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PrP genotypes of captive and free-ranging Rocky Mountain elk (*Cervus elaphus nelsoni*) with chronic wasting disease

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The PrP gene encodes the putative causative agent of the transmissible spongiform encephalopathies (TSEs), a heterogeneous group of fatal, neurodegenerative disorders including human Creutzfeldt–Jakob disease, bovine spongiform encephalopathy, ovine scrapie and chronic wasting disease (CWD) of North American deer and elk. Polymorphisms in the PrP gene are associated with variations in relative susceptibility, pathological lesion patterns, incubation times and clinical course of TSEs of humans, mice and sheep. Sequence analysis of the PrP gene from Rocky Mountain elk showed only one amino acid change (Met to Leu at cervid codon 132). Homozygosity for Met at the corresponding polymorphic site (Met to Val) in humans (human codon 129) predisposes exposed individuals to some forms of Creutzfeldt–Jakob disease. In this study, Rocky Mountain elk homozygous for PrP codon 132 Met were over-represented in both free-ranging and farm-raised CWD-affected elk when compared to unaffected control groups.

Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) of captive and free-ranging elk (*Cervus elaphus nelsoni*), mule deer (*Odocoileus hemionus hemionus*) and white-tailed deer (*O. virginianus*) in a small area of the western United States (Williams & Young, 1992; Spraker *et al.*, 1997). Clinical signs of CWD in elk include weight loss, behavioural changes, polydipsia and polyuria, excessive salivation and teeth grinding (Williams & Young, 1982). Pathological changes in the central nervous system include spongiform encephalopathy and neuronal degeneration (Williams & Young, 1993). CWD is a member of a heterogeneous group of fatal, neurodegenerative disorders characterized by accumulation of an abnormal isoform (PrP-Sc) of a cellular sialoglyoprotein (PrP-C) (Bolton *et al.*, 1982). PrP-Sc is a major component of infectious tissue extracts and is thought to be the transmissible agent, catalysing the conversion of PrP-C to PrP-Sc in the susceptible host (Prusiner, 1982). TSEs occur naturally in ruminant herbivores including sheep, goats, cattle, deer and elk. TSEs of mink and cats occur rarely, possibly from exposure to ruminant-derived infectious material (Marsh & Bessen, 1993; Bradley & Wilesmith, 1991). The human TSEs include a broad range of familial, sporadic and iatrogenic diseases, notably Creutzfeldt–Jakob disease (CJD) and a new variant of CJD (nvCJD) that probably originated from exposure to TSE-contaminated bovine products (Hill *et al.*, 1997; Bruce *et al.*, 1997).

Relative susceptibility to sporadic, iatrogenic and nvCJD is associated with the primary sequence of the host PrP-C. A nonpathogenic polymorphism at codon 129 of the human PrP

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gene encodes Met or Val. Homozygosity at codon 129 predisposes individuals to iatrogenic and sporadic CJD (Collinge *et al.*, 1991; Palmer *et al.*, 1991). nvCJD has been diagnosed to date only in 129 Met/Met homozygotes (Zeidler *et al.*, 1997). We have described a polymorphism (Met to Leu) at the equivalent PrP site in elk, cervid codon 132 (O'Rourke *et al.*, 1998). In this study, we determined the PrP genotypes of CWD-affected elk and of unaffected elk in several free-ranging and captive populations to determine whether this codon modulates susceptibility to CWD.

Methods

■ **Animals.** Tissues or peripheral blood samples were obtained from captive and free-ranging Rocky Mountain elk from several locations and were grouped by disease status, possible exposure to CWD and geographical location (Table 1). The ages of CWD-affected elk ranged from 17 months to 12 years. All unaffected farm-raised elk were adults over 2 years of age when tested; all free-ranging unaffected elk were over 15 months when sampled.

Group 1 included 20 CWD-affected elk born in free-ranging herds in north-central Colorado (CO) or southern Wyoming (WY). Three of the 20 elk were born in or near Rocky Mountain National Park, CO, in June 1986, and transported at approximately 1 week of age to a research facility. These three elk were diagnosed with CWD between May 1991 and February 1995 (Miller *et al.*, 1998). The remaining 17 elk were free-ranging animals from Larimer County, CO ($n = 12$) and southern WY ($n = 5$).

Group 2 consisted of 88 healthy, hunter-harvested elk from Larimer County, CO. The incidence of CWD in elk in this region, based on examination of brain tissue from hunter-harvested elk, is less than 1% (M. W. Miller, unpublished data). Group 3 consisted of healthy, hunter-harvested elk collected near Jackson, WY, an area with no reported cases of CWD. Brain tissue collected from elk in groups 2 and 3 showed neither histological nor immunohistochemical evidence of CWD.

Group 4 included live, healthy elk from South Dakota (SD) that were anaesthetized, radiocollared and released for a habitat evaluation project by the US Department of Agriculture, Forest Service, Rocky Mountain Research Station. Brain tissue will be collected if a radiocollared elk is subsequently hunter-harvested. There are no reported cases of CWD in free-ranging elk in SD. The elk in group 4 range the entire Black Hills region of SD and WY encompassing an area of 4500 square miles. This area includes a privately owned game farm with a high incidence of CWD (groups 5 and 6, as follows).

Groups 5 and 6 included farm-raised elk originating from a single SD facility that houses elk in several different subpopulations, grouped by sex, breeding status and age. CWD was diagnosed in an elk in this herd in 1997. Examination of archived tissues suggests that a case occurred as early as 1995. Forty-two cases have been confirmed subsequently. Frozen tissues or blood samples collected at the time of euthanasia were submitted from 23 elk subsequently diagnosed with CWD by histology and/or immunohistochemistry (group 5). The youngest CWD-affected animal was 17 months old when diagnosed; all other CWD-positive elk were 2 years of age or older. Blood samples were collected from 59 elk (group 6) which had been penned with the CWD-affected elk. Group 6 elk are alive at the time of this writing or were healthy and free of microscopic lesions and PrP-Sc immunostaining in brainstem tissue when culled.

Blood samples were collected from healthy, live elk from a farm in Minnesota with no history of CWD (group 7). These animals are observed regularly and necropsies will be performed on elk that are culled or die from injury or illness.

Samples were collected from 47 elk on a game farm in Montana (MT) (group 8). A single animal originating from this herd was diagnosed with CWD in 1998 after being housed in two facilities in other states between 1996 and 1998. CWD has not been diagnosed in any MT game farm animals or MT wildlife at this time.

Group 9 consisted of 30 samples from farm-raised elk originating in North Dakota and Nebraska and transported to a facility in SD. These animals shared a short length (approximately 150 m) of fenceline with a paddock housing 10 elk from the infected herd (groups 5 and 6). Elk were housed in these adjoining paddocks for 90 days. A separation of groups was re-established so that no fenceline contact was possible. Ninety days of separation had occurred when a group 5 elk died from CWD. The remaining nine elk from group 5 were removed from the facility. The 30 elk in group 9 remain in SD under quarantine with a mandatory CWD laboratory diagnosis on all adult cervid deaths. No cases of CWD have been diagnosed in this herd. Samples were collected from animals that are alive at the time of this writing or were free of histological lesions and PrP-Sc immunostaining when culled.

■ **CWD diagnosis.** Free-ranging affected elk were usually observed by local residents who reported dead or sick animals. Clinical signs included emaciation, loss of fear of humans, ataxia, inability to stand, dehydration, excessive salivation, drooping of the head and ears and

Table 1. PrP codon 132 genotypes of Rocky Mountain elk with CWD (groups 1 and 5) and unaffected control herds

Group	CWD status	No. sampled	132Met/Met	132Met/Leu	132Leu/Leu
1	Positive	20	20 (100%)	0	0
2	Negative	88	60 (68%)	26 (30%)	2 (2%)
3	Negative	55	44 (80%)	11 (20%)	0
4	Negative	42	35 (83%)	7 (17%)	0
5	Positive	23	17 (74%)	6 (26%)	0
6	Negative	56	20 (36%)	30 (54%)	6 (10%)
7	Negative	26	13 (50%)	12 (46%)	1 (4%)
8	Negative	47	29 (62%)	15 (32%)	3 (6%)
9	Negative	30	29 (97%)	1 (3%)	0

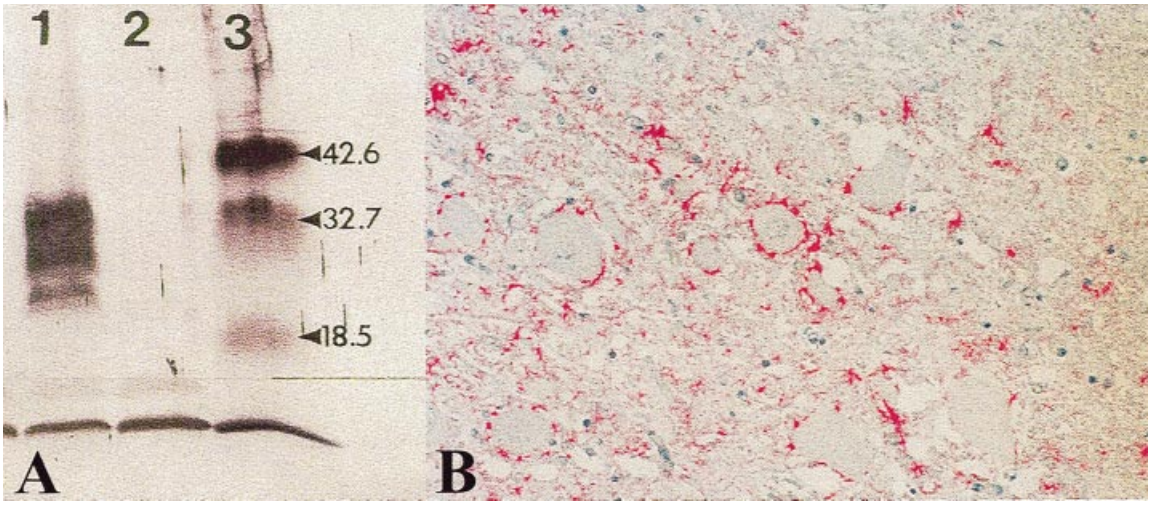

Fig. 1. (A) Western immunoblot, immunostained with polyclonal antisera to murine PrP-Sc, of proteinase-K-treated brain homogenate from a CWD-affected elk (lane 1) showing proteinase-K-resistant glycoproteins characteristic of TSE infection. Samples from a clinically normal elk (lane 2) processed under the same conditions show no immunoreactive proteins. Molecular mass markers (lane 3) in kDa. (B) MAb F89/160.1.5 immunohistochemistry assay of brainstem of a CWD-affected elk showing PrP-Sc antigen (red) accumulations within a brainstem nucleus.

rough dull haircoat. Captive elk were observed regularly by caretakers familiar with the animals' behaviour and euthanized when they exhibited clinical signs of CWD (Miller *et al.*, 1998). CWD was confirmed by histopathological lesions, most notably spongiform encephalopathy characterized by microcavitation, primarily of the grey matter, with single or multiple intracytoplasmic vacuoles in neuronal perikarya and neuronal degeneration (Spraker *et al.*, 1997; Miller *et al.*, 1998). Diagnosis was confirmed by immunohistochemistry using MAb F89/160.1.5 (VMRD and ABR) (O'Rourke *et al.*, 1998) (Fig. 1B) or rabbit polyclonal anti-PrP antiserum R78295, provided by R. Rubenstein to the National Veterinary Services Laboratories (Miller *et al.*, 1994). When fresh brain was available, tissues were examined for the presence of the disease-associated isoform of PrP (PrP-Sc or scrapie-associated fibrils) via negative stain electron microscopy (Guiroy *et al.*, 1991) or by Western immunoblot (Rubenstein *et al.*, 1986) using a polyclonal anti-mouse (ME7) serum provided by R. Rubenstein (Fig. 1A).

■ **PrP sequence analysis.** The amino acid sequence of the full-length PrP gene product was deduced from the DNA sequence of PCR-amplified genomic DNA, using PCR primers C.e.19fwd 5′ ATT TTG CAG ATA AGT CAT C 3′ and C.e.778rev 5′ AGA AGA TAA TGA AAA CAG GAA G 3′. These primers flank the open reading frame and are based on the DNA sequence of molecular clones of the mule deer PrP gene (O'Rourke *et al.*, 1998). This primer pair fails to amplify at least one mule deer PrP allele (unpublished data); therefore, sequence analysis of samples for this study was performed using internal primers spanning codons 24–243: forward primer C.e.bp70fwd 5′ TGC AAG AAG CGA CCA AAA CCT 3′ and reverse primer C.e.bp729rev 5′ CAC AGG AGG GGA GGA GAA GAG GAT 3′ in PCR buffer (Qiagen) using 2·5 mM Mg^{+2} (final concentration). Following a hot start (95 °C), samples were amplified using 10 cycles of 95 °C for 30 s, 59 °C for 30 s, 72 °C for 45 s, followed by 20 cycles of 95 °C for 30 s, 59 °C for 30 s, 72 °C for 60 s. The amplified products were sequenced by dideoxynucleotide chain termination by the Molecular Genetics Facility at the University of Georgia using sequencing primers C.e.315fwd 5′ CAG TAA ACC AAA AAC CAA C 3′ and C.e.583rev 5′ TGG TGG TGA CTG TGT GTT GCT TGA 3′, yielding sequence data for both strands between codons 112 and 180, for the sense strand only between codons 24 and 111, and for the antisense strand from codon 181 to 243.

■ **Statistical analysis.** The χ^2 test was used with the Yates correction for continuity to compare genotype frequencies of animals with and without CWD. For these analyses, animals homozygous for Met at codon 132 were compared with the combined frequencies of animals homozygous for Leu and heterozygous Met/Leu animals, in order to eliminate most instances of analyses containing cells with no observations or with more than 20% of the cells containing less than five expected observations, situations where the χ^2 test is considered unreliable. For those comparisons where there were less than five expected observations in one cell of a 2×2 table even after combining Met/Leu and Leu/Leu animals, genotype frequencies were compared by determining whether or not there was overlap between the exact 95% confidence intervals of both groups' collapsed genotype frequencies based on the binomial distribution. Genotype frequencies of CWD-affected elk born in the wild in CO and WY (group 1) were compared to those of CWD-affected elk of game farm origin (group 5). Genotype frequencies of the two CWD-affected groups were compared separately to their appropriate control groups. Group 1 elk, born wild in CO or WY, were compared to CWD-unaffected elk from herds in CO, WY and SD (groups 2, 3 and 4 pooled). Genotype frequencies of CWD-affected elk born and raised on game farms (group 5) were compared to unaffected elk from the same facility (group 6) and from pooled data from four game farms (groups 6, 7, 8 and 9). Genetic frequencies of CWD-affected elk in free-ranging and game farm populations compared using Fisher's exact test differed significantly ($P = 0{\cdot}026$).

Results

The PrP gene sequence of free-ranging Rocky Mountain elk in this study was highly conserved, encoding a single amino acid polymorphism (Met to Leu) at codon 132 and a silent change (aag to aaa) at codon 104. In three geographically distinct free-ranging populations (Table 1, groups 2, 3 and 4), the mean frequencies of the codon 132 genotypes Met/Met,

Met/Leu and Leu/Leu were 0·751, 0·238 and 0·011 respectively (Table 1). The frequencies of these three genotypes did not differ significantly among the groups. Therefore, data from groups 2, 3 and 4 were pooled for comparison with the genotypes of free-ranging, CWD-affected elk. All free-ranging elk with CWD (group 1, $n = 20$) were genotype Met/Met at codon 132. Thus, this genotype was significantly over-represented in the CWD-affected elk compared to clinically normal or CWD-negative free-ranging elk ($P < 0{\cdot}01$, exact binomial distribution).

Genotypic frequencies varied widely among the farm-raised, unaffected elk (Table 1, groups 6, 7, 8, 9). Codon 132 genotype frequencies were significantly different among the four captive elk herds sampled ($\chi^2 = 5{\cdot}8$, $P < 0{\cdot}05$). Pairwise testing showed that, with the exception of group 8, the genotypes in each herd differed significantly from the genotypes of the healthy free-ranging elk (pooled data from groups 2, 3 and 4) ($\chi^2 = 5{\cdot}8$, $P < 0{\cdot}05$). Therefore, the genotype frequencies of farm-raised elk with CWD were compared to non-affected elk from the same facility (group 5 vs group 6) rather than elk from the other game farms. The frequency of Met/Met elk with CWD differed significantly from that of non-affected elk from the same farm ($\chi^2 = 8$, $P < 0{\cdot}01$) (Table 1). Met/Met homozygosity was therefore significantly over-represented in the CWD-affected farm-raised elk at this facility.

Discussion

Conversion of PrP-C to PrP-Sc following exposure to exogenous PrP-Sc is thought to initiate the TSE disease process. The conversion mechanism is hypothesized to be a two step nucleation–polymerization reaction in which the rate-limiting step is the slow formation of the nucleus or seed (Come *et al*., 1993). Nucleation rate is dependent on the primary sequence of the monomer, homogeneity between native PrP-C and exogenous PrP-Sc at critical residues, and the concentration of heterogeneous peptides which favour dis-aggregation of the nucleus (Come & Lansbury, 1994; Priola *et al*., 1994). Human codon 129, encoding Met or Val, affects the relative susceptibility, pathological lesion profile and clinical course of several forms of CJD. Iatrogenic CJD, which probably arises from exposure to tissues from humans with sporadic CJD, occurs in all three codon 129 genetic groups. However, individuals of either homozygous genotype are more susceptible than heterozygotes (Collinge *et al*., 1991). Sporadic CJD, presumably arising from somatic mutation or low-level exposure to the transmissible agent environmentally, occurs most frequently but not exclusively in Met/Met129 homozygotes (Palmer *et al*., 1991). nvCJD, which presumably arises from exposure to PrP-Sc-contaminated products from cattle, has been reported only in Met/Met129 homozygotes at this time (Zeidler *et al*., 1997). The Rocky Mountain elk is the first non-human species with a reported polymorphism at the corresponding site (cervid codon 132) (O'Rourke *et al*., 1998).

In this study, we first analysed samples from free-ranging elk with CWD. All 20 cases were homozygous for Met/Met132, although the mean genotypic frequency of Met/Met132 was 0·75 in the free-ranging populations. Twenty-three cases of CWD in elk on a game farm in SD provided a second sample group. A total of 74% of the captive CWD-affected elk sampled were homozygous for Met/Met132 although only 47% of the sampled elk from this herd were Met/Met132 homozygotes. Taken together, these data suggest that Met/Met132 homozygosity predisposes exposed elk to CWD.

CWD was diagnosed in Met/Leu132 heterozygotes in the game farm population but not in free-ranging elk. This observation may be an artefact of small sample size of free-ranging CWD-affected elk and the low frequency of the 132Leu allele in free-ranging elk. Alternatively, this difference may reflect varying routes or doses of the transmissible agent under the game farm husbandry conditions or the presence of a novel CWD strain. Biochemical profiles of PrP-Sc from different populations and different genetic groups will provide additional insight into the range of CWD disorders in elk. The relationship between heterozygosity and incubation time in natural disease cannot be determined in CWD-infected herds because the time of infection is not known. However, two of the CWD-affected Met/Leu elk were diagnosed at age 2 years, indicating that heterozygosity is not invariably associated with prolonged incubation time.

The absence of CWD in the Leu/Leu132 elk population is of interest. Homozygosity for PrP Ala136 is associated with resistance to strain A scrapie in sheep (Goldmann *et al*., 1994*a*) and a single copy of the ovine PrP allele encoding Arg171 is sufficient to protect most sheep from strain C scrapie (Goldmann *et al*., 1994*b*). Insufficient numbers of elk with the Leu/Leu genotype were available to allow evaluation of their relative susceptibility to CWD. Extended observations on the surviving elk from the CWD-affected herd and experimental infection of Leu/Leu132 elk with infectious material from Met/Leu132 elk will be necessary to determine the relative resistance of Leu/Leu132 elk to CWD.

Selective breeding for TSE resistance is a valuable adjunct to epidemiology and diagnostic testing in control of sheep scrapie (Dawson *et al*., 1998) if the scrapie strains endemic in the population are known. Our data suggest that large proportions of many free-ranging and farmed Rocky Mountain elk populations are susceptible to CWD. The interaction of PrP genetic polymorphisms and CWD strain types in clinical disease progression, relative susceptibility, transmission efficiency and incubation time in elk warrants further investigation.

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