Chronic Wasting Disease (CWD) Susceptibility of Several North American Rodents That Are Sympatric with Cervid CWD Epidemics[▽]

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Received 18 March 2009/Accepted 6 October 2009

Chronic wasting disease (CWD) is a highly contagious always fatal neurodegenerative disease that is currently known to naturally infect only species of the deer family, *Cervidae*. CWD epidemics are occurring in free-ranging cervids at several locations in North America, and other wildlife species are certainly being exposed to infectious material. To assess the potential for transmission, we intracerebrally inoculated four species of epidemic-sympatric rodents with CWD. Transmission was efficient in all species; the onset of disease was faster in the two vole species than the two *Peromyscus* spp. The results for inocula prepared from CWD-positive deer with or without CWD-resistant genotypes were similar. Survival times were substantially shortened upon second passage, demonstrating adaptation. Unlike all other known prion protein sequences for cricetid rodents that possess asparagine at position 170, our red-backed voles expressed serine and refute previous suggestions that a serine in this position substantially reduces susceptibility to CWD. Given the scavenging habits of these rodent species, the apparent persistence of CWD prions in the environment, and the inevitable exposure of these rodents to CWD prions, our intracerebral challenge results indicate that further investigation of the possibility of natural transmission is warranted.

Chronic wasting disease (CWD) is a contagious transmissible spongiform encephalopathy (TSE), or prion protein (PrP) disease, naturally transmitted among members of the deer family *Cervidae* (29). The putative infectious agent of prion diseases is a misfolded isoform of the PrP referred to as PrP^d that typically possesses unusual resistance to protease degradation. A hallmark of prion diseases is the accumulation of PrP^d in the brain, along with associated pathologies. CWD epidemics are occurring in free-ranging cervid populations at several locations in North America and involve several *Cervidae* species/subspecies (deer, Rocky Mountain elk, and moose [*Odocoileus* spp., *Cervus elaphus nelsoni*, and *Alces alces*, respectively]) (3, 29). In cervids, the potential for direct transmission via saliva has been demonstrated (13, 21), and indirect transmission via environmental contamination has been observed (22). Once in the environment, the agent remains infectious to cervids for extended periods (22). Secretions, excreta, and the tissues of dead animals are potential sources for environmental contamination; their relative importance is unknown. Other wildlife species are inevitably exposed to infectious material in the environment (25).

To examine the potential for noncervid species to support CWD transmission, we intracerebrally challenged four species of native North American rodents: meadow voles (*Microtus pennsylvanicus*), red-backed voles (*Myodes gapperi*), white-footed mice (*Peromyscus leucopus*), and deer mice (*P. maniculatus*). These species all occur in locations undergoing cervid CWD epidemics (Fig. 1), and their opportunistic scavenging behaviors make exposure to infectious material highly likely.

We selected meadow voles because early studies by Chandler and Turfrey (6, 7) demonstrated efficient intracerebral transmission of scrapie to the field vole *Microtus agrestis*. Agnini's group (1, 10) reported similar findings for scrapie transmission into the European bank vole *Myodes glareolus*, motivating us to look at the North American red-backed vole, which some consider to be a semispecies with respect to bank voles (11). We included *Peromyscus* spp. because of their abundance and likelihood of coming into contact with infected cervid tissue in the field.

MATERIALS AND METHODS

Animals and challenges. The voles came from colonies maintained at the USGS-National Wildlife Health Center (Madison, WI). The *Peromyscus* spp. were obtained from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC). All live animal procedures were reviewed and approved by the USGS-National Wildlife Health Center Animal Care and Use Committee.

Inocula (10% [wt/vol] obex tissue in phosphate-buffered saline [PBS]) were

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[▽] Published ahead of print on 14 October 2009.

FIG. 1. Overlap of rodent distributions and CWD epidemics. Black dots show known locations of CWD in free-ranging cervids, and the green is the rodent's distribution. (A) Meadow voles; (B) red-backed voles; (C) white-footed mice; (D) deer mice.

prepared from individual hunter-harvested CWD-positive white-tailed deer (*O. virginianus borealis*) from southern Wisconsin (17, 18). To be used for an inoculum, an obex had to show relatively uniform immunohistochemistry (IHC) staining for PrP^d throughout the cross-section (18). Deer in Wisconsin that possess the G96S polymorphism of the gene coding for PrP have a reduced incidence of CWD infection (16). We prepared two inocula from two deer with the homozygous G96G/G96G (GG) genotype and two inocula from two deer with the heterozygous G96S/G96G (GS) genotype to assess the genotype effect on transmission. Each inoculum was prepared from a single deer source; no pooling was performed. The inoculum bolus was adjusted to approximately reflect differences in body weight: 20 μ l for meadow voles and 10 μ l for the other species. To serve as controls, several animals of each species were inoculated with homogenate prepared from the obex tissue of a CWD-negative deer. Second passages were performed in a similar manner, with 10% inocula prepared from single representative animals shown to be positive in the first passage challenges.

Voles have a strong burrowing habit and needed to be excavated for observation. We strove to minimize disturbances that would stress the animals but tried to directly observe each animal at least weekly, and the monitoring frequency was increased upon the onset of clinical signs. Animals were euthanized when they exhibited significant clinical impairment; they were typically markedly lethargic and appeared to have generally lost motivation for acquiring food and water. A few animals developed health problems within 14 days postchallenge and were not included in the study.

Diagnostic procedures: immunoblots and IHC. PrP^d was tested for by either immunoblotting or IHC or both in almost all endpoint animals, the few exceptions being due to tissue quality problems. Briefly, for immunoblotting a 20% (wt/vol) brain homogenate was prepared in PBS. Brain homogenate was diluted 1:1 in PBS and digested with proteinase K (PK; 50 μ g/ml, final) for 60 min at 37°C, after which Pefabloc SC (2 mM, final; Roche Diagnostic) was added. Digested homogenates were incubated at 100°C for 10 min with NuPage (Invitrogen) sample buffer (2 $\times$, final) and reducing agent (1 $\times$, final) and then centrifuged 2 min at 13,000 rpm. The samples were run on a 12% NuPage Bis-Tris gel (50 min at 200 V) and then transferred to a polyvinylidene fluoride membrane (60 min at 25 V). The primary probe was monoclonal antibody (MAb) SAF83 (Cayman), and the secondary antibody was sheep anti-mouse immunoglobulin G conjugated with horseradish peroxidase (GE Healthcare), both diluted 1:10,000 in Tris-buffered saline and Tween 20. Visualization was performed with an Amersham ECL Western blotting analysis kit using 5- to 10-min exposures on Amersham Hyperfilm ECL (GE Healthcare) or EC3 Imaging System (UVP). The same procedure was used for the deer inocula except for using MAb 6H4 (Prionics) as the primary probe.

For IHC, whole brains or hemispheres were fixed for ≥ 48 h in 10% buffered formalin, embedded in paraffin, sectioned to 5 μ m, and mounted on slides. After deparaffinization, the procedures used previously were followed (14) with minor modifications. Antigen retrieval was performed in three steps using hydrated autoclaving treatments in citrate buffer (1 h), formic acid (88%, 10 min), and guanidine isothiocyanate (4 M, 2 h). This was followed by inactivation of endogenous peroxidases (3% H₂O₂, 5 min) and then blocking with normal horse serum (1%, 1 h). Sections were then incubated overnight at 4°C with MAb SAF83 (1:3,000). Visualization was performed with biotinylated horse anti-mouse immunoglobulin G, avidin-biotin-horseradish peroxidase, and NovaRed substrate

according to the manufacturer's instructions (Vectastain ABC Elite kit; Vector Laboratories). Sections were counterstained with Harris hematoxylin (Fisher HealthCare), dehydrated, and coverslipped.

Glycoform analysis. TIFF-formatted immunoblot images were imported into NIH ImageJ software (9). Representative lanes were examined; lanes that were very faint with respect to background were not used. The ImageJ gel analysis tools were used to obtain digitized optical density plots. These plots were modeled as mixtures of three Gaussian (normal) densities to obtain glycoform proportions. Specifically, the optical density at mobility x was modeled as: $f(x) = c[p_d\phi_d(x) + p_m\phi_m(x) + p_u\phi_u(x)]$, where the subscripts d , m , and u refer to diglycosylated, monoglycosylated, and unglycosylated, respectively, p_i is the glycoform proportion for glycoform i , $\phi_i(x)$ is the Gaussian probability density function for glycoform i , and c is a scaling constant. The glycoform proportions were constrained to sum to 1. In addition to the glycoform proportions p_i , which were the parameters of primary interest, it was necessary to estimate c and the parameters of the Gaussian probability density functions μ_i and σ_i^2 as well. Parameter estimation was performed with a program written in the Bayesian WinBUGS programming language (20). The model $f(x)$ appeared to generally give excellent fits to the density plots. Glycoform percentages were displayed in ternary plot, or triplot, format (12). The results for individual immunoblot lanes are displayed to give a meaningful representation of variability.

Prion protein gene (PRNP) sequencing. We isolated genomic DNA from skin or muscle tissue by using the DNA IQ system (Promega) according to the manufacturer's instructions. PCR and sequencing conditions were as described previously (15). Direct sequencing of meadow voles was complicated by the sporadic occurrence of pseudogenes, the sequencing and analysis of which we will describe in detail in a future publication. Briefly, we started by identifying conserved sequences of the related species *Myodes glaeolus* (GenBank accession no. EF45012) and *Microtus agrestis* (GenBank accession no. AF367625) to construct primers AACTGGTGAAGCCGATA and GCTTGAGGTGATGT TGACG, which after amplification and sequencing led to the construction of new step-down PCR primers (30). After trial-and-error, we found the primers GAGGAGGGCTGCACCATGACTATCACA and GGGCGGCTTCCCTCA TCCCCTATCAG efficiently amplified the ORF of meadow voles and red-backed voles and performed satisfactorily on the *Peromyscus* spp. Only meadow voles appeared to have pseudogenes, so these primers were sufficient for the other species. For meadow voles, the primers CCCTGACTGGACCCTGACTC and GCATTAGCCTATGGGGGACA were constructed to amplify only the expressed gene using conserved 5' untranslated region sequence. Lasergene software (DNASTAR, Inc.) was used for primer design and sequence analysis. For analysis here, we focused on amino acid (aa) polymorphisms of the mature PrP protein (aa 23 to 231) only.

RESULTS

Disease transmission and progression. In all rodent species, the initial clinical sign of disease was usually a very subtle waddle. As time progressed, the animals became less agile, less able to maintain balance and kyphotic, and their fur became dull and poorly groomed. In the most advanced state, the animals were quite lethargic and unresponsive, but they were generally euthanized before this point. The presentation of the disease phenotype was quite variable from animal to animal. Some displayed gradual, subtle declines that spanned 4 or more weeks, with brief, apparent rallies. Others progressed from apparently healthy sign-free states to end-stage disease in a matter of days.

CWD was efficiently transmitted to all species. Meadow voles proved to be the most susceptible, with the earliest presentation of disease signs after about 7 months and with 100% mortality by 374 days and a median survival of 270 days (Fig. 2). Of 18 tested for PrP^d, all were positive. Red-backed voles exhibited a moderately increased incubation period with a median survival time of 351 days. Of the seven animals tested for PrP^d, only one did not have detectable PrP^d, which was also the longest survivor (552 days). Deer mice exhibited a median survival time of 595 days; 1 of the initial 20 has not yet suc-

FIG. 2. Survival curves for CWD-challenged meadow voles (black; $n = 18$), red-backed voles (red; $n = 10$), deer mice (blue; $n = 20$), and white-footed mice (green; $n = 17$). Of 53 endpoint animals tested for PrP^d by either immunoblotting or IHC, only 4 had undetectable levels: 1 red-backed vole and 3 white-footed mice. The genotypes of the four deer used for the inocula did not appear to have any effect on the incubation distribution (square = GG, circle = GS; an asterisk [*] means the observation was censored).

cumbed (>1,035 days). Of the 17 tested for PrP^d, all had detectable PrP^d. White-footed mice exhibited a median survival time of 786 days; 4 of the initial 17 have not yet succumbed (>931 days). Of the 11 white-footed mice tested for PrP^d, 3 did not have detectable PrP^d, for which the survival times were 347, 762, and 799 dpi. Several control-challenged animals of each species have been tested and PrP^d was consistently not detected. The inoculum genotype, GG or GS, did not have any consistent association with the time until endpoint (log-rank statistic = 2.33, $P = 0.51$; Fig. 2).

Second passages were performed for meadow voles ($n = 12$), deer mice ($n = 8$) and red-backed-voles ($n = 6$). For meadow voles, the median survival time shortened by ca. 30% upon second passage, from 270 to 190 dpi. For deer mice, the median survival time shortened by ca. 43% upon second passage, from 595 to 340 dpi. For red-backed voles, the median survival time shortened by ca. 35% upon second passage, from 351 to 226.5 dpi. Second passages have not yet been performed with white-footed mice.

Immunoblots/glycoform analysis. Immunoblots of the deer inocula showed strong staining for PrP^d (Fig. 3). There were no conspicuous differences due to the deer's genotypes, either with respect to general overall concentration, glycoform proportions, or glycoform mobility.

Immunoblot staining for PrP^d in the challenged rodents was

FIG. 3. Immunoblots of four of the white-tailed deer inocula used for challenges. Each inoculum used obex tissue from only a single deer. Each inoculum is shown without (-PK) and with (+PK) PK digestion. The two inocula on the left are from deer with the GG PrP genotype, and the two on the right are from deer with the GS (resistant) genotype. No consistent differences are obvious.

FIG. 4. Representative immunoblots for white-footed mice (PI), deer mice (Pm), red-backed voles (Mg), and meadow voles (Mp). The two left-most lanes are for a meadow vole that was challenged with inoculum prepared from CWD-negative deer brain tissue. The right control lane was digested with PK (+PK), and the left control lane was not (-PK). The other eight lanes are for animals that received inoculum from CWD-positive deer; these lanes were PK digested, and all show the presence of PK-resistant PrP^d. Within each species, the left column labeled GS is for a subject that received inoculum from a CWD-positive deer with the GS genotype; the right column is a subject that received the GG genotype.

generally strong; we observed the characteristic increase in mobility of PrP^d after PK digestion (Fig. 4). The immunoblot banding patterns showed no conspicuous differences with respect to either the rodent species or the inoculum genotype (GG or GS; Fig. 4). Among the numerous animals tested, PrP^d was never detected in immunoblots of animals that received control inoculum prepared from a CWD-negative deer (Fig. 4).

In the deer used for inocula and all first passage rodents, the immunoblots displayed the ordering of PrP^d glycoform proportions as diglycosylated > monoglycosylated > unglycosyl-

FIG. 5. Glycoform proportions for a sample of first-passage (A) and second-passage (B) subjects. Symbols: red square, deer used for inoculum; black circle, meadow vole; blue star, red-backed vole; green plus, white-footed mouse; purple diamond, deer mouse. The solid black dot is for reference; it is the centroid for the monoglycosylated-dominated phenotype of mouse-adapted 139A scrapie expressed in murid hosts (from Fig. 2 of Agrimi et al. [1]).

FIG. 6. PrP^d deposition in the thalamus. (A) Meadow voles; (B) red-backed voles; (C) white-footed mice; (D) deer mice. The scale is 10 μ m.

ated (Fig. 5A). It appears that there might be some subtle clustering by species; this requires a larger sample to resolve. The general patterns observed were consistent with those observed in other cricetid rodents challenged with either CWD, scrapie, or mouse-adapted 139A scrapie (1, 24) and contrasts with the *Muridae* (Old World rats and mice, gerbils) 139A pattern of monoglycosylated > diglycosylated > unglycosylated (1). The first passage patterns appeared to be preserved upon second passage (Fig. 5B).

IHC. PrP^d IHC staining was intense in all of the examined brains. Staining was found in most regions of the brain, but there was a good deal of local variability in staining between animals. The thalamus was one region of the brain that was heavily labeled in all species (Fig. 6). Compared to the other rodents, which have punctate and diffuse patterns of staining (Fig. 6A to C), deer mice (Fig. 6D) consistently presented with larger, more well-defined plaques. Control animals from all species had little PrP immunostaining and no PrP plaques (not shown). We performed both immunoblots and IHC for a number of controls and subjects for all species; the results were always consistent, and we observed no false negatives or false positives.

Sequencing. Our amino acid number convention follows that of Kurt et al. (19), which shifts *Microtus* sequences right by 1 at aa 56 to account for a deletion. For mature PrP (aa 23 to 231), the most common amino acid genotype for our meadow voles (GenBank accession no. GQ850539.1) matched the genotype reported for the European field vole *Microtus agrestis* (1; GenBank accession no. AF367625.1) with the exception that field voles have a histidine at aa 230, whereas meadow voles have a tyrosine. Most of our sequenced meadow voles were homozygous glycine/glycine at aa 64, but two were heterozygous glycine/glycine. These animals survived until dpi 245 and 270; there was no suggestion that this polymorphism influenced survival (log-rank statistic, $P = 0.25$). No amino acid polymorphisms were observed within the red-backed voles. The mature PrP amino acid sequence for our red-backed voles (GenBank accession no. GQ850538.1) matched that for Euro-

pean bank voles (1; GenBank accession no. AF367624.1) with the exception that bank voles have asparagine (N) at aa 170, whereas our challenged red-backed voles had a serine (S).

This is noteworthy because aa 170 has been hypothesized to play a pivotal role in the trans-species transmission of prion diseases (1, 8, 19), and a serine at aa 170 appears previously unprecedented in a rodent from the family *Cricetidae*, which includes hamsters, voles, and New World rats and mice (1, 19). For independent confirmation of this unusual result, we sent samples from five nonstudy animals to a sequencing service (GeneTyper, New York, NY). We specified the primer sequences GTGGAACAAGCCCAGTAAGCCAAA and ATG GTGATGTTGACGCAATCGTGC from Kurt et al. (19). The two animals with pure Wisconsin parentage returned SS at aa 170. The one animal from north-central Pennsylvania returned NN. Two F₁ Wisconsin/Pennsylvania hybrids returned SN. All of our challenged red-backed voles were of Wisconsin descent. Our *Peromyscus* spp. came from the same source as those in the study by Kurt et al. (19), so our sequences (*P. maniculatus* GenBank accession no. GQ850542.1; *P. leucopus* GenBank accession no. GQ850543.1) matched theirs (GenBank accession no. FJ232958.1 and FJ232959.1), although ours were more complete. In light of the role conjectured for aa 155 (1), it is noteworthy that *P. leucopus* had a tyrosine (Y) at aa 155, whereas the other three species had asparagine. As noted, only red-backed voles had a serine at aa 170, whereas the other three species had asparagine. When the species' genotypes are ranked by their median survival times, we see 155N/170N < 155N/170S < 155N/170N < 155Y/170N (meadow vole < red-backed vole < deer mouse < white-footed mouse).

DISCUSSION

CWD was efficiently transmitted to the species we tested via intracerebral challenge. For voles the incubation duration was comparable to that for transgenic mice (*Mus musculus*) engineered to express white-tailed deer PrP (27). The onset of clinical disease was generally delayed in the *Peromyscus* spp. relative to the voles. The two species of *Peromyscus* we examined are noteworthy for their long maximum life spans of ~8 years. Various protective cellular mechanisms have been proposed for this longevity (28); perhaps similar mechanisms help delay the onset of prion disease. Experience with our breeding colony suggests the life span for voles is about 3.5 to 4 years.

The shortening of median survival times upon second passage indicates the occurrence of adaptation to its new host. Glycoform proportions remained the same from the first to the second passage, maintaining its "CWD phenotype." Despite the unusual (for a cricetid rodent) serine at aa 170, red-backed voles displayed a disease onset phenotype intermediate to meadow voles and *Peromyscus* spp.

Our in vivo results are generally consistent with the Kurt et al. Protein misfolding cyclic amplification (PMCA) results for *Microtus* sp. and *Peromyscus* spp. (19). It has been suggested that asparagine at aa 170 supports trans-species amplification of PrP^{CWD} (19) via the "rigid loop" hypothesis (26). It has been noted that ferrets, which have a serine at aa 170 but which support amplification, are an exception, but this was explained by a unique leucine at aa 176 (19). Christen et al. (8) specifi-

cally suggested that bank vole susceptibility to TSEs arises from a cervid-like structured loop resulting from an asparagine at aa 170. Red-backed voles, which are very closely related to bank voles (11) and which have the aa 170 to 176 sequence SNQNNF consistent with species observed to not support amplification (19), are an interesting contradiction. Our results clearly demonstrate that the aa 170 to 176 segment alone is not sufficient to substantially suppress in vivo amplification. Agrimi et al. (1) suggest that 155Y 170S leads to in vivo scrapie resistance and that 155N 170N leads to in vivo scrapie susceptibility, but in vitro results are contradictory (23). Clearly, aa 155 and aa 170 appear to be important, but their effects do not seem to be consistent from species to species or in vivo versus in vitro. As Agrimi et al. note (1), this suggests an important role for species-specific cofactors in addition to a PrP genotype effect.

During the course of our challenges, Agrimi's group noted that elk CWD was readily transmitted to European bank voles *Myodes glareolus* (2). All other in vivo studies to date (4, 5, 24) suggest CWD transmission to rodents is inefficient. Transmission to nontransgenic lab mice (*Mus musculus*) was quite inefficient (5). Of various hamster species challenged, only Chinese hamsters (*Cricetulus griseus*) acquired CWD with more than very modest efficiency and then only with elk and mule deer CWD (4, 24). Curiously, no transmission occurred with white-tailed CWD (24). During the course of our challenges Agrimi's group also observed that scrapie was efficiently transmitted to a European *Peromyscus* species, *P. polionotus*, via the intracerebral route (1).

In light of our findings, the possibility of natural transmission to rodents cannot be dismissed. This is concerning because of a TSE's ability to change its properties and host affinities after being passaged (4). Cannibalism and scavenging are common among small rodents, and small rodents are a very important food source for many predators and scavengers. Small rodent tissue also enters the domestic livestock and human food chain by accidental inclusion in grain and forage. Further investigation of these species as potential hosts, bridge species, and reservoirs of CWD is warranted. Even in its natural cervid hosts, the mechanisms of natural transmission and infection of CWD are not well understood. However, the ability to support amplification of PrP^d would seem to be a prerequisite, which all of our rodent species have demonstrated. We have initiated studies to examine the susceptibility of these rodent species via more natural routes of infection.

ACKNOWLEDGMENTS

We thank J. Lambert-Newman and the animal care staff at the USGS—National Wildlife Health Center, the staff of the Wisconsin Department of Natural Resources, C. Acker, J. Aiken, R. Bessen, C. Bunck, K. DeJesus, C. Dejoia, S. Dubay, E. Heisey, L. Heisey, D. McKenzie, S. Nolden, K. O'Connell, E. Osnas, and B. Richards.

Thanks to the staff at the Treehaven Field Station of the University of Wisconsin—Stevens Point for support in collecting red-backed voles. Rodent range data were provided by NatureServe in collaboration with Bruce Patterson, Wes Sechrest, Marcelo Tognelli, Gerardo Ceballos, The Nature Conservancy—Migratory Bird Program, Conservation International—CABS, World Wildlife Fund—US, and Environment Canada—WILDSpace.

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