

# Patterns of PrP<sup>CWD</sup> accumulation during the course of chronic wasting disease infection in orally inoculated mule deer (*Odocoileus hemionus*)

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Patterns of abnormal prion protein (PrP) accumulation during the course of chronic wasting disease (CWD) infection were studied and the distribution and timing of disease-associated PrP (PrP<sup>CWD</sup>) deposition and lesions in 19 mule deer (*Odocoileus hemionus*) 90–785 days after oral inoculation were described. PrP<sup>CWD</sup> deposition occurred relatively rapidly and widely in lymphoid tissues, later in central and peripheral nervous tissues and sporadically in a variety of tissues and organs in terminal disease stages. Development of spongiform encephalopathy lagged behind PrP<sup>CWD</sup> deposition in the central nervous system (CNS), but occurred in the same neuroanatomical locations. PrP<sup>CWD</sup> deposition in the lymphatic and nervous systems tended to be consistent and progressive in specific organs and tissues. Locations of PrP<sup>CWD</sup> deposition were similar between deer of two PrP genotypes (225SS and 225SF), but the time course differed between genotypes: in 225SF deer, PrP<sup>CWD</sup> accumulated more slowly in lymphatic tissues than in 225SS animals, but that disparity was small in comparison to the disparity between genotypes in timing of deposition in CNS tissue. These data confirm retropharyngeal lymph node and medulla oblongata at the level of the obex as early sites of PrP<sup>CWD</sup> accumulation in mule deer with CWD. Data on the relative time frames for and genetic influences on PrP<sup>CWD</sup> accumulation may also offer insights about epidemic dynamics and potential control strategies.

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## INTRODUCTION

Chronic wasting disease (CWD) is a prion disease of North American deer (*Odocoileus* spp.) and wapiti (*Cervus elaphus nelsoni*) (Williams & Young, 1980, 1982, 1992). The neuropathology of clinical CWD has been well described (Williams & Young, 1980, 1982, 1992, 1993; Spraker *et al.*, 1997, 2002b), but the progression of lesions from preclinical stages until death remains poorly characterized. Among clinical CWD cases, the nucleus of the vagus nerve is affected most consistently and severely by spongiform changes in all three host species (Williams & Young, 1993; Spraker *et al.*, 1997; Williams & Miller, 2002). Immunohistochemistry (IHC) has been used to demonstrate accumulation of CWD-specific prion protein (PrP<sup>CWD</sup>) in brain, spinal cord, lymphoid tissues, nerves and ganglia of the peripheral nervous system, and in endocrine tissues in clinical cases (Spraker *et al.*, 1997, 2002b, c; Sigurdson *et al.*, 2001).

Accumulation of PrP<sup>CWD</sup> in brain and several lymphoid tissues, particularly retropharyngeal lymph node and tonsil, also can occur in the absence of spongiform lesions or clinical signs (Sigurdson *et al.*, 1999; Miller *et al.*, 2000; Miller & Williams, 2002).

Current understanding about the distribution and progression of PrP<sup>CWD</sup> accumulation and lesions in mule deer (*Odocoileus hemionus*) is based primarily on observations from naturally infected animals (Miller *et al.*, 2000; Miller & Williams, 2002; Spraker *et al.*, 2002c) and a study of early lymphoid tropism of PrP<sup>CWD</sup> accumulation (Sigurdson *et al.*, 1999), and by inferences drawn from studies of scrapie in sheep. From these limited studies, observed patterns of PrP<sup>CWD</sup> accumulation in cranial lymphoid tissues of preclinical cases provided the basis for an ante-mortem CWD test in mule deer (Wild *et al.*, 2002; Wolfe *et al.*, 2002) and improved methods for large-scale CWD surveillance (Hibler *et al.*, 2003). It follows that more complete data on CWD pathogenesis, including the chronology, distribution

†Deceased 29 December 2004.

and relative intensity of PrP<sup>CWD</sup> accumulation, as well as the potential effect of genotype, could provide further insights into CWD diagnosis and epidemiology. Here, we describe the tissue distribution and progression of PrP<sup>CWD</sup> deposition and lesions in mule deer examined post-mortem 90–785 days after oral inoculation with infectious brain material.

## METHODS

**Animals and inoculation.** Twenty 5- to 6-month-old mule deer fawns (15 male, five female) were captured from the Rocky Mountain Arsenal National Wildlife Refuge (RMANWR) on 2 and 3 December 1997. Following capture, the fawns were transported to the Colorado Division of Wildlife's Foothills Wildlife Research Facility (FWRF) in Fort Collins, CO, USA. Previous and subsequent surveys revealed that deer residing at RMANWR were not infected with CWD (Miller *et al.*, 2000, 2004; Miller & Williams, 2003), thereby ensuring that fawns were not exposed to CWD until inoculated experimentally. For capture, fawns were anaesthetized with tiletamine HCl and zolazepam (Telazol; about 5 mg kg<sup>-1</sup>) and xylazine HCl (about 2.5 mg kg<sup>-1</sup>) delivered intramuscularly (IM) via projectile syringe. Additional xylazine [20–100 mg intravenously (IV) or IM] was administered as needed to keep fawns sedated until they arrived at FWRF. Fawns were sexed and marked individually in the field with paired alphanumeric ear tags.

Upon arrival at FWRF, residual sedation was antagonized with IV yohimbine HCl (0.25 mg kg<sup>-1</sup>). Once fawns recovering from anaesthesia could swallow effectively, each received about 5 g pooled, homogenized brain tissue delivered *per os*. This inoculum pool comprised brain tissues collected from 28 captive mule deer previously diagnosed with spongiform encephalopathy and was shown to be infectious in a study of early CWD pathogenesis in mule deer (Sigurdson *et al.*, 1999). Presence of scrapie-associated fibrils in this homogenate was confirmed via negative-stain electron microscopy and Western blot; the homogenate contained about 3 µg PrP<sup>CWD</sup> (g homogenate)<sup>-1</sup> (Raymond *et al.*, 2000). Homogenate was deposited as an unencapsulated bolus into the posterior oropharynx by using a modified syringe. Once fawns swallowed the homogenate, they were released into a 3 ha paddock where no CWD-infected deer had resided for at least 12 years. Alfalfa hay, pelleted supplemental diets (high-energy and 'browser' rations), mineralized salt blocks and water were provided to deer *ad libitum* throughout the study. All deer were observed daily by animal caretakers and evaluated monthly for signs of CWD by an attending veterinarian.

For this study, we randomly selected and sacrificed two deer at 90, 189, 272, 362, 482 and 603 days after oral challenge (days p.i.). At the time of sacrifice, we anaesthetized deer with tiletamine HCl and zolazepam (Telazol; 5 mg kg<sup>-1</sup>) and xylazine HCl (2.5 mg kg<sup>-1</sup>) delivered via projectile syringe. We then administered 400 mmol KCl, 400 mg ketamine HCl and 200 mg xylazine HCl IV to induce cardiac arrest. Deer that developed clinical signs suggestive of end-stage CWD (severe depression or ataxia and emaciation with estimated >20% loss in body weight) were euthanized as described above. We collected two age- and sex-matched control deer from RMANWR within 13 days of each date when challenged deer were sacrificed; in all, 12 control deer were examined. Control deer were anaesthetized in the field, sampled and euthanized as described above. All research protocols were reviewed and approved by the Colorado Division of Wildlife's Animal Care and Use Committee.

**Tissues collected.** We collected a standard set of tissues at necropsy from each animal. Duplicate samples of non-central nervous system (CNS) tissues were taken, of which one was fixed in

10% neutral-buffered formalin and the other was stored at -20 °C. The fresh brain was sectioned parasagittally; one cerebral hemisphere was frozen and the remainder, which included brainstem, was formalin-fixed. Samples from cervical, thoracic and lumbar spinal cord were frozen and the remainder was formalin-fixed. After sampling, carcasses were incinerated.

**IHC and histopathology.** Representative subsamples of formalin-fixed tissues were cut into slices 2–3 mm thick, immersed in 98% formic acid for 1 h and rinsed under flowing tap water for at least 4 h. These tissues were embedded in paraffin blocks and sectioned at 5–6 µm for staining. Sections were stained by IHC using minor modifications of the technique of van Keulen *et al.* (1995), using mAb F99/97.6.1 (Cell line F99/97.6.1; VMRD Inc.) (O'Rourke *et al.*, 2000; Spraker *et al.*, 2002a), a biotinylated secondary antibody and alkaline phosphatase–streptavidin conjugate, a substrate chromogen and a haematoxylin and bluing counterstain (Ventana Medical Systems) as described previously (Miller & Williams, 2002); a standard set of 11 levels of brain and three levels of spinal cord was examined from each animal. Positive- and negative-control tissues from mule deer were included in each staining batch. One of us (K. A. F.) evaluated each tissue for the presence of IHC staining and categorized it as PrP<sup>CWD</sup>-positive or PrP<sup>CWD</sup>-negative in the absence of knowledge about the CWD exposure status of individual deer. Tissues were considered PrP<sup>CWD</sup>-positive based on characteristic staining detectable at ×200 magnification. Positive staining was differentiated from background staining based on comparison with IHC control tissues. A section cut from each block and stained with haematoxylin and eosin was used as a reference control and to examine CNS tissues for spongiform change (Williams & Young, 1993).

Unfixed tissue samples also were tested for PrP<sup>CWD</sup> by using an ELISA (Bio-Rad Laboratories) (Hibler *et al.*, 2003); assay methods followed those established for lymphatic and brain tissue from mule deer.

**PrP genotyping.** We determined the deduced amino acid sequence of the PrP protein for all deer in the study by PCR amplification of genomic DNA and commercial sequencing using primers and protocols described previously (Jewell *et al.*, 2005). Assessment of genetic effects on patterns of PrP<sup>CWD</sup> accumulation was based on the occurrence of sequences for the amino acids serine (S) or phenylalanine (F) at codon 225 (Brayton *et al.*, 2004; Jewell *et al.*, 2005); we denote these genotypes here as 225SS, 225SF and 225FF.

**Statistical analysis.** Among deer surviving long enough to develop clinical CWD, we calculated mean time to onset of clinical signs (incubation) and mean clinical course (clinical onset to end-stage CWD). Because our study was descriptive, statistical analyses of pathology data were not attempted.

## RESULTS

### Genotypes of subject deer

Genetic variation in the mule deer prion protein (Heaton *et al.*, 2003; Brayton *et al.*, 2004; Jewell *et al.*, 2005) had not been described when our study began in 1997, and possible effects of genetic differences between subjects were not considered in our original study design. We learned subsequently that, of the 20 inoculated deer in our study, 12 were genotype 225SS and eight were genotype 225SF; none was genotype 225FF. Throughout the study, we selected subjects randomly for euthanasia at predetermined time intervals after inoculation and, consequently, the

distribution of the 225SS and 225SF genotypes across sampling frames was relatively uniform (Tables 1 and 2). Although the PrP genotypes of subject deer were determined *post hoc* and were not part of the original study plan, their influences on CWD pathogenesis became apparent during the course of the study. Consequently, we elected to describe and compare results for genotype 225SS and 225SF individuals, even though the study was not originally designed to examine differences between genotypes.

### Disease course

Nineteen of 20 inoculated deer survived for longer than 90 days p.i. and were examined as described; one fawn died of capture-related complications <1 day p.i. and was not evaluated here. One of the 19 died from a cervical fracture at 97 days p.i.; 12 others were euthanized at 90–603 days p.i. according to the study schedule. The remaining six survived to develop terminal CWD or to study termination.

Six of the eight inoculated deer that survived for longer than 482 days p.i. developed end-stage clinical CWD. All six were genotype 225SS; three were male and three were female. The two deer that survived for longer than 482 days p.i. and did not show obvious clinical signs were both genotype 225SF; both appeared clinically normal when euthanized (603 and 785 days p.i.). One of the six deer that developed clinical

CWD was showing marked clinical signs when euthanized at 603 days p.i. according to the established sampling schedule; the remaining five either died or were euthanized in end-stage clinical CWD at 630–785 days p.i.

Based on observations of the six deer that developed clinical CWD, earliest signs (dullness in eyes, diminished alertness, misdirected behaviours, piloerection) were first documented in individuals at 442–578 days p.i. (mean  $\pm$  SEM = 526  $\pm$  21 days p.i.). Initial clinical signs were subtle and inconsistent. As clinical disease progressed, behavioural changes (e.g. blank staring, uncharacteristic or subdued responses to aversive stimuli, lowered head or other unusual postures, ataxia, inefficient foraging activity) and loss of body condition became more pronounced and consistent. Ptyalism, polydipsia and polyuria occurred relatively late in clinical courses and were not seen in all cases. Among the five affected deer that lived long enough to develop terminal CWD, clinical courses ranged from about 106 to 289 days (mean  $\pm$  SEM = 173  $\pm$  37 days); the shortest clinical course (106 days) was complicated by acute aspiration pneumonia.

### PrP<sup>CWD</sup> deposition in tissues

**90 days p.i.** Two genotype 225SS deer were sacrificed or died at 90 or 97 days p.i. Tissues from these deer had staining consistent with PrP<sup>CWD</sup> deposition in the

**Table 1.** PrP<sup>CWD</sup> distribution by time after inoculation and PrP genotype in lymphoid tissues from mule deer inoculated orally with brain tissue from CWD-infected mule deer

PrP<sup>CWD</sup> was detected by IHC with mAb F99/97.6.1. ND, Not done.

Tissue	Genotype 225SS (n=11): days p.i. (no. examined)							Genotype 225SF (n=8): days p.i. (no. examined)						
	90 (2)	189 (1)	272 (1)	362 (0)	482 (1)	603 (1)	≥630 (5)	90 (1)	189 (1)	272 (1)	362 (2)	482 (1)	603 (1)	≥630 (1)
Tonsil	1 <sup>(1)*</sup>	1	1	ND	1	1	5	0	1	1	2	1	1	1
Lymph nodes:														
Submandibular	2	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Retropharyngeal	2	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Bronchial	2	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Mediastinal	2	1	1	ND	1	1	2 <sup>(2)</sup>	0	0 <sup>(0)</sup>	1	2	1	1	1
Hepatic	2	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Mesenteric	1 <sup>(1)</sup>	0 <sup>(0)</sup>	1	ND	1	1	2 <sup>(2)</sup>	0 <sup>(0)</sup>	1	1	2	1	1	1
Ileocaecal	1	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Superficial cervical	1	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Popliteal	2	1	1	ND	1	1	2 <sup>(2)</sup>	0	1	1	2	1	1	1
Ileal Peyer's patches	0	1	1	ND	1	1	4 <sup>(4)</sup>	0	0	0	1 <sup>(1)</sup>	1	0 <sup>(0)</sup>	1
Spleen	0	1	1	ND	1	1	5	0	0	0	1 <sup>(1)</sup>	1	0 <sup>(0)</sup>	1
Thymus	0	0	0	ND	0	0	0 <sup>(2)</sup>	0 <sup>(0)</sup>	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0 <sup>(0)</sup>
GALT of colon	0	0	1	ND	0	0	1	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
GALT of oesophagus	0	0	0	ND	0	0	2 <sup>(4)</sup>	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Lymphoid aggregates:														
Soft palate	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>	ND	0 <sup>(0)</sup>	0 <sup>(0)</sup>	2 <sup>(2)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0 <sup>(0)</sup>
Lung	0	0	0	ND	1	0	0 <sup>(4)</sup>	0 <sup>(0)</sup>	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Renal pelvis	0	0	1	ND	0	1	1	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0

\*Some tissues were not available for examination; superscript number in parentheses represents the number of tissues available.

**Table 2.** PrP<sup>CWD</sup> distribution by time after inoculation and PrP genotype in nervous-system and endocrine tissues from mule deer inoculated orally with brain tissue from CWD-infected mule deerPrP<sup>CWD</sup> was detected by IHC with mAb F99/97.6.1. ND, Not done.

Tissue	Genotype 225SS ( <i>n</i> =11): days p.i. (no. examined)							Genotype 225SF ( <i>n</i> =8): days p.i. (no. examined)						
	90 (2)	189 (1)	272 (1)	362 (0)	482 (1)	603 (1)	≥630 (5)	90 (1)	189 (1)	272 (1)	362 (2)	482 (1)	603 (1)	≥630 (1)
Myenteric plexuses:														
Oesophagus	0	0	0	ND	1	0	2 <sup>(4)</sup>	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Rumen	0 <sup>(1)*</sup>	0	1	ND	0	1	4	0 <sup>(0)</sup>	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Omasum	0	0	1	ND	0	1	5	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Abomasum	0	0	1	ND	1	0	5	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Duodenum	0	0	1	ND	0	1	5	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0 <sup>(0)</sup>
Ileum	0	0	1	ND	1	1	3 <sup>(4)</sup>	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Colon	0	0	1	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Submucosal plexuses:														
Ileum	0	0	1	ND	1	1	3 <sup>(4)</sup>	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Colon	0	0	1	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Vagus nerve	0	0 <sup>(0)</sup>	1	ND	1	1	2 <sup>(3)</sup>	0 <sup>(0)</sup>	0	0	0	0 <sup>(0)</sup>	0 <sup>(0)</sup>	0
DMNV	0	1	1	ND	1	1	5	0	0	0	0	1	1	1
Cord/medulla junction	0	0	1	ND	1	1	4 <sup>(4)</sup>	0	0	0	0 <sup>(1)</sup>	0	0	0 <sup>(0)</sup>
Other nuclei of medulla†	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	1
Nuclei of midbrain/pons‡	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	1
Nuclei of thalamus	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	1
Hypothalamic nuclei	0	0	0	ND	0 <sup>(0)</sup>	1	5	0	0	0	0 <sup>(1)</sup>	0	0	1
Basal ganglia	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Cerebellum – roof nuclei	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Throughout cerebellum	0	0	0	ND	0	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Cerebral cortex – grey	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Cerebral cortex – white	0	0	0	ND	0	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Olfactory cortex	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	1
Hippocampus	0	0	0	ND	0	0	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Cervical spinal cord	0	0	0	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Thoracic spinal cord	0	0	1	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Lumbar spinal cord	0	0	1	ND	1	1	5	0	0	0	0 <sup>(1)</sup>	0	0	0
Neuroendocrine:														
Islets of Langerhans	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	ND	0	0	1	0	0	0	0 <sup>(1)</sup>	0	0 <sup>(0)</sup>	0
Pituitary – pars nervosa	0	0	0 <sup>(0)</sup>	ND	1	1	3 <sup>(3)</sup>	0	0 <sup>(0)</sup>	0	0 <sup>(1)</sup>	0	0	0
Pituitary – pars intermedia	0	0	0 <sup>(0)</sup>	ND	0	0	3 <sup>(3)</sup>	0	0 <sup>(0)</sup>	0	0 <sup>(1)</sup>	0	0	0
Adrenal medulla	0	0	0	ND	0	1	2 <sup>(2)</sup>	0 <sup>(0)</sup>	0	0	0 <sup>(1)</sup>	0	0	0

\*Tissue not available for examination; superscript number in parentheses represents number of tissues examined at time of writing.

†Cuneate nucleus, nucleus solitarius, hypoglossal nucleus, nucleus of the spinal tract of the trigeminal nerve, nucleus ambiguus, reticular formation, olivary nuclei.

‡Periaqueductal grey matter, oculomotor nucleus, superior colliculus, pontine nucleus, tegmental nucleus.

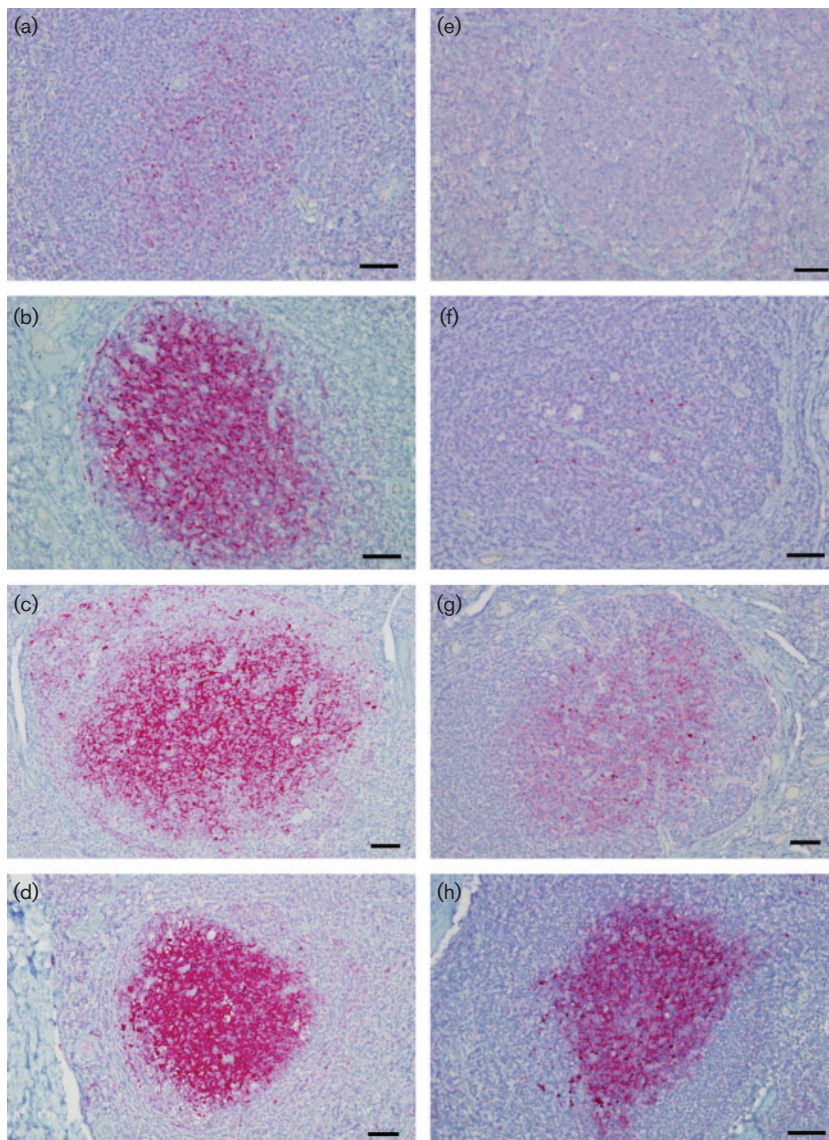
germinal centres of the tonsil (Fig. 1a) and all lymph nodes examined (Table 1), with the exception of the ileocaecal and superficial cervical lymph nodes, which stained positively in one animal, but not in the other. In contrast, no PrP<sup>CWD</sup> was detected in any tissues of the genotype 225SF animal sacrificed at 90 days p.i. (Fig. 1e).

**189 days p.i.** Tissues from the genotype 225SS deer sacrificed at 189 days p.i. had PrP<sup>CWD</sup> deposits in tonsil and lymph nodes, Peyer's patches of the ileum, lymphoid

follicles of the spleen and as bilaterally symmetrical granular chromagen deposits in neuropil and surrounding neurons of the dorsal motor nucleus of the vagus nerve (DMNV). Tissues from the genotype 225SF deer sacrificed at 189 days p.i. had PrP<sup>CWD</sup> deposits in the germinal centres of lymphoid follicles of the tonsil and all lymph nodes examined (Table 1).

**272 days p.i.** Tissues from the genotype 225SS deer sacrificed at 272 days p.i. had additional sites of PrP<sup>CWD</sup>



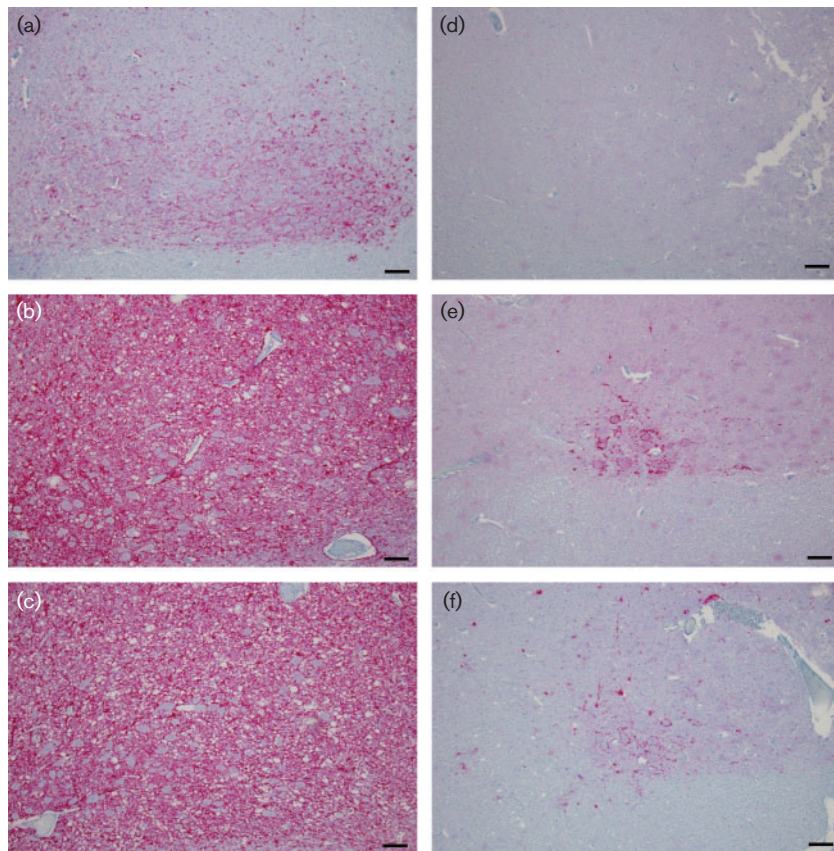


**Fig. 1.** Immunohistochemical detection of PrP<sup>CWD</sup> using mAb F99/97.6.1 in tonsil tissue from PrP genotype 225SS and 225SF mule deer at various time intervals after inoculation. (a) 225SS deer at 90 days p.i.; (b) 225SS deer at 272 days p.i.; (c) 225SS deer at 482 days p.i.; (d) 225SS deer at 785 days p.i.; (e) 225SF deer at 90 days p.i.; (f) 225SF deer at 272 days p.i.; (g) 225SF deer at 482 days p.i.; (h) 225SF mule deer at 785 days p.i. Panels (a–d) and (f–h) show positive staining (red stain) for PrP<sup>CWD</sup>. Bars, 50  $\mu$ m.

deposition in gut-associated lymphoid tissue (GALT) of the colon and a lymphoid aggregate in the renal pelvis (Fig. 3a). In the peripheral nervous system, perineuronal staining occurred within the myenteric plexuses of the ileum, colon, duodenum, rumen, omasum and abomasum and in the submucosal plexuses of the ileum and colon, as well as within and surrounding axons of the vagus nerve. In the CNS, staining was observed in DMNV (Fig. 2a; Table 2), in the lateral aspect of the intermediate grey matter at the junction of rostral cervical cord with medulla oblongata, bilaterally in the lateral aspect of the intermediate grey matter of the lumbar spinal cord and unilaterally in the intermediolateral column of the thoracic spinal cord. The genotype 225SF deer sacrificed at 272 days p.i. had PrP<sup>CWD</sup> deposits in the germinal centres of lymphoid follicles of the tonsil and all lymph nodes examined, but there was no peripheral nervous system or CNS involvement (Fig. 2d).

**362 days p.i.** The two deer sacrificed at 362 days p.i. were both genotype 225SF. In addition to staining in tonsil and lymph nodes similar to that noted at earlier time points, tissues from these deer also demonstrated staining in the Peyer's patches of the ileum and lymphoid follicles of the spleen, with no observed peripheral nervous system or CNS involvement.

**482 days p.i.** Tissues from the genotype 225SS deer sacrificed at 482 days p.i. had PrP<sup>CWD</sup> deposited extensively in both lymphatic- and nervous-system tissues. Additional areas of PrP<sup>CWD</sup> accumulation included a lymphoid aggregate in the lung, the myenteric plexus of the oesophagus and all major nuclei of medulla oblongata, as well as in midbrain, thalamus, basal ganglia, hypothalamic nuclei, roof nuclei of the cerebellum, olfactory cortex, grey matter of the cerebral cortex, lateral aspects of the intermediate grey matter of the cervical spinal cord



**Fig. 2.** Immunohistochemical detection of PrP<sup>CWD</sup> using mAb F99/97.6.1 in dorsal motor nucleus of the vagus nerve (DMNV) from PrP genotype 225SS and 225SF mule deer at various time intervals after inoculation. (a) 225SS deer at 272 days p.i.; (b) 225SS deer at 603 days p.i.; (c) 225SS deer at 785 days p.i.; (d) 225SF deer at 272 days p.i.; (e) 225SF deer at 603 days p.i.; (f) 225SF deer at 785 days p.i. Panels (a–c), (e) and (f) show positive staining (red stain) for PrP<sup>CWD</sup>. Bars, 100  $\mu$ m.

bilaterally, dorsal horns of the cervical, thoracic and lumbar spinal cord, the white matter of the cervical, thoracic and lumbar spinal cord and in pars nervosa of the pituitary gland. Tissues from the genotype 225SF deer sacrificed at 482 days p.i. had no additional PrP<sup>CWD</sup> deposits in the lymphoid tissues sampled. There was positive staining in the DMNV bilaterally (Fig. 2e), with no staining elsewhere in brain, spinal cord or pituitary gland.

**603 days p.i.** Tissues from the genotype 225SS deer sacrificed at 603 days p.i. had extensive PrP<sup>CWD</sup> deposition in both lymphatic- and nervous-system tissues. Additional sites of PrP<sup>CWD</sup> deposition occurred throughout the molecular, Purkinje cell and granular layers of the cerebellum, in the white matter of the cerebral cortex and in ventral horns of the cervical, thoracic and lumbar spinal cord. The distribution of PrP<sup>CWD</sup> deposits in tissues from the genotype 225SF deer sacrificed at 603 days p.i. was comparable to that seen in the 225SF deer at 482 days p.i. and, in the CNS, was restricted to the DMNV.

**630–785 days p.i.** The remaining five deer with a 225SS genotype died or were euthanized in end-stage CWD at 630, 671, 721, 733 or 785 days p.i. Tissues from all five had extensive PrP<sup>CWD</sup> deposition throughout both lymphatic- and nervous-system tissues. We observed additional sites of PrP<sup>CWD</sup> staining in GALT in the oesophagus, lymphoid aggregates in the submucosa of the soft

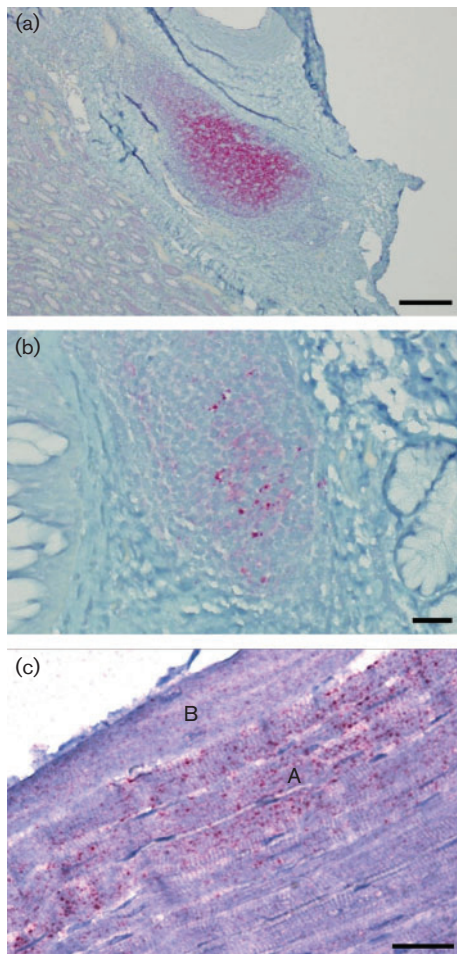
palate of the caudal pharynx, pars intermedia of the pituitary gland, hippocampus and islets of Langerhans. Submucosa of the soft palate of the caudal pharynx was sampled in only two animals and was positive in both (Fig. 3b). In the 225SS deer sacrificed at 785 days p.i., we also observed staining in one group of myocytes in left ventricular myocardium (Fig. 3d). Tissue from the 225SF deer sacrificed at 785 days p.i. had additional sites of CNS PrP<sup>CWD</sup> deposition throughout the nuclei of the medulla, midbrain, thalamus and hypothalamus and in the olfactory cortex. Deposition of PrP<sup>CWD</sup> in brain of this 225SF individual was less extensive than in the brains of the five 225SS animals examined at >630 days p.i. (Table 2).

No staining was detected in trigeminal ganglion, stellate ganglion, dorsal root ganglion, nodose ganglion, radial nerve, sciatic nerve, thymus, bone marrow, triceps muscle, semitendinosus muscle, latissimus dorsi muscle, diaphragm, tongue, submandibular salivary gland, parotid salivary gland, liver, urinary bladder, gonad/reproductive tract, skin (from head) or nasal mucosa samples examined from any inoculated deer, regardless of post-exposure interval. None of the 12 uninoculated control deer collected from RMANWR showed evidence of PrP<sup>CWD</sup> deposition in any of the tissues examined.

Analyses of tissues by ELISA (data not shown) revealed essentially the same PrP<sup>CWD</sup> distribution patterns in lymphatic and nervous tissues as was shown with IHC.



Discrepancies tended to arise either from early sampling periods or from organs where PrP<sup>CWD</sup> deposition was patchy or sporadic, or where samples were small or used preferentially for IHC. Because PrP<sup>CWD</sup> distribution could not be assessed microscopically, 'suspect' results for dorsal root and stellate ganglia, sciatic nerve and submandibular and parotid salivary gland could not be interpreted further to specify the site of PrP<sup>CWD</sup> accumulation or eliminate non-specific reactions. Neither cardiac nor skeletal muscle (from three sites) yielded 'suspect' results.



**Fig. 3.** Immunohistochemical detection of PrP<sup>CWD</sup> using mAb F99/97.6.1 in mule deer tissues where PrP<sup>CWD</sup> deposits were previously undocumented. (a) Renal tissue from 225SS deer at 272 days p.i.; note red staining in a lymphoid aggregate in renal pelvic connective tissue. (b) Soft palate tissue of caudal pharynx from 225SS deer at 660 days p.i.; note red staining of a lymphoid aggregate in submucosa. (c) Left ventricular myocardium tissue from 225SS deer at 785 days p.i.; note deep-red granular staining (A) of a small focus of myocytes. Light-pink staining (B) is background and found in control left ventricular myocardium. Bars, 150  $\mu$ m (a); 25  $\mu$ m (b); 25  $\mu$ m (c).

## Histopathology

Spongiform changes were consistent with those described previously (Williams & Young, 1980, 1993; Spraker *et al.*, 1997). The earliest spongiform changes occurred within the DMNV; subsequent distribution and progression throughout the grey matter of the CNS followed the pattern seen with PrP<sup>CWD</sup> deposition. In the 225SS deer, the first spongiform changes in the DMNV were detected in samples collected at 603 days p.i., 414 days after the first detection of PrP<sup>CWD</sup> accumulation in the DMNV by IHC. No spongiform changes were detected in spinal cord, with an interval of 513 days between the earliest detection of PrP<sup>CWD</sup> in the spinal cord and the last tissue-sampling date. However, mild autolysis of tissues arising from up to a 12 h delay in fixation may have prevented accurate detection of subtle spongiform changes in both the brain and spinal cord. No lesions suggestive of spongiform encephalopathy were seen in any of the 225SF deer or in any of the negative-control deer.

## DISCUSSION

The findings described here extend our knowledge about the progressive accumulation of PrP<sup>CWD</sup> in tissues of CWD-infected mule deer. To complement data on early PrP<sup>CWD</sup> distribution in lymphoid and nervous tissue 10–80 days p.i. (Sigurdson *et al.*, 1999), we assayed an array of tissues for PrP<sup>CWD</sup> accumulation from 90 to 785 days p.i., covering periods of progressive lymphoid-system involvement, CNS invasion and terminal stages of the disease. As in the earlier study, we examined a limited number of animals (one or two) at each time point and thus cannot report on potential variability among individuals attributable to factors including sex, age and exposure dose. Our data do, however, provide insights about the influence of PrP genotype on patterns of PrP<sup>CWD</sup> accumulation and perhaps CWD pathogenesis in mule deer. The data presented here are perhaps best viewed as a broad framework for the temporal progression of PrP<sup>CWD</sup> accumulation and CWD pathogenesis in mule deer that may be useful for interpreting observations from natural cases of CWD for which the incubation period is unknown (Williams & Young, 1993; Miller *et al.*, 2000; Sigurdson *et al.*, 2001; Miller & Williams, 2002; Spraker *et al.*, 2002b, c).

The general pattern of PrP<sup>CWD</sup> accumulation in mule deer is characterized by relatively rapid and widespread involvement of lymphatic tissues, followed by progressive involvement of and lesions in central and peripheral nervous tissues, with involvement of a wider variety of tissues and organs, including endocrine system and heart, as animals become terminally ill (Tables 1, 2, 3). The pattern of PrP<sup>CWD</sup> deposition in the lymphatic and nervous systems tended to be consistent and progressive within specific organs and tissue types; that is, once a tissue was found to be IHC-positive, it tended to be positive at subsequent time intervals among individuals of the same genotype. Exceptions to this trend were neurons of the myenteric and submucosal plexuses, the vagus nerve (Tables 1 and 2)

**Table 3.** Effect of PrP genotype on the number of lymphoid and nervous tissue sites with detectable PrP<sup>CWD</sup> at different times after inoculation in mule deer inoculated orally with brain tissue from CWD-infected mule deer

See text and Tables 1 and 2 for detailed anatomical site names and locations. +, 1–5 sites; ++, 6–10 sites; +++, 11–15 sites; + + + +, 16–20 sites; + + + + +, 21–25 sites; ND, not done.

System	Genotype	Time after inoculation (days p.i.)						
		90	189	272	362	482	603	> 603
Lymphoid	225SS	++	+++	+++	ND	+++	+++	++++
	225SF	0	++	++	+++	+++	+++	+++
Nervous	225SS	0	+	++	ND	++++	+++++	+++++
	225SF	0	0	0	0	+	+	++

and perhaps other organs or sites yielding 'suspect' ELISA results. This pattern is similar to those described for both scrapie (Andréoletti *et al.*, 2000; Heggebø *et al.*, 2000, 2002, 2003a, b; van Keulen *et al.*, 2000; Jeffrey *et al.*, 2001a) and bovine spongiform encephalopathy (BSE) infection in sheep (Jeffrey *et al.*, 2001b; Jeffrey & González, 2004). It differs substantially from the pattern seen in BSE in cattle (Wells *et al.*, 1998).

Studies of the early stages of scrapie in sheep identified lymphoid aggregates of the intestinal mucosa, particularly Peyer's patches in distal ileum, as the likely site for initial uptake and accumulation of scrapie prions (Andréoletti *et al.*, 2000; Heggebø *et al.*, 2000, 2003b; van Keulen *et al.*, 2000). Mule deer fawns inoculated orally with CWD also showed early accumulation of PrP<sup>CWD</sup> in Peyer's patches and ileocaecal lymph nodes, as well as the retropharyngeal lymph node, at 42 days p.i. (Sigurdson *et al.*, 1999). However, the proportion of positive follicles was higher in retropharyngeal lymph node (3–27%) than in Peyer's patches and ileocaecal lymph nodes (0–0.6%) from 42 to 80 days p.i. (Sigurdson *et al.*, 1999), suggesting that retropharyngeal lymph nodes may play a more important role as a portal of entry for CWD in early propagation and accumulation of PrP<sup>CWD</sup> in deer compared with Peyer's patches in scrapie-exposed sheep.

In the mule deer that we sampled at  $\geq 90$  days p.i., PrP<sup>CWD</sup> deposits appeared in tonsils and lymph nodes prior to detectable accumulation in intestinal lymphoid aggregates (Table 1). In 225SS individuals, PrP<sup>CWD</sup> was widespread in palatine tonsil and lymph nodes throughout the body by 90 days p.i., but was not detected in lymphoid aggregates in the ileum until the next sampling (189 days p.i.). Because lymphoid tissues of the mouth, pharynx and oesophagus would probably be exposed first to infectious prions after oral exposure and might be re-exposed through repeated mastication and deglutition during rumination, these seem plausible entry sites for the CWD prion. The lateral retropharyngeal lymph nodes in ruminants are collection centres for lymph fluid from other lymph nodes that drain the head (Dyce *et al.*, 2002); they empty via the tracheal and thoracic ducts directly into the venous supply. This provides a plausible mechanism for rapid, widespread dissemination

of prions to lymph nodes throughout the body, as well as to the spleen, that could account for the appearance of PrP<sup>CWD</sup> in bronchial, mediastinal, superficial cervical and popliteal lymph nodes relatively soon after its appearance in nodes that drain the alimentary tract. The pattern of PrP<sup>CWD</sup> deposition observed after oral inoculation closely resembles patterns seen in presumed early cases of natural CWD (Miller & Williams, 2002; Spraker *et al.*, 2002c). Because deer in our study were infected experimentally, however, it is possible that some aspects of the distribution and timing that we observed may have been influenced by our inoculation methods.

Following detection in lymph nodes, we observed PrP<sup>CWD</sup> in GALT and ganglia of the enteric nervous system, as well as the DMNV, the intermediolateral column of the spinal cord and the vagus nerve. The appearance of PrP<sup>CWD</sup> in myenteric and submucosal plexuses of the gastrointestinal tract and in brain and spinal cord is consistent with the hypothesis that autonomic innervation, particularly of enteric tissues, plays a role in the spread of PrP<sup>CWD</sup> from peripheral tissues to the CNS (Sigurdson *et al.*, 2001), as was suggested previously for scrapie in hamsters (Beekes *et al.*, 1996, 1998; Beekes & McBride, 2000) and sheep (van Keulen *et al.*, 1999; Heggebø *et al.*, 2003a). Moreover, apparently simultaneous deposition of PrP<sup>CWD</sup> in DMNV and thoracolumbar spinal-cord segments without evidence of concurrent deposition in cervical spinal cord supports the model for scrapie pathogenesis (van Keulen *et al.*, 2000; Jeffrey *et al.*, 2001a) and observations in CWD (Sigurdson *et al.*, 2001) that prion invasion of the CNS can occur by two routes: one via parasympathetic preganglionic nerve fibres to the DMNV, as shown in orally infected hamsters (Baldauf *et al.*, 1997; Beekes *et al.*, 1998), the other via sympathetic fibres to the intermediolateral column of the spinal cord (McBride *et al.*, 2001).

PrP<sup>CWD</sup> in the brain was detected first in the DMNV. Spread from the DMNV to the rest of the brain initially affected nuclei in the medulla, thalamus, hypothalamus, midbrain and olfactory cortex. Involvement of cerebellum, hippocampus and the rest of the cerebral cortex occurred later in the disease course (Table 2). A similar pattern, inferred from the relative frequency of observing PrP<sup>CWD</sup> deposits at



different neuroanatomical sites, has been observed in brains from free-ranging, CWD-infected mule deer with varying degrees of CNS involvement (Spraker *et al.*, 2002c). In the spinal cord, PrP<sup>CWD</sup> first appeared in the intermediolateral column followed by spread to dorsal horns, particularly substantia gelatinosa, and the intermediate grey matter of the entire cord; the ventral horns and white matter became involved later in the disease course. The appearance of PrP<sup>CWD</sup> deposits in endocrine tissues occurred in late stages of infection, suggesting spread from the hypothalamus (in the case of the pituitary) or dissemination by peripheral nerves of the autonomic nervous system (in the cases of the islets of Langerhans and adrenal medulla). Endocrine-gland involvement has been described in naturally infected mule deer that had died of CWD (Sigurdson *et al.*, 2001).

The foregoing patterns of PrP<sup>CWD</sup> deposition appeared to be similar between animals of the two prion protein genotypes available for comparison, but the time scale for these patterns differed between genotypes (Table 3). In the 225SF animals, accumulation of PrP<sup>CWD</sup> (as determined subjectively by intensity of staining) in lymphatic tissues developed more slowly compared with the 225SS animals (Fig. 1). However, the earliest detection and spread of PrP<sup>CWD</sup> in lymphatic tissues was delayed only slightly (one sampling interval) compared with detection and spread in the CNS (Table 3). Of eight 225SF animals included in our study, none had detectable PrP<sup>CWD</sup> in the enteric or other peripheral nerves, spinal cord or endocrine tissues sampled; only one had detectable PrP<sup>CWD</sup> accumulation in the brain outside the DMNV. The one 225SF animal with observed PrP<sup>CWD</sup> deposition in brain tissue outside the DMNV showed a pattern of staining that varied from that seen in 225SS deer: although PrP<sup>CWD</sup> did accumulate in the major nuclei of the medulla, midbrain, thalamus and hypothalamus of that animal, the staining in these nuclei was noticeably more scattered than was observed in 225SS deer (Fig. 2). Similar influences of genotype on PrP<sup>Sc</sup> deposition in CNS have been reported in sheep (González *et al.*, 2002). No spongiform changes were apparent in the DMNV of the 225SF animal sacrificed at 785 days p.i. However, because 225SF mule deer can develop clinical CWD (Jewell *et al.*, 2005; M. W. Miller, unpublished data), additional longer-term studies that are designed to investigate PrP<sup>CWD</sup> accumulation in 225SF (and 225FF) individuals appear warranted, to determine the full extent of genotype effects on CWD pathogenesis in mule deer. From our data, the primary effect of the 225SF genotype probably is only to delay, rather than to prevent, clinical CWD in infected deer.

Our findings have implications for current methods used to diagnose CWD. Retropharyngeal lymph-node tissue has been considered the tissue of choice when sampling for early detection of CWD in mule deer (Sigurdson *et al.*, 1999; Miller & Williams, 2002; Hibler *et al.*, 2003). Even in the earliest cases that we examined, follicles of the retropharyngeal lymph nodes stained more consistently and intensely than follicles in lymph nodes elsewhere in both

225SS and 225SF deer. Based on patterns described here, PrP<sup>CWD</sup> should be detectable in the retropharyngeal lymph nodes (and tonsils) of either 225SS or 225SF mule deer shortly after natural infection is established. Similarly, the patterns observed here confirm that the DMNV is an appropriate site in the brain to examine for early PrP<sup>CWD</sup> deposits in the CNS of mule deer. In contrast to retropharyngeal lymph nodes, PrP genotype may influence the likelihood of detecting early natural CWD infections via examination of mule deer medulla oblongata tissue.

Observations of PrP<sup>CWD</sup>-specific staining in kidney, lung, soft palate and heart suggest that a variety of other tissues can support prion replication and accumulation. In kidney, lung and soft palate, the presence of hyperplastic lymphoid tissue may provide a nidus for PrP<sup>CWD</sup> accumulation. This resembles a phenomenon described in scrapie-infected transgenic mice, wherein PrP<sup>Sc</sup> accumulated in inflamed organs, including kidney, liver and spleen (Heikenwalder *et al.*, 2005). In contrast, the presence of PrP<sup>CWD</sup> in left ventricular myocardium was probably due to dissemination by peripheral nerves, as this phenomenon also has been seen in CWD-infected white-tailed deer and elk (Jewell *et al.*, 2006). Given the demonstrable infectivity of skeletal muscle tissue inoculated into transgenic mice (Angers *et al.*, 2006), PrP<sup>CWD</sup> deposits might be expected to occur in CWD-infected mule deer, as they do in scrapie-infected sheep (Andréoletti *et al.*, 2004). In the present study, no PrP<sup>CWD</sup> deposits were detectable by IHC in skeletal muscle, corroborating similar negative observations made by other investigators (Spraker *et al.*, 2002b; Jewell *et al.*, 2006). Taken collectively, data from various studies show that, although PrP<sup>CWD</sup> may be detectable by bioassay in skeletal muscle (and perhaps other tissues), it is present in amounts too small to be detected by IHC. Nevertheless, PrP<sup>CWD</sup> has now been documented in a variety of organs other than lymphoid and CNS tissues and may be distributed widely throughout the body, albeit in small amounts, during terminal disease stages. These findings underscore the wisdom of longstanding advice to keep cervids with clinical CWD out of animal and human food chains.

In the context of CWD epidemiology, we view the two main venues of PrP<sup>CWD</sup> propagation as largely competitive in nature: propagation in peripheral lymphatic tissues, particularly GALT, seems likely to be tied to disease transmission (Sigurdson *et al.*, 1999; Andréoletti *et al.*, 2000; Williams & Miller, 2002; Miller & Williams, 2003; Miller & Wild, 2004), whereas nervous-system propagation leads to clinical disease and ultimately the death of infected individuals, thereby interrupting shedding and at least some pathways of transmission (Miller *et al.*, 2004). In this context, individuals (e.g. 225SF mule deer) showing PrP<sup>CWD</sup> propagation in lymphoid tissues relatively early in the course of infection, but with an extended time to onset of clinical signs, may represent a source of chronic prion shedding within populations. It follows that improved understanding of the relative time frames for these two main

processes in CWD pathogenesis may also lead to insights about epidemic dynamics and potential control strategies.

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