a focal geographical region encompassing north-eastern Colorado and south-eastern Wyoming (Williams and Young, 1980, 1982), wherein its prevalence in free-ranging mule deer has reached up to 30% (Williams, 2005). More recently, this transmissible spongiform encephalopathy (TSE) was discovered in isolated foci over 100 miles beyond this endemic area, most recently as far east as New York (Sigurdson and Miller, 2003). Transmission among cervids is believed to occur horizontally, based on observations with captive deer sharing common pastures and epidemiological models of prevalence and population at risk (Miller *et al.*, 1998, 2000; Miller and Williams, 2003). The mechanism(s) and route(s) of natural

Correspondence to: E. A. Hoover (e-mail: edward.hoover@colostate.edu).
[¶] Current address: Department of Pathology, 9500 Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0612, USA.
[#] Current address: Institute of Neuropathology, University Medical Center Hamburg, D-20246 Hamburg, Germany.

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agent transmission remain unclear, although infectious prions have been demonstrated in saliva and blood of CWD-infected deer (Mathiason *et al.*, 2006). Moreover, whether other species are susceptible to, and/or can act as reservoirs for, CWD has been minimally investigated and thus remains uncertain.

A CWD-susceptible small animal model is crucial in order to study CWD prevention schemes and therapeutics, and to serve as a bioassay for potential transmission vehicles such as saliva, urine or faeces. Animal prion diseases such as scrapie in sheep and goats have been extensively modelled in mice and hamsters, enabling mapping of prion infectivity during the course of disease (Brandner *et al.*, 2000). Wild-type mice have been shown to be resistant to CWD (Williams and Young, 1992; Browning *et al.*, 2004). However, transgenic mice expressing cervid prion protein (PrP) in the central nervous system (CNS) have been shown to be susceptible to CWD after intracerebral inoculation, supporting the importance of the cervid PrP gene in disease susceptibility (Browning *et al.*, 2004; Kong *et al.*, 2005; Tamguney *et al.*, 2006). Bartz *et al.* (1998) showed that ferrets (*Mustela furo*; European polecats) are susceptible to CWD, and that CWD-resistant hamsters became susceptible to ferret-passaged CWD. These results suggest that ferrets may be a surrogate small animal model and may provide insights into the potential for CWD to cross species barriers. The aim of the present study was to determine the susceptibility of ferrets to CWD following exposure by a natural route (orally; PO) and by intracerebral (IC) inoculation.

Materials and Methods

Animals and Experimental Inoculation

Brain homogenate from terminally ill, naturally CWD-infected mule deer was prepared in physiological saline with penicillin-streptomycin (100 U/ml) added.

Nine domestic ferret kits (*Mustela furo*) were administered anaesthesia and intracerebrally-inoculated with 300 µl of a 10% homogenate of CWD-infected mule deer brain given into the left parietal cortex. Two of these ferrets were killed at 3, 6 and 12 months post-inoculation, and the three remaining ferrets were maintained until terminal disease developed. Brain from these terminally diseased ferrets was then pooled and inoculated into three further ferrets.

Nine ferrets were syringe-fed once daily for 4 days with 1.8 ml of a 40% CWD brain homogenate (total dose equivalent to 2.9 g of brain from infected mule deer). Two of these ferrets were killed at 3, 6 and 12 months post-inoculation. Control ferrets ($n = 9$) were inoculated with uninfected mule deer brain by the intracerebral and oral routes described above, and were killed at equivalent time points.

Histology and Immunohistochemistry

Eight sections of brain were examined from each of five intracerebrally-inoculated and four control animals. These sections were taken to include the basal ganglia, cerebral cortex, hippocampus, thalamus, midbrain, pons, cerebellum, and medulla oblongata. Tissue sections were mounted onto positively charged glass slides, dewaxed, and hydrated in preparation for immunohistochemistry (IHC). For exposure of PrP epitopes, sections were pre-treated by hydrated autoclaving for 10 min in a citrate-based antigen retrieval solution, pH 6.0 (Dako, Carpinteria, CA), and cooled for 30 min. The reagents employed sequentially in the immunohistochemical reaction included: monoclonal antibody (Mab) L42, which is specific for the ovine 145–163 PrP epitope (R-Biopharm AG, Darmstadt, Germany) (Vorberg *et al.*, 1999), biotinylated secondary anti-mouse antibody, alkaline phosphatase-streptavidin conjugate, substrate chromogen (Ventana Medical Systems, Tucson, AZ), and haematoxylin counterstain. CWD positive and negative control tissue sections were included in each experiment.

Immunofluorescence Labelling

Tissue sections (6 µm) were mounted onto positively charged glass slides, dewaxed and hydrated. To expose PrP epitopes, sections were heated in a citrate buffer solution (pH 6.0) for 5 min using a pressure cooker, cooled for 5 min, and then treated with 2 µg/ml proteinase K (Roche Applied Science, Indianapolis, Indiana) for 10 min at room temperature (RT) and washed in distilled water. Tissues were next immersed in 3% H_2O_2 in methanol for 15 min to quench endogenous peroxidase, blocked (TSA Kit blocking reagent; PerkinElmer, Boston, MA) and incubated in a mixture of antibody specific for glial fibrillary acidic protein (GFAP; rabbit polyclonal, 14.5 µg/ml; Dako) and anti-PrP monoclonal antibody L42 (0.05 µg/ml) for 30 min. Secondary antibodies were then added sequentially to "visualize" the location of GFAP and PrP within the tissues. For GFAP, the secondary antibody was goat anti-rabbit IgG-Alexa 546 (Invitrogen, Carlsbad, CA) and for PrP the secondary antibody was goat anti-mouse IgG-horseradish peroxidase (HRP) (Zymed, San Francisco, CA) followed by amplification with biotinyl tyramide and detection using

streptavidin-Alexa488 (Invitrogen). Controls included CWD-negative ferret tissues and use of an isotype- and concentration-matched mouse antibody of irrelevant specificity (mouse myeloma IgG_1; Zymed) on selected positive tissues. The fluorescent images were collected using an Olympus microscope equipped with a cooled CCD F-view camera (Soft Imaging System, Munich, Germany), through spectrum orange (excitation 559, emission 588) and spectrum green (excitation 497, emission 524) filters. The two fluorescent images were collected separately using each filter and then merged using the Analysis software program (Soft Imaging Systems).

Western Blotting

Brain samples were prepared as 10% homogenates in Tris-HCl buffer (pH 7.5) containing 0.5% nonidet P40 and 0.5% deoxycholate or as 5% homogenates in phosphate buffered saline (PBS; pH 7.4) with 2% sarcosyl. Homogenates were treated with proteinase K at 50 μg/ml or at a ratio of 1:50 (proteinase K: protein) for 1 h at 37°C. Following digestion, pefabloc (Sigma-Aldrich; St. Louis, MO) was added to a final concentration of 4 mM. Proteins were separated on a 12% bis-tris NuPAGE™ gel (Invitrogen) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) overnight by wet blotting (Biorad, Hercules, CA). Blots were then blocked with 1% casein/0.05% Tween 20, incubated in the anti-PrP mouse monoclonal antibody BAR224 (0.1 μg/ml) specific for epitope 141–151 of ovine PrP (Feraudet *et al.*, 2005) or mouse monoclonal antibody POM1 (0.25 μg/ml) specific for an epitope 121–230 of mouse PrP (Polymenidou *et al.*, 2005), and finally incubated in goat anti-mouse IgG antibody conjugated to HRP (0.045 μg/ml). Blots were developed using ECL plus™ reagent (Pierce, Rockford, IL).

Sodium Phosphotungstic Acid Precipitation of PrP^{Sc}

Sodium phosphotungstic acid (NaPTA) precipitation was used to enrich for PrP^{CWD} prior to western blotting using published methods (Wadsworth *et al.*, 2001). Briefly, a 500-μl tissue homogenate in PBS (10% w/v) was incubated with benzonase (100 U/ml) and $MgCl_2$ (1.5 mM) (Sigma-Aldrich) at 37°C for 45 min. An equal volume of 4% N-lauroylsarcosine (NLS) in PBS was added to the homogenates and incubated for 30 min at room temperature. Samples were incubated with proteinase K (50 μg/ml) for 1 h at 37°C followed by the addition of proteinase inhibitors (pefabloc; Sigma-Aldrich; 4 mM). Samples were incubated in NaPTA (4% NaPTA in 170 mM $MgCl_2$, pH 7.4) at 37°C for 30 min and centrifuged at 18,000 *g* for 30 min. Pellets were resuspended in loading buffer (Invitrogen) containing 0.1% NLS and were processed as described above for western blot analysis.

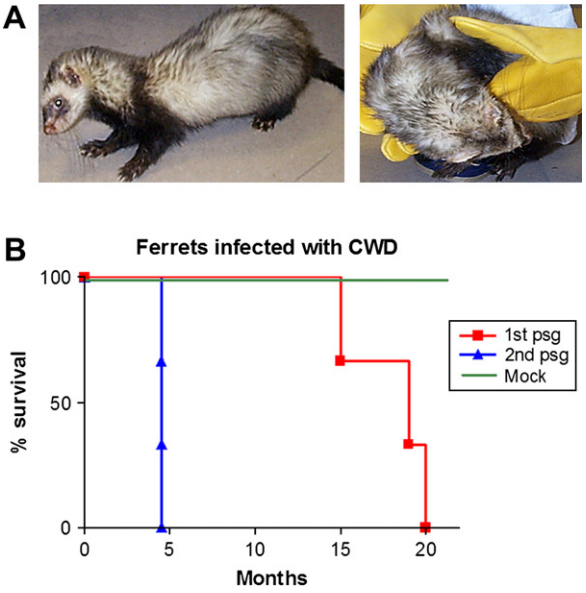
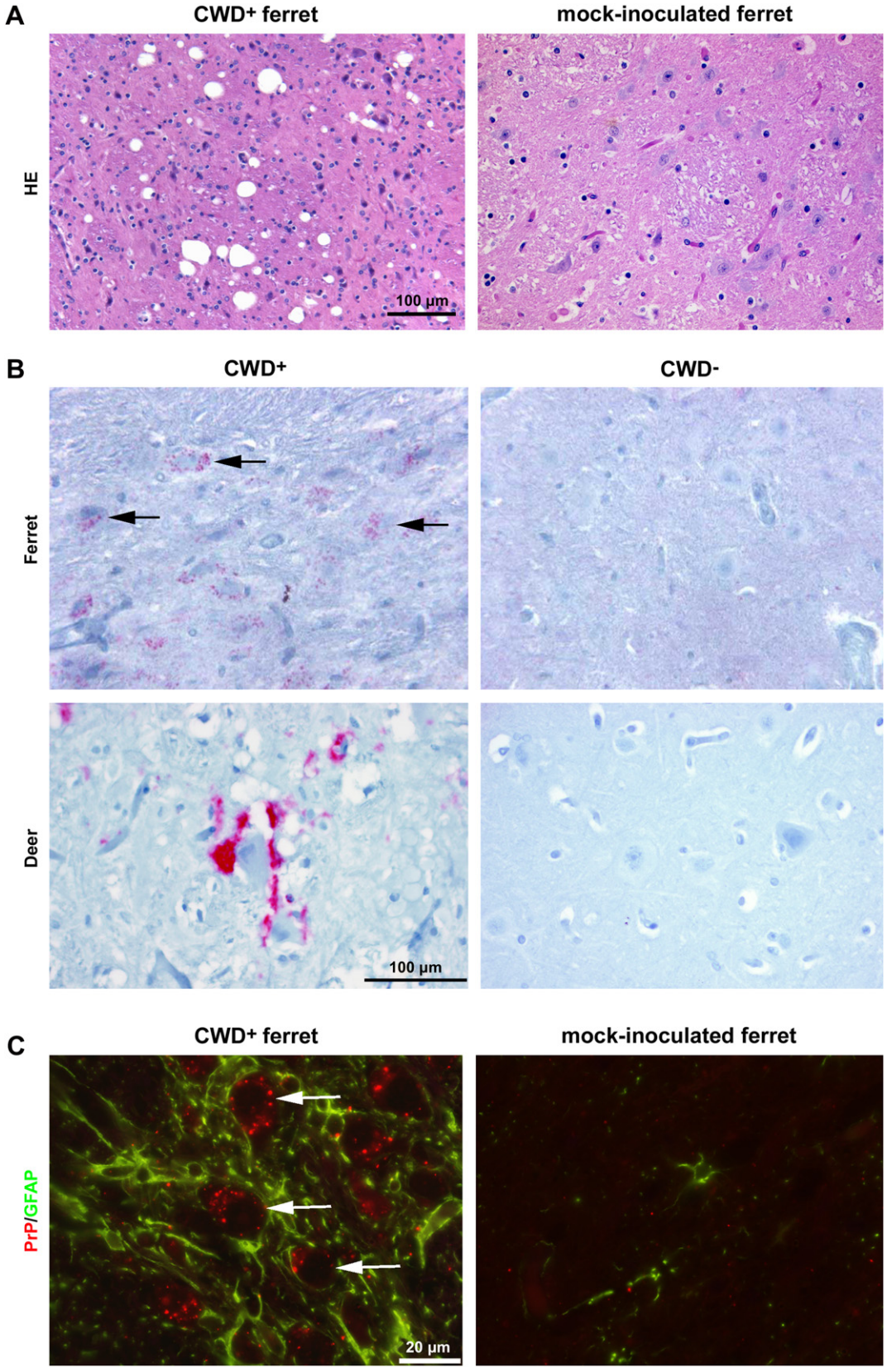


Fig. 1. (A) Clinical features of CWD in ferrets. Ferrets inoculated IC with CWD-infected brain developed clinical neurological disease characterized by piloerection, lordosis, torticollis and ataxia between 15 and 20 months post-infection. (B) Survival curve shows the marked decrease in incubation period between the first (deer CWD) and second passages (ferret CWD) indicating that strain adaptation has occurred.

Table 1
Experimental protocol

Inoculum	*Route of exposure*	*Number infected/ number inoculated*	*Time to terminal disease (months)*
CWD^+ deer brain	Intracerebral	3/9*	15, 19, 20
CWD^+ deer brain	Oral	0/9*,†	ND
Mock deer brain	Intracerebral/ oral	0/9*	ND
CWD^+ ferret brain	Intracerebral	3/3	4.6, 4.6, 4.8
Mock ferret brain	Intracerebral	0/2	ND
Saliva from CWD^+ deer	Intracerebral	0/4	ND
PBMC from CWD^+ deer	Intracerebral	0/3	ND
Saliva from CWD^- deer	Intracerebral	0/4	ND
PBMC from CWD^- deer	Intracerebral	0/4	ND

ND, no disease.
*Six ferrets were killed at early time points (3, 6 and 12 months post-inoculation) to study the kinetics of the infection.
†One ferret died at 13 months post-inoculation of causes unrelated to TSE.


A
CWD+ ferret
mock-inoculated ferret
HE
100 μm
B
CWD+
CWD-
Ferret
Deer
100 μm
C
CWD+ ferret
mock-inoculated ferret
PrP/GFAP
20 μm

Results

Induction of Disease

All ferrets maintained beyond 14 months ($n = 3$) developed clinical TSE after IC inoculation of primary passage CWD-infected deer brain homogenate. The incubation period was 15–20 months. This interval decreased to only 5 months (more than a 65% decrease of the initial incubation period) upon serial passage of the CWD-infected ferret brain (Fig. 1). The clinical syndrome began with isolation behaviour from other ferrets, polyphagia and somnolence. As the disease progressed, intention tremors, hyperreflexia and ataxia were evident. Animals in later stages of disease developed piloerection, lordosis, torticollis and lethargy (Fig. 1). However, after oral CWD inoculation, no ferret (0/3) developed TSE. Mock brain-inoculated ferret controls also remained uninfected with observation for up to 2.5 years post-inoculation (Table 1).

Microscopical Lesions

After primary IC passage of deer CWD, mild spongiform lesions were present in the brain of ferrets in the terminal stages of disease. Spongiosis was most prominent in the basal ganglia, thalamus, optic chiasm, midbrain and pons (trapezius nucleus) and occurred predominantly in the neuropil, but also in white matter tracts. Perikaryonic vacuoles were rarely observed. CNS lesions were not seen in ferrets killed at earlier time points. Upon subsequent passage, similar TSE lesions were present; however, spongiosis and gliosis increasingly targeted the cerebral cortex and cerebellum, and lesions were more intense and destructive (Fig. 2A).

Deposition of PrP^{CWD}

PrP^{CWD} deposits detected by IHC were small (1–2 μm) and granular, affecting neurons and the neuropil. Overall, very little PrP^{CWD} was detected upon primary passage. The PrP^{CWD} deposits were notable in the basal ganglia, thalamus and cerebellum, and were associated with gliosis (Fig. 2B and C). Coarse PrP^{CWD} aggregates were closely associated with neurons in the pontine nuclei in all three ferrets with terminal prion disease. This pattern of deposition was unlike CWD in deer in which there are often large extracellular plaque-like deposits (Fig. 2B). In contrast, animals inoculated with ferret-adapted CWD brain homogenate developed diffuse, fine PrP^{CWD} aggregates in the cerebral cortex. No PrP^{CWD} aggregates or other microscopical evidence of prion infection were seen in ferrets killed at earlier time points (3–12 months post-inoculation), or in mock-inoculated controls (Fig. 2B).

PrP^{CWD} Biochemistry

PrP^{CWD} was detected in the brains of all three IC inoculated ferrets that developed clinical neurological disease, but not the mock-inoculated ferrets. The glycoform profile was characterized by a predominant diglycosylated PrP, similar to deer and elk with CWD (Fig. 3). In ferrets exposed orally to primary CWD inoculum, PrP^{CWD} was not detectable in the brains by western blotting, either directly or after sodium phosphotungstic acid (NaPTA) precipitation of up to 40 mg tissue equivalents followed by western blotting.

Discussion

Upon primary IC inoculation of brain from CWD-infected deer, ferrets proved susceptible to infection and developed a TSE, consistent with the findings of Bartz *et al.* (1998). The primary incubation period ranged from 15–20 months, similar to that in mule deer and white-tailed deer inoculated with CWD (Williams and Young, 1992; Mathiason *et al.*, 2006). Prion infectivity was demonstrated in the brain of affected ferrets by subsequent passage into naive ferrets, leading to a shorter incubation period upon secondary passage of 4.5–5 months, equivalent to a 66% reduction in incubation period. This shortening of the incubation period is consistent with a species barrier between deer and ferret, and suggests that adaptation to a new, ferret-modified prion strain had taken place. This latter interpretation was supported by an altered pattern of PrP^{Sc} deposition in the brain by immunohistochemistry; the primary passage PrP^{Sc} deposits were primarily coarse and associated with neurons, whereas by the second passage, deposits consisted of

Fig. 2. Brain pathology in CWD-infected ferrets. (A) Microscopical lesions were characterized by spongiform vacuolation, gliosis and focal neuronal necrosis, typical for transmissible spongiform encephalopathy. Spongiform vacuolation was most prominent in the basal ganglia, thalamus, optic chiasm, midbrain and pons. (B) Immunohistochemistry for PrP^{CWD}. PrP labelling by immunohistochemistry of brain revealed punctuate, neuron-associated PrP^{CWD} deposits in ferrets receiving primary deer CWD (arrows), whereas deer with CWD show large extracellular PrP^{CWD} aggregates. (C) Dual immunofluorescence labelling with antibodies against GFAP (label astrocytes green) and PrP (red) show gliosis and PrP^{CWD} accumulation on or within neurons (arrows). No PrP expression was evident in mock-inoculated control ferrets.

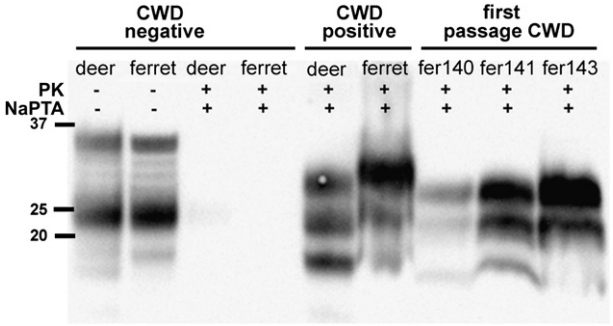


Fig. 3. Western blot of brain from CWD-infected and uninfected deer and ferrets. In uninfected deer and ferrets, PrP^{C} is visible in the undigested samples, but not following sodium phosphotungstic acid (NaPTA) concentration and proteinase K (PK) digestion. In CWD-infected positive control samples, di-, mono- and unglycosylated PrP^{Sc} is visible after concentration by NaPTA precipitation and digestion with PK, which cleaves the amino-terminus of PrP^{Sc}. In CWD-infected ferrets, PK-resistant PrP^{Sc} shows a dominant diglycosylated band as seen in CWD-infected deer and elk.

finer, more diffuse aggregates. Also indicative of a species barrier was the fact that ferrets appeared to resist oral prion infection, even after a 2.5-year observation period. However, ferrets may have other barriers to oral TSE infection, and it will be important to assess oral susceptibility to the ferret-adapted CWD in further studies. Sequence comparison between ferret and mule deer PrP genes reveals 91% sequence identity, although even three amino acid differences between the host PrP^{C} and agent PrP^{Sc} molecules can prevent the formation of new PrP^{Sc} (Kocisko *et al.*, 1995).

Clinically, TSE-infected ferrets developed a progressive neurological disease over several weeks. Ferrets progressively developed social isolation from other ferrets, polyphagia and somnolence, and later developed piloerection, lordosis, torticollis and ataxia. In contrast, mink (also family Mustelidae) with transmissible mink encephalopathy (TME) display behavioural changes including increased aggression and hyperaesthesia, which progresses to ataxia, occasional tremors or circling, and compulsive biting of self or objects (Eckroade *et al.*, 1979). Thus the clinical disease among Mustelids appears to vary depending on the strain.

In ferrets, first IC passage CWD was characterized by mild spongiform lesions in the basal ganglia, thalamus, optic chiasm, midbrain and pons (trapezius nucleus), with a variable degree of vacuolation in the cerebral cortex and cerebellum. On second passage the vacuolation was more intense in all regions, with more vacuolation evident in the cerebral cortex and cerebellum, similar to mink with TME and cats with feline spongiform encephalopathy (FSE) (Sigurdson and Miller, 2003). The most salient histologic feature in the TME-infected brain was the extensive neuropil vacuolation in the cerebral cortex, corpus striatum, thalamus and hypothalamus, being less severe in the midbrain, pons and medulla oblongata (Eckroade *et al.*, 1979). Domestic cats with FSE have brain lesions typified by spongiform degeneration in the neuropil, with the most severe lesions localized to the medial geniculate nucleus of the thalamus and the basal nuclei (Ryder *et al.*, 2001).

By comparison, the CWD spongiform lesions in deer and elk predominately arise in the olfactory cortex, thalamus and hypothalamus, pons and medulla oblongata (Spraker *et al.*, 2002), and thus have some overlap with sites affected in the ferrets. Perikaryonal neuronal vacuoles are common in cervids, but were exceedingly rare in the ferret. Therefore, the lesion profile of CWD in ferrets is more similar to TSEs in other carnivores than to lesions of CWD in cervids, although differences in exposure route may have influenced lesion profiles.

In ferrets inoculated with primary source CWD, PrP^{CWD} deposits were demonstrated immunohistochemically associated with neurons, particularly in the pontine nuclei, but in subsequent passages PrP^{CWD} aggregates were more diffusely distributed throughout the cerebral cortex. The dense, large (>50 μm) PrP^{CWD} plaques and perivascular aggregates common in deer were not apparent in the brains of CWD-infected ferrets.

The glycoform profile of protease-resistant PrP^{CWD} in the brains of ferrets was characterized by a predominant diglycosylated band; a profile common to deer with CWD, cattle with bovine spongiform encephalopathy (BSE) and sheep with scrapie (Raymond *et al.*, 2000). Therefore, some aspects of the CWD strain may be maintained in the ferret.

Consumption of CWD-infected deer and elk by predators and scavengers is certain to occur; however, the CWD susceptibility of these predatory species remains largely unknown. Of interest is that raccoons have been shown to be resistant to mule deer CWD after IC inoculation (Hamir *et al.*, 2003, 2007). Although clearly ferrets are CWD susceptible by the most extreme exposure route, it would be worthwhile to perform CWD IC inoculation and feeding studies with additional species such as felids, coyote and opossum to begin to understand whether CWD is a promiscuous TSE, as is BSE, able to infect several species by the oral route, or conversely, will be restricted to cervids. Studies are ongoing to assess the oral susceptibility of ferrets to ferret-adapted CWD, as this route would be of greater relevance to CWD in nature. If orally susceptible to the

ferret-adapted CWD strain, ferrets may be useful to evaluate potential preventative strategies for oral CWD exposure.

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