Low frequency of PrP genotype 225SF among free-ranging mule deer (Odocoileus hemionus) with chronic wasting disease

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The prion protein (PrP) gene was characterized in 1482 free-ranging mule deer (Odocoileus hemionus) from Wyoming and Colorado. Using DNA sequences from 363 deer, dimorphisms at codons 20 (aspartate/glycine) and 225 [serine (S)/phenylalanine (F)] were found; silent changes occurred at codons 131 (tyrosine) and 247 (isoleucine). The remaining samples were surveyed for codon 225 genotype and all were characterized for chronic wasting disease (CWD) infection status. A total of 112 deer with the genotype 225SF or FF were found, of which one was CWD-positive; 1370 were 225SS, with 289 positive for CWD. Among CWD-negative deer, the frequency of 225SF/FF genotypes was 9·3 % but among CWD-positive deer it was only 0·3 %. For all samples combined, CWD status was not independent of codon 225 genotype (P<0·0001). The odds that a deer of the 225SS genotype was CWD-infected were 30 times greater (95 % confidence intervals = 4–213) than for a 225SF deer. The proportion of 225SF animals in sampled subpopulations varied from 0 to 18 %; the CWD prevalence varied from 0 to 25 %. However, no relationship was observed between genotype frequency and CWD prevalence in different areas. The PrP sequences of experimentally infected mule deer were analysed from pre-existing projects and 10 animals were found with 225SF genotypes, all of which were positive for CWD. Data available from some of these animals suggest that the 225SF genotype could be associated with longer incubation periods in CWD infection compared with the 225SS genotype.

INTRODUCTION

Chronic wasting disease (CWD) is an infectious and fatal neurodegenerative disease of cervid species in North America (Williams & Young, 1980, 1982). One of the transmissible spongiform encephalopathies (TSEs) (Prusiner, 2004), also known as prion diseases, CWD occurs endemically and epidemically (Miller et al., 2000; Williams & Miller, 2002) in wild mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus) and Rocky Mountain elk or wapiti (Cervus elaphus nelsoni) in several states of the USA as well as in captive or farmed cervids outside these areas (Williams, 2002; Miller & Williams, 2004). Although CWD is not perceived as an immediate major threat to deer or elk populations, it is of concern and interest for several reasons: (i) the possibility that wild herds could be gradually destroyed by CWD (Gross & Miller, 2001) due to the persistence of the infectious agent in the environment (Miller et al., 1998, 2004; Williams et al., 2002) and the lethality of the disease; (ii) the unknown nature of circumstances, if any, under which this disease could affect a human; (iii) the greater understanding of human TSEs that could accrue by knowing more about related mammalian prion diseases that have not been well understood before; (iv) the economic impact of CWD on game farming; and (v) the possible spread of CWD to other members of the cervid family worldwide through unregulated commerce and transport of captive animals (Williams & Miller, 2003). In other acquired prion diseases, notably scrapie of sheep and variant Creutzfeld–Jakob disease (vCJD) in humans, amino acids encoded at certain key positions in the...
endogenous host prion protein (PrP) are strongly associated with susceptibility to TSE infection (Goldmann et al., 1994; Hunter et al., 1994; Collinge et al., 1996; O’Rourke et al., 1997). A study of CWD in Rocky Mountain elk (O’Rourke et al., 1999) suggested that the PrP sequence at position 132 influences likelihood of infection in that species. To what extent similar influences affect deer (Odocoileus spp.) has been unclear (Johnson et al., 2003; Brayton et al., 2004; O’Rourke et al., 2004). If heritable characters influence susceptibility to CWD in free-ranging mule deer, which are the most abundant of the three natural hosts within the Rocky Mountain endemic area (Miller et al., 2000), it could have important implications for understanding the epidemiology of this disease.

We investigated the possibility that PrP sequences may vary between CWD-infected and uninfected deer in the wild. To that end we determined PrP polymorphisms in select populations of free-ranging mule deer in Wyoming and Colorado, and estimated the relative abundance of one particular polymorphism among CWD-infected and uninfected animals. Specifically, we tested whether CWD status (positive or negative) was independent of genotype at codon 225, and constructed a regression model to test whether there was a population-level relationship between CWD prevalence and 225 genotype among the herds sampled. We also retrospectively analysed experimentally infected deer for PrP genotype to look for possible effects of genotype during the course of the disease in these animals.

**METHODS**

**Animal and tissue samples.** We genotyped 1482 free-ranging mule deer from 13 herd units (HUs) in south-eastern Wyoming and one data analysis unit (DAU) in Colorado (Fig. 1) and categorized them according to CWD infection status. For analytical purposes, we regarded both HUs and DAUs as representing biologically distinct deer populations with <10% ingress and egress between populations. Deer from Wyoming were sampled in conjunction with the Wyoming Game and Fish Department’s 2001, 2002 and 2003 CWD harvest surveys; tissues used were frozen brain (2001 and 2002) or homogenates of retropharyngeal lymph node (2003). For the Colorado samples, we extracted genomic DNA from whole blood samples obtained from live deer that were captured and released during 2002–2004 in the course of various Colorado Division of Wildlife research and management programmes.

**DNA extractions, PCR amplifications and sequencing.** We extracted total genomic DNA from approximately 30–50 mg frozen brain tissue using a FastPrep F120 homogenizer and FastDNA kit (Q-Biogene), from 0–5 ml of 15% w/v homogenates of retropharyngeal lymph nodes using a DNeasy kit (Qiagen), or from 0–3 ml EDTA-treated whole blood using an Aqua-Pure Genomic DNA kit (Bio-Rad Laboratories), all according to manufacturers’ instructions. The complete open reading frame (ORF) plus 25 bp of 5’ flanking sequences and 53 bp of 3’ flanking sequences in the PrP coding region were amplified using PCR in 50 µl reaction volumes containing 200 µM each dNTP, 2.5 units Taq polymerase (Qiagen), 200 nM each of a forward (MD582F, 5’TGGTGGGTGACTGTTGCTTGA-3’) and a reverse (MD1479RC, 5’AGAAGATAATGAAACAGGAGG-3’) primer, 2.5 mM MgCl₂, 1 × Qiagen PCR buffer and approximately 70–150 ng total genomic DNA. Reactions were thermal-cycled in a PTC 200 (MJ Research) at 94 °C for 5 min and then 32 cycles of 94 °C for 7 s, 62 °C for 15 s, 72 °C for 30 s and a final cycle of 72 °C for 5 min, and kept at 4 °C until inspected. Occurrence of the phenylalanine (F) codon TTC at position 132 in the PrP ORF (Fig. 2a) from 363 samples that included 244 mule deer (95 CWD-negative, 149 CWD-positive) from throughout the south-eastern quarter of Wyoming, and 119 mule deer (105 CWD-negative, 14 CWD-positive) from the Estes Valley, Colorado. We viewed sequence chromatograms and checked base calls using Chromas v.2.23 software (Technelysium Pty), and made alignments of DNA sequences for comparisons by using the SeqMan module of LaserGene software (DNASTar).

**Codon 225 genotyping by EcoRI digestion.** To increase sample size for statistical analysis, PrP genotypes of 1119 additional samples selected from HUs in the study areas were analysed by EcoRI restriction digestion of the PCR-amplified PrP region, produced as described for samples to be sequenced. This assay detects only the codon 225 polymorphism, which is the only one we found in mule deer PrP that encoded different amino acids in the mature polypeptide. Aliquots (10 µl) of completed PCR reactions were incubated with 10 U EcoRI (New England Biolabs) in a total volume of 12 µl containing 50 mM NaCl, 100 mM Tris/HCl, 10 mM MgCl₂, 0-025% Triton X-100 (pH 7.5) at 37 °C for 2–16 h followed by the addition of 2-5 µl 6 × concentrated gel loading solution (Sigma-Aldrich) per sample, and the inspection of products by agarose gel electrophoresis. Occurrence of the phenylalanine (F) codon TTC at...
position 225 creates the EcoRI recognition sequence and cleavage site GAATTC from codons 224–225, whereas the serine (S) codon TTC creates the DNA sequence GAATCC, which is not cut by EcoRI. When PCR products were incubated with EcoRI, the presence of a TTC codon at position 225 resulted in cleavage fragments of predictable sizes, and a search of the PrP ORF DNA sequence verified that no other sites were potentially transformable to GAATTC. When an EcoRI restriction fragment was digested with HaeIII, the presence of a HaeII site at position 225 results in cleavage fragments of predictable sizes, and a search of the PrP ORF DNA sequence verified that no other sites were potentially transformable to GAATTC with one base change, supporting the use of this as a valid genotyping method. Samples that showed evident cleavage were sequenced to verify the change.

**CWD status and prevalence.** The 1482 deer that were genotyped and tested for CWD were a subset of deer tested from the 2001–2003 CWD hunter harvest surveys in Wyoming or from 2002–2004 field studies in north-central Colorado (Wolfe et al., 2002, 2004). For Wyoming data, tissues from sampled deer were screened for CWD infection by immunohistochemistry (IHC) (Miller & Williams, 2002) at the Wyoming State Veterinary Laboratory, Laramie, WY (2001 and 2002 samples) or by CWD-ELISA (Bio-Rad Laboratories) at the Wildlife Disease Laboratory of the Wyoming Game and Fish Department with IHC confirmation of ELISA-positive samples (2003 samples). For Colorado data, IHC was performed on tonsil biopsies from live deer sampled from Estes Park during 2002–2004 (Wolfe et al., 2002, 2004). CWD prevalence for Wyoming mule deer HUs was estimated using data obtained from the Wyoming Game and Fish Department (H. Edwards, personal communication); CWD prevalence for Colorado DAU 10, which includes Estes Park, was from harvest samples taken during 2001–2003 by the Colorado Division of Wildlife (http://wildlife.state.co.us/CWD/).

**Statistical analysis.** Because this was essentially a case–control study, we used odds ratios rather than relative risk to analyse statistically the association between CWD status and genotype. In these analyses, we used diploid genotypes rather than allele frequencies to avoid artificially inflating sample sizes and potentially biasing estimates of $\chi^2$ and odds ratio statistics (Agresti, 1996; Sasieni, 1997). We combined genotype 225SF with 225FF due to the low proportion of the latter genotype in our samples (0.2%). We used 2×2 contingency tables to test whether CWD status (positive or negative) was independent of 225 genotype (PROG FREQ; SAS OnlineDoc 8, SAS Institute). If we failed to reject the null hypothesis, it would conclude that genotype was unrelated to CWD status. We conducted the analysis for all data combined and then for Wyoming and Colorado separately. We did not analyse the data by individual Hu or DAU because we lacked sample size at that level and could not meet the assumptions of the $\chi^2$ analysis.

To test for a relationship between CWD prevalence and frequencies of 225 genotypes among sampled populations, we conducted a regression model of logit-transformed CWD prevalence versus proportion of 225SF genotype (PROG GENMOD; SAS Institute). Our null hypothesis was that there was no relationship between CWD prevalence and proportion of 225SF genotype. Type III sum of squares was used to test our hypothesis (SAS Institute).

**Observations of experimentally infected mule deer.** Two pre-existing projects provided us with the opportunity to assess whether the course of CWD in captive, experimentally infected deer was different between 225SF and 225SS genotypic groups. From one project (E. S. Williams and others, unpublished data), survival time and genotype data were obtained for eight hand-reared mule deer fawns, which had been exposed to CWD at 3 or 4 months of age by a single oral dose of 1 g pooled brain homogenate as previously described (Sigurdson et al., 1999; Raymond et al., 2000). The animals were later euthanized upon presenting with severe clinical signs of CWD (Williams & Young, 1992), and late-stage CWD infection at death was confirmed by necropsy and IHC of fixed tissues. We isolated genomic DNA from blood samples collected during the course of the project, and obtained PrP sequences as described above. From the second project (M. W. Miller, unpublished data), we isolated genomic DNA from archived frozen tissues that were collected at necropsy from experimentally infected mule deer and obtained PrP sequences as described above.
RESULTS

Polymorphisms in PrP of free-ranging mule deer

DNA sequences of the protein coding region in the Prnp gene of 363 deer sampled from Wyoming and Colorado during 2001–2003 showed four recurring variable codons with two alleles each (Fig. 2), in agreement with polymorphisms previously reported for mule deer PrP (Heaton et al., 2003; Brayton et al., 2004). Of these, only codon 225 [serine (S)/phenylalanine (F)] encoded different amino acids in the mature polypeptide (Fig. 2a). A single base change in codon 20 created a substitution polymorphism (aspartate/glycine) in the amino-terminal signal sequence that is removed during membrane translocation (Bendtsen et al., 2004). Variations in codon 131 (tyrosine1/tyrosine2) and codon 247 (isoleucine1/isoleucine2) did not change the encoded amino acid. At all four loci, one allele was more abundant and one was infrequent [Fig. 2b(iv–v)]. A comparison of the genotypes found in CWD-positive and CWD-negative samples showed only one 225SF among the CWD-positive group. This suggested that the 225SF allele might be under-represented in CWD-infected deer, but the low frequency of 225F among all samples (allele frequency 0.033; SF/FF genotype frequency 6.3 %) and the non-random nature of the sample set made these data insufficient to determine if a significant bias existed. We therefore analysed 1119 additional samples from all units in the study area and categorized them according to CWD infection status. We used a simplified method of DNA analysis to determine only the codon 225 genotype in these samples (Fig. 3).

Statistical analysis

The results, which are presented in Table 1, continued to show lower than expected numbers of 225SF genotypes among CWD-infected animals. \( \chi^2 \) analysis performed with all data combined returned the result that CWD status was not independent of codon 225 genotype \( (P<0.0001) \). In a total sample of 290 free-ranging, CWD-positive mule deer, we observed only one 225SF individual (0.3 %); 22 were expected based on the overall genotype frequency (7.6 %) in the sample. The odds that a deer of the 225SS genotype was CWD-infected were 30 times greater (95 % confidence intervals = 4–213) than for deer of the 225SF genotype. This pattern also held when Wyoming \( (P<0.0001) \) and Colorado \( (P=0.012) \) samples were analysed separately. For the Wyoming samples, the odds that a deer of the 225SS genotype was CWD-infected were 16 times greater (95% confidence intervals = 2–119) than for deer of the 225SF genotype; for the Colorado samples the odds ratio was 13 (95 % confidence intervals = 1–209).

With odds ratios this high, we considered it possible that a relationship might be detected between CWD prevalence and genotype frequencies. If the 225SF genotype provided a degree of resistance to CWD infection, then areas with high 225SF frequency might be expected to show low CWD prevalence. Alternatively, if CWD provided selective pressure for the resistant genotype, then the SF genotype might be more common in areas of high prevalence. However, neither relationship was observed. Although both the proportion of 225SF individuals and CWD prevalence varied among the mule deer populations sampled (Table 1), the slope of the regression model shown in Fig. 4 was not significantly different from 0 \( (P=0.228) \).

Observations of experimentally infected mule deer and PrP genotypes

Retrospective sequence analysis of DNA from two groups of experimentally infected captive mule deer revealed 10 225SF deer, as well as some differences in disease development that appeared to group according to codon 225 genotypes. In the first experimental group, six of the eight deer were of the 225SS genotype and two were 225SF. All eight were CWD-infected. The time until development of severe clinical CWD in the 225SS deer ranged from 19 to 23 months post-inoculation (p.i.), and the two 225SF deer developed severe symptoms at 36 months p.i. In the second group of deer, we identified eight 225SF animals, all of whom had been CWD-infected. Preliminary results for a small number of deer infected for 16 months or longer indicated that PrP\(^\text{CWD} \) was detectable by CWD-ELISA in brain tissue by 16 months p.i. in 225SS deer but not until >25 months p.i. in 225SF deer (M. W. Miller and others, unpublished data).
Low PrP genotype 225SF frequency in mule deer with CWD

**DISCUSSION**

**Genetics or geography?**

Our data show a large deficit of the 225SF genotype among CWD-infected mule deer in the wild, demonstrating that genotype and CWD infection are strongly associated and also implying a low probability of CWD infection among free-ranging 225SF deer. It cannot be ruled out that this might reflect a low probability of exposure to CWD for 225SF animals due to possible distribution characteristics of mule deer population units in the endemic area such as chance clustering of related 225SF deer away from highly localized infectious sites or animals. The low percentage of 225SF genotypes relative to 225SS genotypes combined with a possibly limited total area actually containing infection sources could also reduce exposure probability. Alternatively, the lower than expected occurrence of 225SF genotypes among free-ranging CWD-infected animals could be due to a lower susceptibility of those deer to natural CWD. Although 225SF deer are fully susceptible to experimental challenge, orally exposed captive mule deer with the SF genotype were slower to develop clinical symptoms of CWD, and neuroinvasion by PrP<sub>CWD</sub> took place later than in 225SS deer. This SF genotype effect could manifest as slower disease development (lengthened incubation period) in experimental CWD, and as lower probability of infection in the wild, although these may not necessarily be manifestations of the same phenomena. Lengthened survival times and apparently slower disease development have also been observed in experimentally infected elk with PrP codon 132ML and 132LL genotypes (T. J. Kreeger and others, unpublished results).Comparable observations are difficult to make for natural infections among free-ranging animals, and a test of the relative susceptibility of

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**Table 1. CWD prevalence and genotype data by deer management unit**

Estimated CWD prevalence by HU and DAU in harvest samples taken during 2001–2003 from Wyoming (Wyoming Game and Fish Department) and Colorado (http://wildlife.state.co.us/CWD/). Proportion of 225SF in the genotyped samples by HU and DAU, and observed (Obs.) and expected (Exp.) numbers of 225SF per unit among CWD-positive samples are also shown.

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<th>State</th>
<th>HU or DAU</th>
<th>No. CWD tested</th>
<th>CWD prevalence (%)</th>
<th>No. genotyped</th>
<th>Proportion of 225SF (%)</th>
<th>No. CWD-positive genotyped</th>
<th>Obs. no. CWD positive 225SF</th>
<th>Exp. no. CWD positive 225SF</th>
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*Includes three 225FF animals.
†Proportion of all genotyped deer used in this study that were 225SF or 225FF (112/1482).
§7.6 % x 290.

**Fig. 4.** Regression of logit-transformed CWD prevalence versus proportion of 225SF genotypes by HU or DAU for mule deer in Wyoming and Colorado. CWD prevalence based on harvest samples taken during 2001–2003 from Wyoming (Wyoming Game and Fish Department) and Colorado (http://wildlife.state.co.us/CWD/). Proportion of analysed samples with 225SF genotype from each HU or DAU as given in Table 1. CL, Confidence limits.
225SF deer to CWD, in the sense of amount of infectious agent required for infection, have not been carried out.

Nevertheless, the precedent of genetic factors affecting susceptibility to infectious prion diseases is well-established (Prusiner et al., 2004). The high odds ratios reported here for a possible genetically linked differential susceptibility to CWD in mule deer are consistent with the high degree of influence of genotype reported for scrapie in sheep (Hunter, 1997; Detwiler & Baylis, 2003). Some differences between genetics of scrapie in sheep and CWD in cervids are notable: CWD is unique as a TSE that occurs in wild populations subject to genetic drift and natural selection, rather than in selectively bred domestic animals. Also, the number of polymorphic codons in deer and elk PrP [two in mule deer, one in elk and three in white-tailed deer, all dimorphisms (Raymond et al., 2000; Heaton et al., 2003; Johnson et al., 2003; O’Rourke et al., 2004)] is low compared with the number of different amino acid positions that vary and the number of alleles that occur at these positions in domestic sheep breeds worldwide (Bossers et al., 2000; Baylis & Goldmann, 2004). It should be noted that the odds ratios relating 225SF genotype and CWD in mule deer stand in contrast to the low odds ratios previously reported by O’Rourke et al. (2004) for genetic susceptibility to CWD in white-tailed deer based on the three different polymorphisms found in that species.

Implications for CWD transmission

If the effects observed that suggest the 225SF genotype is associated with longer incubation times in experimentally infected deer also apply to deer with naturally acquired CWD, this could affect transmission of CWD in areas where there is a high proportion of the 225SF genotype among mule deer, if they become infected. Increased survival time for infected, asymptomatic animals might increase the period during which infectious agent is shed into the environment, although it should be pointed out that all but one of the 225SF deer in this study were not infected, rather than undergoing an asymptomatic, protracted subclinical phase. The possibility of ‘dormant infections’ or carrier state in TSEs has been raised in connection with scrapie (O’Rourke et al., 1997) and more recently with inapparent vCJD infections in PrP codon 129VM heterozygous humans and 129VV transgenic mice (Carrell, 2004; Wadsworth et al., 2004). Alternatively, slower development of CWD could mean 225SF deer require longer times of infection to reach the shedding stage. This situation could be neutral for transmission in the wild, or it could lead to a decrease in CWD prevalence due to infected deer dying of other causes before becoming infectious. This issue will remain unresolved until we learn when the infectious agent is shed during CWD infections and are able to measure its specific infectivity.

Genotypes 225SF and 225FF do not confer complete resistance

Even though the 225SF genotype may reduce susceptibility or affect the course of the disease, it clearly does not confer complete resistance. Experimental infection of 225SF deer appears to take place readily at the oral doses typically administered. In addition, we detected one free-ranging infected 225SF deer from Wyoming in our analysis, and subsequent genotyping of 234 additional CWD-positive samples from north-central Colorado revealed five 225SF individuals (M. W. Miller, unpublished data). It is also important to note that 225FF homozygous deer are not resistant, as evidenced by two 225SF deer also found among the additional positive samples genotyped. The 225F allele is present in such low frequencies in the deer herds sampled that free-ranging 225SF deer are not often found in surveys (e.g. only three of the 1482 samples used in this analysis were 225FF), and accurate assessment of their relative susceptibility to CWD will depend on work with animals currently being bred in captivity.

CWD prevalence and codon 225 genotype frequencies

If free-ranging 225SF mule deer are less likely to be infected with CWD than 225SS deer because of genetic causes, an increase of this genotype among deer populations over time could contribute to a decrease of CWD. Alternatively, selection against the 225SS genotype by high CWD prevalence could produce areas of both high disease prevalence and high frequency of the SF genotype. On an overall basis we saw no relationship between the proportions of SF animals estimated in HUs and the prevalence of CWD, but it may be necessary to compare proportions of SF and CWD prevalence in the same unit over time to detect such trends. Whether the time scales required for such observations would be feasible is not known. CWD is estimated to have been present in wild cervids for 50 years or more (Miller et al., 2000), so the time over which such effects could have been at work in CWD endemic areas may be too short to show reduced disease prevalence in areas of high SF frequencies. Also, the effect could be small compared with drift or mutation if CWD exerts only a low level of genetic selection because of its normally long incubation period, which allows for continued reproduction or genetic replacement, despite disease status. The fact that we did not observe a relationship may also be due to the scale at which our observations have been made (i.e. HUs), which are large relative to the population units most affected by variations in factors involved in the disease, including genetics (Conner & Miller, 2004).

Variable distribution of the 225F allele

The proportion of 225SF genotypes among sampled HUs in this analysis varied from 0 to 18%, with the highest frequency found in DAU 10 (Table I) and the lowest frequencies throughout the Wyoming HUs. An additional
203 Colorado samples that were genotyped only from DAU's 7, 9 and 16 west and south of D-10, showed 225SF sample proportions of 3, 11 and 22%, respectively (J. E. Jewell, unpublished data). This widely variable frequency of the 225SF allele could be localized founder effects that are a legacy of demographic bottlenecks created in the historic past by intense market hunting of this species. An intriguing alternative is that it does reflect selection of genotype by CWD (directional or balancing) without detectable trends in disease prevalence, or is an example of population genetic differentiation such as isolation by distance. Characterization of the mule deer population genetic structure using frequency data for selectively neutral loci such as microsatellites should allow us to distinguish the underlying causes of this variability.

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