Increased Attack Rates and Decreased Incubation Periods in Raccoons with Chronic Wasting Disease Passaged through Meadow Voles

S. Jo Moore, Christina M. Carlson, Jay R. Schneider, Christopher J. Johnson, Justin J. Greenlee

Chronic wasting disease (CWD) is a naturally-occurring neurodegenerative disease of cervids. Raccoons (Procyon lotor) and meadow voles (Microtus pennsylvanicus) have previously been shown to be susceptible to the CWD agent. To investigate the potential for transmission of the agent of CWD from white-tailed deer to voles and subsequently to raccoons, we intracranially inoculated raccoons with brain homogenate from a CWD-affected white-tailed deer (CWDwtd) or derivatives of this isolate after it had been passaged through voles 1 or 5 times. We found that passage of the CWDwtd isolate through voles led to a change in the biologic behavior of the CWD agent, including increased attack rates and decreased incubation periods in raccoons. A better understanding of the dynamics of cross-species transmission of CWD prions can provide insights into how these infectious proteins evolve in new hosts.

Transmission spongiform encephalopathies, or prion diseases, are a group of fatal neurodegenerative diseases that include chronic wasting disease (CWD) in cervids, scrapie in sheep and goats, bovine spongiform encephalopathy (mad cow disease) in cattle, and Creutzfeldt-Jakob disease and Kuru in humans. As of January 2020, CWD has been reported in free-ranging and farmed cervids in 26 states in the United States and 3 provinces in Canada (1). CWD-affected cervids shed infectious prions into their environment during both the preclinical and clinical stages of disease (2–8), and infectivity persists in soil (9–13), on the surface of contaminated plant leaves and roots (14), and in association with mineral licks (15). Environmental contamination with CWD prions represents a source of infectious material to which noncervid wildlife species, including raccoons and other small mammals, can be exposed.

We previously reported the transmission of the agent of CWD from white-tailed deer (Odocoileus virginianus borealis) and elk to raccoons through experimental intracranial inoculation (16). Raccoons are able to propagate CWD prions from white-tailed deer and elk but with low attack rates (25%) and with disease-associated prion protein distribution restricted to the brain (16).

Successful transmission of the agent of CWD from white-tailed deer to 4 species of native North America rodents has been reported previously, and meadow voles (Microtus pennsylvanicus) were found to be the most susceptible species (17). Meadow voles are known to opportunistically scavenge carcasses and engage in cannibalistic behavior (18), providing a plausible route for exposure to CWD and the possibility of continued disease transmission. Small rodents are a food source for predators and scavengers, including raccoons, and meadow voles and raccoons inhabit overlapping geographic ranges that also overlap with locations undergoing cervid CWD epidemics (Figure 1). Therefore, the potential for direct exposure of meadow voles and raccoons to CWD infectivity in the environment exists. Indeed, studies in Wisconsin have shown that raccoons are present at deer carcasses and gut piles with a high frequency (19). In addition, because raccoons are mesopredators and scavengers, there is the potential for secondary exposure of raccoons through consumption of contaminated rodents.
To examine the potential for noncervid species to support CWD transmission, we intracranially inoculated raccoons with the agent of CWD from a white-tailed deer or with derivatives of the same inoculum after it had been passaged through meadow voles 1 or 5 times. In this study, we report the successful transmission of the agent of CWD from a white-tailed deer and vole-passaged CWD to raccoons through experimental intracranial inoculation. Our findings suggest passage of the CWD agent through voles results in a CWD agent with altered phenotypic properties.

**Materials and Methods**

We sourced 17 raccoon kits (8 weeks of age) that had no previous history of prion disease from a commercial breeder and challenged them by intracranial inoculation using 0.1 mL of a 10% brain homogenate (\(10^{\text{th}}\)). Brain material from 3 CWD-affected donor animals generated in a previous study (17) were used as inocula: 1 hunter-harvested (year of harvest 2001) CWD-positive white-tailed deer that was heterozygous for glycine and serine at codon 96 of the prion protein (GS96) (CWD\(^{\text{Wtd}}\)), 1 meadow vole that had been inoculated intracranially with the CWD\(^{\text{Wtd}}\) inoculum (first passage, CWD\(^{\text{Vole-P1}}\)), and 1 meadow vole that had been inoculated intracranially with brain material from a fourth passage vole (fifth passage, CWD\(^{\text{Vole-P5}}\)) (Table; Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-0271-App1.pdf). We inoculated raccoons in the negative control groups with brain material from a vole that had been intracranially inoculated with obex tissue from a CWD-negative deer (CWD\(^{\text{Neg}}\)) (Table 1; Appendix). We prepared each inoculum from a single donor animal; no pooling was performed. We monitored raccoons daily and euthanized them when they showed unequivocal signs of prion disease (such as ataxia, inability to climb, or recumbency), when intercurrent illness or injury was present that could not be remedied by veterinary care, or at the end of the experiment at 35 months after inoculation. At raccoon death, we performed a full necropsy on all raccoons. We fixed 1 set of tissue samples in 10% buffered formalin, embedded in paraffin wax, and sectioned at 5 μm for microscopy examination.
Table. Summary of results of experimental inoculation of raccoons with the agent of CWD from white-tailed deer or vole-passaged CWD isolates*

| Raccoon no. | Inoculum       | Incubation time, mpi | Clinical signs | EIA OD | Spongiform change | Immunohistochemistry |
|-------------|----------------|----------------------|---------------|--------|-------------------|----------------------|
| 1           | WTD CWD        | 21                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 2           | (CWD<sub>WTD</sub>) | 22                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 3           |                | 27                   | +             | 3.244  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 4           |                | 32                   | −             | 0.095  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |
| 5           | 1st passage    | 13                   | −             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 6           | CWD<sub>WTD</sub> in vole | 18                   | −             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 7           | (CWD<sub>vole-P1</sub>) | 22                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 8           |                | 22                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 9           |                | 24                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 10          | 5th passage    | 3                    | −             | 0.093  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |
| 11          | CWD<sub>WTD</sub> in vole | 17                   | −             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 12          | (CWD<sub>vole-PS</sub>) | 18                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 13          |                | 21                   | +             | 4.000  | +                 | Brain: + Retina: − Pituitary: − ENS: − LRS: − |
| 14          |                | 21                   | +             | 4.000  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |
| 15          | CWD-negative  | 35                   | −             | 0.097  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |
| 16          | WTD in vole    | 35                   | −             | 0.095  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |
| 17          | (CWD<sub>NEG</sub>) | 35                   | −             | 0.090  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |
| 18          |                | 35                   | −             | 0.109  | −                 | Brain: − Retina: − Pituitary: − ENS: − LRS: − |

*CWD, chronic wasting disease; EIA, antigen-capture enzyme immunoassay; ENS, enteric nervous system; LRS, lymphoid tissues; mpi, months postinoculation; NA, not available; OD, optical density; WTD, white-tailed deer.

after hematoxylin and eosin staining or immunohistochemical staining for detection of disease-associated prion protein (PrP<sup>Sc</sup>) by using a cocktail containing 2 monoclonal antibodies, F89/160.1.5 and F99/97.6.1 (Appendix). We froze the second set of tissues, comprising subsamples of all tissues collected into formalin, and examined selected samples for the presence of disease-associated prion protein (PrP<sup>Sc</sup>) by using a commercially available antigen-capture enzyme immunoassay or in-house Western blotting (Appendix).

Ethics Statement
This experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC, USA) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL, USA). The Institutional Animal Care and Use Committee at the National Animal Disease Center reviewed and approved the animal use protocols (approval no. ARS-2778).

Results
In the CWD<sub>WTD</sub> group, 3/4 raccoons demonstrated clinical signs consistent with prion disease (ataxia, inability to climb, recumbency); the average survival time was 23 months postinoculation (mpi) (Table). The remaining raccoon was euthanized at 32 mpi because of bilateral eye lesions; PrP<sup>Sc</sup> was not detected in any tissues examined. We detected PrP<sup>Sc</sup> in all raccoons in the CWD<sub>vole-P1</sub> group. Two raccoons were euthanized or found dead because of urinary tract disease, and 1 was euthanized at 22 mpi because of lameness that was not responsive to treatment. The remaining 2 raccoons exhibited ataxia and inability to climb and were euthanized at 22 and 24 mpi (Table). In the CWD<sub>vole-PS</sub> group, 2 raccoons were euthanized because of urinary tract disease at 3 mpi (PrP<sup>Sc</sup> not detected) and 17 mpi (PrP<sup>Sc</sup>-positive). During 18–21 mpi, the remaining 3 raccoons demonstrated ataxia and inability to climb; 2 of these animals also showed head tremors (Table). All 4 raccoons in the CWD<sub>NEG</sub> control group were clinically normal when they were euthanized at the end of the study at 35 mpi (Figure 2). By using antigen-capture enzyme immunoassay, we detected PrP<sup>Sc</sup> in the brains of 3/4 raccoons in the CWD<sub>WTD</sub> group, 5/5 raccoons in the CWD<sub>vole-P1</sub> group, and 4/5 raccoons in the CWD<sub>vole-PS</sub> group.

Figure 2. Survival curves for raccoons inoculated intracranially with the agent of CWD from white-tailed deer or vole-passaged CWD. CWD, chronic wasting disease; CWD<sub>WTD</sub>, CWD negative white-tailed deer; CWD<sub>vole-P1</sub>, first passage (white-tailed deer to vole); CWD<sub>vole-PS</sub>, fifth passage (vole to vole); CWD<sub>NEG</sub>, CWD from white-tailed deer.
4/4 raccoons in the CWD\textsuperscript{Vole-P5} group (not including the raccoon that was euthanized because of urinary tract disease at 3 mpi), and 0/4 raccoons in the CWD\textsuperscript{Neg} group (Table).

When we analyzed brain samples by Western blot by using monoclonal PrP antibody P4, migration patterns for all animals within a treatment group were similar to each other and to the original inoculum (data not shown). When we compared samples across groups, migration patterns for vole-passaged groups were similar to each other with the unglycosylated band at \( \approx 19 \text{ kDa} \). The unglycosylated band of the sample from the CWD\textsuperscript{Vid} group migrated slightly higher, at 20 kDa, and that of the original donor white-tailed deer migrated slightly higher again, at \( \approx 21 \text{ kDa} \) (Figure 3).

We examined hematoxylin and eosin–stained sections to assess pathologic changes in the brain (Figure 4). Immunohistochemical staining for PrP\textsuperscript{Sc} was applied to the brain and peripheral tissues to investigate the distribution of PrP\textsuperscript{Sc} throughout the body (Figure 4). In raccoons in the CWD\textsuperscript{Vid} group, spongiform change of the neuropil was mild caudally (medulla at the level of the obex and midbrain) and moderate rostrally (thalamus and basal nuclei). Spongiform change was not observed in the dorsal motor nucleus of the vagus nerve (Figure 4, panel E) and neocortex. In contrast, spongiform change in the vole-passaged CWD groups was moderate to marked throughout the brain, including in the dorsal motor nucleus of the vagus nerve (Figure 4, panel B), basal nuclei (Figure 4, panel F) and neocortex, and mild in the cerebellum. Intraneuronal vacuolation was only observed in 2 raccoons, both of which were from the vole-passaged CWD groups. A single intraneuronal vacuole was seen in the red nucleus of raccoon 6 (CWD\textsuperscript{Vole-P1}) and the dorsal motor nucleus of raccoon 14 (CWD\textsuperscript{Vole-P5}).

We detected immunoreactivity for PrP\textsuperscript{Sc} in the brain, spinal cord, retina, optic nerve, and/or pituitary in \( \geq 2 \) raccoons per group (Table). We did not detect PrP\textsuperscript{Sc} in any lymphoid tissues sampled but was observed in the enteric nervous system of the stomach, jejunum, ileum and colon of raccoon 3 (CWD\textsuperscript{Vid}).

In the brains of raccoons in the CWD\textsuperscript{Vid} group, the overall amount of PrP\textsuperscript{Sc} immunoreactivity was less in the caudal parts of the brain (Figure 4, panel C) and greater in the rostral parts of the brain (thalamus and basal nuclei) (Figure 4, panel G). Extracellular PrP\textsuperscript{Sc} accumulation in the neuropil and on neurons was more prominent than intraneuronal accumulation (Figure 4, panels C, G). In contrast, the pattern of PrP\textsuperscript{Sc} immunoreactivity was similar in raccoons in the vole-passaged CWD groups and characterized by PrP\textsuperscript{Sc} immunoreactivity throughout the brain with intracellular PrP\textsuperscript{Sc} accumulation in microglia, astrocytes, and neurons (Figure 4, panels D, H).

To enable objective comparisons of the distribution and severity of spongiform change between inoculation groups, we scored the severity of vacuolation on a scale of 0–4 for 17 neuroanatomical areas and used the score to generate vacuolation lesion profiles as described previously (21). We made modifications to include the red nucleus and dorsal motor nucleus of the vagus nerve, which resulted in a total of 19 neuroanatomical areas examined. The distribution of vacuolation in raccoons was similar in the vole-passaged CWD groups, although the overall severity of vacuolation was greater in the CWD\textsuperscript{Vole-P5} group than the CWD\textsuperscript{Vole-P1} group (Figure 5). The pattern of vacuolation observed in raccoons in the CWD\textsuperscript{Vid} group was different from raccoons in the vole-passaged groups. A trend for less severe vacuolation overall was particularly noticeable in the medulla (Figure 5, neuroanatomical areas 1–4), midbrain (Figure 5, areas 8–9), frontal cortex (Figure 5, area 13), and claustrum (Figure 5, area 17) (Appendix).

**Discussion**

We demonstrated that clinical disease developed in raccoons inoculated intracranially with the agent of CWD from white-tailed deer (CWD\textsuperscript{Vid}); the average incubation period was \( \approx 23 \text{ mpi} \). Passage of the CWD\textsuperscript{Vid} isolate through meadow voles before inoculation of raccoons with this vole-passaged CWD resulted in slightly shorter incubation periods (\( \approx 20 \text{ mpi} \)) and different neuropathology and western blot migration pattern as compared with the original CWD\textsuperscript{Vid} isolate.
We previously reported that experimental intracerebral inoculation of raccoons with an inoculum prepared from pooled brainstems from 11 CWD-affected white-tailed deer (CWD\textsuperscript{Wtd-pool}) resulted in disease in 1/4 raccoons with an incubation period of 73 mpi and restricted distribution of PrP\textsubscript{Sc} accumulation in the brain (16). The low attack rate and prolonged incubation period produced by the CWD\textsuperscript{Wtd-pool} inoculum compared with the CWD\textsuperscript{Wtd} inoculum reported in this study could be due to differences in the titer of PrP\textsubscript{Sc} in the donor inocula. However, we consider this scenario unlikely, because all donor deer used to prepare the CWD\textsuperscript{Wtd-pool} inoculum and the single donor deer used to prepare the CWD\textsuperscript{Wtd} inoculum.
inoculum were positive by immunohistochemistry for PrPSc. Another point of difference in disease expression produced by the CWDWtd-pool compared with the CWDWtd inoculum is the pattern of neuropathology observed in the brain: vacuolation of neuronal perikarya was widespread in the brain of the raccoon inoculated with the CWDWtd-pool (16) but was not observed in raccoons inoculated with CWDWtd. The differences in biologic behavior of these 2 CWD isolates are most likely associated with differences in the prion protein (PRNP) genotype of the donor deer. Four PRNP polymorphisms exist in white-tailed deer: Q95H, G96S, A116G, and Q226K (reviewed in S.J. Robinson et al. [22]). At codon 96, the S96 allele is associated with reduced CWD prevalence (23–26) and prolonged incubation periods (27). Donor deer for the CWDWtd-pool inoculum were all GG96 PRNP genotype (16), whereas the donor deer for the CWDWtd inoculum was GS96 PRNP genotype. We did not expect that inoculum containing the S96 allele would produce disease in raccoons more efficiently than inoculum exclusively containing the G96 allele. In addition, sequencing of PRNP from raccoons in a previous study showed that raccoons are homozygous for glycine at codon 96 (GG96) (S.J. Moore, unpub. data). Therefore, our results suggest that patterns of disease susceptibility associated with PRNP polymorphisms at codon 96 in CWD-affected white-tailed deer might not be a useful predictor of disease outcomes in intracranially inoculated raccoons. Further studies are under way to investigate the biologic behavior in raccoons of CWD from a single-source GG96 white-tailed deer.

Cross-species transmission of CWD isolates might result in a change in the biochemical properties of the disease-associated prion protein or the biologic behavior of the prion strain, such as adaptation to its host, or both (16,28–33). The pattern of PrPSc accumulation in the brain of the raccoon inoculated with CWDWtd-pool in our previous study (16) was similar to raccoons inoculated with the CWDWtd inoculum in this study and was characterized by prominent linear and perineuronal PrPSc accumulation, although this comparison is limited by the small number of animals available for examination.

Both vole-passaged CWD isolates produced similar disease phenotypes in raccoons with regards to incubation periods, western blot migration patterns, and neuropathology. The patterns of spongiform change and PrPSc accumulation in the brains of raccoons inoculated with vole-passaged CWD isolates were similar to each other and different from those observed in the brains of raccoons inoculated with the CWDWtd isolate. Inoculum-associated differences in western blot migration patterns were observed (i.e., similar patterns for vole-passaged CWD isolates and a different pattern for the CWDWtd isolate). In addition, the migration pattern of the original CWD-affected white-tailed deer donor (Figure 3, deer CWD, unglycosylated band at ≈21 kDa) was different from both the raccoon-passage CWDWtd isolate (Figure 3, white-tailed deer, 20 kDa) and the vole-passaged CWD isolates (Figure 3, vole-P1 and vole-P5, 19 kDa) after passage through raccoons. Therefore, passage of CWDWtd through voles appears to result in a change in the biologic behavior of this prion isolate when inoculated intracranially.

**Figure 5.** Vacuolation lesion profiles for study raccoons inoculated with the agent of CWD from WTD (blue) or inoculum prepared from the first-passage (orange) or fifth-passage (gray) of CWD WTD in voles. Error bars represent SE of the mean. CWD, chronic wasting disease; WTD, white-tailed deer.
into raccoons. This finding raises the possibility for emergence of novel CWD strains after passage in off-target species through host-driven selection of a strain present in the donor inoculum (29,34). The original inoculum was derived from a white-tailed deer with the GS96 PRNP genotype, and propagation of CWD prions on S96 PrPSc results in the formation of alternative PrPSc conformers (34). We are unsure what role the genotype of the deer in the inoculum might have played in the change in biologic behavior noted after passage through voles. Because intracranial inoculation is not a natural route for exposure of raccoons to CWD infection, oral transmission studies are underway to characterize the biologic behavior of the CWD-Wtd and vole-passaged CWD isolates using a more natural route of exposure.

We observed a single intraneuronal vacuole in the red nucleus of raccoon 6 (CWDVole-P1 group) and the dorsal motor nucleus of raccoon 14 (CWDVole-P5 group). Intraneuronal vacuolation was previously reported as an incidental finding in the brainstem (including facial and pontine nuclei), cerebellar roof nuclei, and cerebrum of raccoons (35,36). In those raccoons, no evidence of concurrent neuropil vacuolation, neuronal degeneration, or astrocytosis was seen. In contrast, widespread neuropil vacuolation throughout the brains of raccoons 6 and 14, and strong PrPSc immunoreactivity in vacuolated neurons was evident; therefore, the intraneuronal vacuoles observed in these raccoons are likely associated with prion infection.

Although PrPSc immunoreactivity was widely distributed throughout the brain and spinal cord, we did not generally observe involvement of the peripheral nervous system, with the exception of 1 raccoon (3) inoculated with CWD-Wtd, in which PrPSc immunoreactivity was present in the enteric plexi of the stomach, jejunum, ileum, and colon. The general lack of peripheral nervous system involvement is likely because raccoons were inoculated through the intracranial route that bypasses centripetal spread of PrPSc from the alimentary tract to the brain along parasympathetic nerves, as is observed in orally infected deer (37). Instead, PrPSc immunoreactivity in the enteric nervous system of raccoon 3 was likely the result of centrifugal spread from the central nervous system. Why PrPSc immunoreactivity was observed throughout the spinal cord in all raccoons is unclear, but enteric nervous system involvement was only seen in raccoon 3. Raccoon 3 was the longest surviving raccoon (27 mpi), so the possibility exists that, had other raccoons not succumbed to clinical disease, there might have been time for transport of PrPSc to the enteric nervous system. Inoculation of raccoons by the oral route is needed to improve our understanding of the pathogenesis of CWD in raccoons when exposure occurs by a more natural route.

The longest surviving CWD-inoculated raccoon (4) was euthanized at 32 mpi because of bilateral eye lesions. Histopathologic examination resulted in a diagnosis of multicentric lymphoma, and PrPSc was not detected in any tissues. Clinical disease and widespread PrPSc accumulation at 21–27 mpi developed in all other raccoons in the CWD-Wtd group (n = 3). The reason for the unexpected negative result for raccoon 4 is unclear but could include experimental error or host factors. With regard to experimental inoculation, all inocula were prepared and all raccoons were inoculated on the same day, so the likelihood is very low that this raccoon did not receive the correct inoculum. The strongest determinant of susceptibility to prion diseases is the host PRNP sequence. No unique single nucleotide polymorphisms were detected in the PRNP open reading frame of raccoon 4 (S.J. Moore, unpub. data). It is tempting to speculate that host, genetic, or immunological factors outside of the PRNP open reading frame that contributed to the development of neoplasia might have had a suppressive effect on PrPSc accumulation.

Prion diseases of free-ranging animals do not exist in isolation. Meadow voles and raccoons are widespread in North America, and their habitat ranges overlap with those of CWD-affected white-tailed deer and other cervids. Therefore, a substantial potential for exposure of these or other off-target species to CWD infectivity in the environment exists. We have demonstrated that CWD-Wtd from a GS96 white-tailed deer transmitted readily to raccoons. Passage of this isolate through voles followed by intracranial inoculation of raccoons with vole-derived inoculum resulted in disease with different biologic characteristics and neuropathology than the original CWD-Wtd isolate. These results provide strong evidence for the emergence of a novel strain of CWD after passage in meadow voles and raccoons. Therefore, interspecies transmission of CWD prions between cervids and noncervid species that share the same habitat might represent a confounding factor in CWD-management programs. In addition, passage of CWD prions through off-target species might represent a source of novel CWD strains with unknown biologic characteristics, including zoonotic potential. Characterization of the biologic behavior of CWD isolates after cross-species transmission will help us develop more effective management strategies for CWD-affected populations.
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About the Author
Dr. Moore performed this work as a postdoctoral research associate at the National Animal Disease Center, US Department of Agriculture, Ames, Iowa. Her research interests include pathogenesis and pathology of animal diseases with a special interest in neuropathology and interspecies transmission of prion diseases.

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Address for correspondence: Justin Greenlee, National Animal Disease Center, ARS, USDA, 1920 Dayton Ave, PO Box 70, Ames, IA 50010, USA; email: justin.greenlee@usda.gov
Increased Attack Rates and Decreased Incubation Periods of Chronic Wasting Disease in Raccoons after Passage through Meadow Voles

Appendix

Materials and Methods

Inoculum

The CWD\textsuperscript{Wtd} inoculum (10\% [wt/vol] obex tissue in phosphate-buffered saline) was prepared from a single hunter-harvested (year of harvest 2001) CWD-positive white-tailed deer (\textit{Odocoileus virginianus}) from southern Wisconsin that was heterozygous for glycine and serine at codon 96 of the prion protein (GS96) as described previously (1). The vole-passaged CWD inocula (10\% [wt/vol] whole brain in phosphate-buffered saline) were prepared from individual meadow voles (\textit{Microtus pennsylvanicus}) as described previously (1). Donor voles had been inoculated intracranially with either the CWD\textsuperscript{Wtd} inoculum (first passage, CWD\textsuperscript{Vole-P1}), brain homogenate from a fourth passage vole (fifth passage, CWD\textsuperscript{Vole-P5}), or brain homogenate from a vole inoculated with homogenate from the obex tissue of a CWD-negative deer (CWD\textsuperscript{Neg}). Each inoculum was prepared from a single vole source; no pooling was performed.

Animals

Eight-week-old raccoon kits were sourced from a commercial breeder that had never had a reported case of prion disease (2). The kits were divided into 4 groups: group 1 (n = 4) was inoculated with CWD\textsuperscript{Wtd}; group 2 (n = 5) was inoculated with CWD\textsuperscript{Vole-P1}; group 3 (n = 4) was inoculated with CWD\textsuperscript{Vole-P5}; group 4 (n = 4) was inoculated with CWD\textsuperscript{Neg} and served as negative controls.
**Animal Housing**

Raccoons were housed in a Biosafety Level 2 containment facility at the National Animal Disease Center (Ames, Iowa, USA) and monitored daily for clinical signs of prion disease. Raccoons were euthanized when they showed unequivocal signs of prion disease such as ataxia, inability to climb, or recumbency; when euthanasia was necessary due to intercurrent illness or injury that could not be remedied by veterinary care; or at the end of the experiment at 35 months after inoculation.

**Sample Collection**

The following samples were collected in postmortem examination: brain, spinal cord, retina, peripheral nervous tissue (ganglia: dorsal root, trigeminal; nerves: optic, sciatic), lymphoid tissues (thymus, 3rd eyelid; tonsils: pharyngeal, palatine, rectal; lymph nodes: mesenteric, popliteal, prescapular, retropharyngeal), adrenal gland, anal gland, gall bladder, gastrointestinal (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum), heart, diaphragm, skeletal muscle (biceps, masseter, psoas, triceps), tongue, kidney, urinary bladder, liver, pancreas, pituitary, salivary gland, skin (lip margin, nose skin, paw skin), spleen, thyroid, turbinate, trachea, and lung.

**Vacuolation Lesion Profiling**

The following neuroanatomical areas were assessed for vacuolation lesion profiling: 1, nucleus of the solitary tract; 2, nucleus of the spinal tract of the trigeminal nerve; 3, hypoglossal nucleus; 4, dorsal motor nucleus of the vagus nerve; 5, vestibular nuclear complex; 6, cochlear nucleus; 7, cerebellar vermis; 8, central gray matter; 9, rostral colliculus; 10, red nucleus; 11, medial geniculate nucleus; 12, hypothalamus; 13, nucleus dorsomedialis thalami; 14, nucleus ventralis lateralis thalami; 15, frontal cortex; 16, septal nuclei; 17, caudate nucleus; 18, putamen; 19, claustrum.

**Immunohistochemistry**

Immunostaining for the detection of disease-associated prion protein (PrP\textsuperscript{Sc}) was performed as described previously (3) using a cocktail containing 2 monoclonal antibodies, F89/160.1.5 and F99/97.6.1, each applied at a concentration of 5 μg/mL with an automated processor.
Antigen-Capture Enzyme Immunoassay (EIA)

Frozen brainstem samples were homogenized in 1X phosphate-buffered saline at a concentration of 20% wt/vol and assayed with a commercially available antigen-capture enzyme immunoassay kit (HerdChek BSE-Scrapie Ag Test Kit; IDEXX Laboratories, https://www.idexx.com) as previously described (4).

Western Blotting

Samples were collected from the brainstem at the level of the obex and western blotting was performed as previously reported (5) after preparing the homogenate in a commercial solution (TeSeE Western Blot; BIO-RAD, https://www.bio-rad.com). Western blots were developed by using mouse anti-prion protein monoclonal antibody 6H4 (ThermoFisher Scientific, https://www.thermofisher.com) at a 1:10,000 dilution.

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