Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation

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Abstract. To determine the transmissibility of chronic wasting disease (CWD) to sheep, 8 Suffolk lambs of various prion protein genotypes (4 ARQ/ARR, 3 ARQ/ARQ, 1 ARQ/VRQ at codons 136, 154, and 171, respectively) were inoculated intracerebrally with brain suspension from mule deer with CWD (CWD<sub>md</sub>). Two other lambs were kept as noninoculated controls. Within 36 months postinoculation (MPI), 2 inoculated animals became sick and were euthanized. Only 1 sheep (euthanized at 35 MPI) showed clinical signs that were consistent with those described for scrapie. Microscopic lesions of spongiform encephalopathy (SE) were only seen in this sheep, and its tissues were determined to be positive for the abnormal prion protein (PrP<sub>res</sub>) by immunohistochemistry and Western blot. Three other inoculated sheep were euthanized (36 to 60 MPI) because of conditions unrelated to TSE. The 3 remaining inoculated sheep and the 2 control sheep did not have clinical signs of disease at the termination of the study (72 MPI) and were euthanized. Of the 3 remaining inoculated sheep, 1 was found to have SE, and its tissues were positive for PrP<sub>res</sub>. The sheep with clinical prion disease (euthanized at 35 MPI) was of the heterozygous genotype (ARQ/VRQ), and the sheep with subclinical disease (euthanized at 72 MPH) was of the homozygous ARQ/ARQ genotype. These findings demonstrate that transmission of the CWD<sub>md</sub> agent to sheep via the intracerebral route is possible. Interestingly, the host genotype may play a notable part in successful transmission and incubation period of CWD<sub>md</sub>.

Key words: Chronic wasting disease; immunohistochemistry; intracerebral transmission; prion protein; sheep; spongiform encephalopathy.

Introduction

Chronic wasting disease (CWD), a prion disease also known as transmissible spongiform encephalopathy (TSE), has been identified in captive cervids in Colorado since 1967.19 Natural cases of CWD have been documented in mule deer (Odocoileus hemionus hemionus), black-tailed deer (Odocoileus hemionus columbianus), white-tailed deer (Odocoileus virginianus), Rocky Mountain elk (Cervus elaphus nelsoni),18 and moose (Alces alces shirasi).1 The disease has been experimentally transmitted by intracerebral inoculation of infected brain material into a variety of domestic, wild and laboratory animal species.18 Among domestic animals, 1 goat18 and several cattle5–7 reportedly developed experimental prion disease after intracerebral inoculation of brain material from affected mule deer. The origin of CWD is not known. However, it has been proposed that the agent may have originated from sheep scrapie,18 and experimental inoculation of elk with scrapie resulted in a TSE that was neuropathologically indistinguishable from CWD.11

The susceptibility of sheep to scrapie is dependent on genetic variation of the host prion protein (PRNP) gene.4 PRNP genotypes are defined by variations in the amino acids encoded at codons 136, 154, and 171 and are termed polymorphisms. At least 5 variant alleles have been found with respect to a risk of contracting scrapie; these are depicted as ARQ, ARR, VRQ, AHQ and ARH. The codes represent polymorphism of amino acids at each codon, that is, A = alanine, R = arginine, and Q = glutamine. The other two amino acids are H = histidine and V = valine. Homozygous and heterozygous pairing of the two alleles inherited from a ram and a ewe therefore results in considerable variation of the PRNP genotype. This level of risk varies depending on breed type and the genotypes found within the flock.4 A study of Suffolk sheep in the United States found that 61% of orally inoculated animals developed scrapie.15 All were homozygous for glutamine (QQ) at allele 171 on the PRNP gene.15

The primary objectives of this study were to determine if the CWD agent could be transmitted to...
sheep that are susceptible (QQ) or relatively resistant (QR) to scrapie to provide information about clinical course, lesions, and suitability of currently used TSE diagnostic procedures for detecting experimental CWD in sheep.

Materials and methods

Animals

Two groups (n = 4) of 4-month-old Suffolk lambs from an in-house scrapie-free flock were obtained. One group of animals was genetically more resistant (QR at codon 171) to scrapie and the other was susceptible (QQ at codon 171) to scrapie. All sheep were RR at codon 154, and, except for 1 sheep (No. 0024; Table 1) that was AV at codon 136, all others were AA at 136 (Table 1). Inoculated lambs were housed in a Biosafety Level 2 isolation barn (2 lambs per pen) at the National Animal Disease Center (NADC) in Ames, Iowa. They were fed pelleted growth and maintenance rations that contained no ruminant protein, and clean water was available ad libitum. Control lambs were housed together in an open shed and fed pelleted growth and maintenance rations and alfalfa hay.

Inoculum

The inoculum was prepared from a pool of 28 CWD-affected mule deer brains. It was the same inoculum that was utilized in the oral cattle inoculation at University of Wyoming.18 The inoculum was positive for scrapie-associated fibrils (SAF) as determined by negative-stain electron microscopy and for abnormal prion protein (PrPSc) as determined by Western blot (WB). In addition, this pool was infective for orally inoculated mule deer2 and intracerebrally inoculated white-tailed deer (Kunkle et al., unpublished data, 2006). The inoculum was ground in a mechanical grinder and gentamicin was added to attain a final concentration of 100 µg/ml of homogenized inoculum. The final concentration of the brain inoculum was a 10% (wt/vol) in phosphate-buffered saline.

Inoculation procedure

Lambs were inoculated intracerebrally with 1 ml of the brain inoculum from mule deer with CWD (CWDsc), as described previously. Briefly, the lambs were sedated with xylazine, a midline incision was made in the skin at the junction of the parietal and frontal bones, and a 1-mm hole was trephined through the calvarium. The inoculum was injected into the midbrain via a 22-gauge 9-cm long needle while withdrawing the needle from the brain. The skin incision was closed with a single suture.

Necropsy and samples

Animals were euthanized with pentobarbital, and a complete necropsy was conducted on each of the carcasses. Representative samples of liver, kidney, spleen, skin, striated muscles (heart, tongue, diaphragm, masseter), thoracic aorta, thyroid gland, turbinates, trachea, lung, tonsils, esophagus, rumen, reticulum, omasum, abomasum, intestines (ileum), salivary gland, adrenal gland, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), nerves (optic, trigeminal), pituitary gland, gasserian ganglion, spinal cord (cervical, thoracic, lumbar), and eye (retina) were immersion fixed in 10% neutral buffered formalin. The brain was cut longitudinal; one half was fixed in formalin for not less than 3 weeks, and the other half was frozen. The formalin-fixed brain was cut into 2- to 4-mm wide coronal sections. Sections of various anatomic sites (a minimum of 5 hemisections of brain per animal) of rostral cerebrum, hippocampus, midbrain (at the level of rostral colliculus), cerebellum, midbrain, brainstem (at the level of obex), and 6 sections of spinal cord (2 each of cervical, thoracic, and lumbar) were processed for routine histopathology, embedded in paraffin wax, and sectioned at 5 µm. The sections were stained with hematoxylin and eosin (HE) and by an immunohistochemical (IHC) method for detecting PrPSc. A cocktail of 2 monoclonal antibodies, F89/160.1.5 and F99/97.6.1, was used for IHC, at a final concentration of

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* Postinoculation period (months).
† SE = spongiform encephalopathy; NAD = neuroaxonal dystrophy; IHC = immunohistochemistry; M = male; F = female; + = lesions/PrPSc present; – = lesions/PrPSc absent.
‡ Controls were not inoculated but were euthanized at these times.

Table 1. Findings in sheep (resistant and susceptible to scrapie) inoculated intracerebrally with chronic wasting disease agent from mule deer.
10 μg/ml protein. Past experience at NADC has indicated that these antibodies will recognize PRNP sequences in livestock species in which experimental TSEs have been reported.1-3,9,11 For detecting PrP\textsuperscript{res} by WB, a method described previously was used on frozen brain (caudal medulla) tissue.16 The antibody used in this technique was 6H4.\textsuperscript{a}

**Results**

Within 36 months postinoculation (MPI), 2 sheep became recumbent and were euthanized (Table 1). Only 1 of the 2 sheep (No. 0024, euthanized at 35 MPI) showed reduced appetite, weight loss, and neurologic signs (fine-muscle twitching and progressive recumbency from sternal to lateral) that were consistent with signs seen with scrapie.\textsuperscript{8} During the next 24 months, 3 other sheep were euthanized because of conditions (lameness problems) unrelated to TSE, and the remaining 3 sheep were euthanized at he termination of the study (72 MPI, Table 1). One control sheep was euthanized at 37 months and the other at termination of the study (72 months).

Except for 1 animal (No. 0017; Table 1) with urethral blockage and 3 animals with hoof abscesses, clinically significant gross lesions were not observed in any of the animals. Microscopically, lesions characteristic of spongiform encephalopathy (SE) (vacuolation of neuronal perikarya and neuropil) were seen in the brain (Fig. 1 and Fig. 2) and spinal cord of the sheep with clinical neurologic signs (No. 0024, euthanized at 35 MPI, Table 1) and in 1 of the 4 sheep (No. 0021, euthanized at 72 MPI, Fig. 3; Table 1) that were euthanized at termination of the study. In the sheep with neurologic signs, SE lesions were present in most areas of the brain (except in the hippocampus and rostral cerebrum) but were more severe and extensive in the thalamus and cerebellum. In the thalamus there was widespread vacuolation in neuropil, but vacuolation of neuronal cytoplasm was not prominent. However, in the cerebellum, besides the presence of variable-sized vacuoles in the neuropil of the molecular layer, there were prominent single or multiple cytoplasmic vacuoles in Purkinje cells (Fig. 2). Examination of brainstem showed characteristic SE in various nuclei, including the dorsal nucleus of the vagus nerve (Fig. 1). However, spongiform change was much more severe and extensive in the pontine nucleus than in the nucleus of the dorsal vagus. At some sites of SE lesions, mild to moderate gliosis was evident. All 3 areas of the spinal cord (cervical, thoracic, and lumbar) had mild to moderate vacuolations in the neuropil of the gray matter.

In the brain of the sheep without clinical signs of disease (No. 0021, euthanized at 72 MPI; Table 1), SE lesions were confined to the obex (Fig. 3). They were comparatively less extensive than for the sheep with clinical signs of disease and were predominantly in the area of the dorsal nucleus of the vagus nerve (Fig. 3).

Non-SE lesions in the brain consisted of discrete foci in the dorsal medulla (usually in the lateral cuneate nucleus) with a few swollen neurons/axons and focal areas of spongiosis in the neuropil (Fig. 4; Table 1). These were present in 9 of 10 sheep (Table 1) and were indicative of neuroaxonal dystrophy, an age-related, histopathologic finding in sheep of this age,\textsuperscript{2} unrelated to prion disease. In 1 sheep (No. 0013) the thoracic spinal cord had mild multifocal dilated myelin sheaths devoid of axons (Wallerian degeneration) in the lateral and ventral funiculi. This was indicative of mild chronic traumatic injury to the spinal cord.

All central nervous system (CNS) and lymphoid tissues of the clinically affected sheep (No. 0024; Table 1) were positive for PrP\textsuperscript{res} as determined by IHC. In the brain, staining was most pronounced in medulla (Fig. 5) and colliculi, primarily appearing as diffuse and particulate, with a few small scattered aggregates, in gray matter. Intracytoplasmic staining of neurons was common but perineuronal staining was not observed in the medulla and colliculi. In cerebellum, staining was present throughout gray matter (Fig. 6), but was most concentrated in the granular layer. A small amount of particulate staining was present multifocally in the white matter of the colliculi and cerebellum. Lesser amounts of staining were present in other parts of the brain, often appearing as subependymal patches. Perineuronal staining was prominent in cerebral cortex. Lymphoid tissue staining (follicular germinal centers) was extensive in tonsils (Fig. 7) and lymph nodes but limited in the spleen. Other tissues showing immunoreactivity were retina, which had diffuse particulate staining in the inner and outer plexiform layers (Fig. 8), and adrenal gland medulla (Fig. 9).

In the sheep without clinical signs of disease (No. 0021; Table 1), a similar pattern of immunoreactivity was present in CNS and tonsil (palatine and pharyngeal) tissue but staining was not observed in other lymphoid tissues. The staining in brain tissue was most prominent in the medulla, where it appeared as diffusely scattered particles in gray matter and perineuronal aggregates. In other parts of the brain, staining was confined to just a few small foci, often in subependymal locations. The hypothalamus was IHC negative. Diffuse staining was observed in the gray matter of all spinal cord sections. Similar to sheep No. 0024, PrP\textsuperscript{res} was present in retina (Fig. 10) and adrenal medulla tissue, but the amount
Figure 1. Medulla oblongata at the level of obex of sheep No. 0024 with clinical disease. There is extensive vacuolation of the neuropil and presence of multiple vacuoles in neuronal perykaria (arrows). Note the paucity of normal-appearing neurons. HE stain. Bar = 70 μm.

Figure 2. Cerebellum of sheep No. 0024 with clinical disease. Many variable-sized clear vacuoles are present in the molecular layer, and a few small vacuoles are in the granular layer. HE stain. Bar = 70 μm.

Figure 3. Medulla oblongata at the level of obex of sheep No. 0021 without clinical disease. There is extensive vacuolation of the neuropil. HE stain. Bar = 70 μm.

Figure 4. Dorsal medulla of sheep No. 0001 with mild neuroaxonal dystrophy. There are scattered swollen neurons (arrowhead) and vacuolar degenerative changes (arrows). HE stain. Bar = 70 μm.
Figure 5. Medulla oblongata at the level of obex of sheep No. 0024 with clinical disease. There is extensive diffuse PrP\textsuperscript{res} labeling in the neuropil. A few neurons show minimal staining. Stained for PrP\textsuperscript{res} labeling (red) by IHC (immunoalkaline phosphatase) and counterstained with HE. Bar = 70 \textmu m.

Figure 6. Cerebellum of sheep No. 0024 with clinical disease. The PrP\textsuperscript{res} diffuse labeling is present in molecular (m), Purkinje cells (p), and granular (g) layers but not in the white matter. Stained for PrP\textsuperscript{res} labeling (red) by IHC (immunoalkaline phosphatase) and counterstained with HE. Bar = 100 \textmu m.

Figure 7. Palatine tonsil of sheep No. 0024 with clinical disease. Most of the PrP\textsuperscript{res} labeling is present in the germinal center of a follicle. Stained for PrP\textsuperscript{res} labeling (red) by IHC (immunoalkaline phosphatase) and counterstained with HE. Bar = 100 \textmu m.

Figure 8. Eye; retina (with artifactual separation) of sheep No. 0024 with clinical disease. Extensive diffuse PrP\textsuperscript{res} labeling is
of staining was much less and was multifocal, rather than diffusely distributed.

Western blot analysis was performed on brainstem samples from all sheep. As shown in Fig. 11, only sheep No. 0021 and No. 0024 were positive for the presence of PrP<sub>res</sub>, whereas the other 6 CWD-inoculated sheep did not show any presence of PrP<sub>res</sub>, even after OIE SAF-Immunoblot enrichment<sup>12</sup> of the samples for the PrP<sub>res</sub> (data not shown). Positive samples showed the typical profile of 3 bands of proteinase K–resistant isoforms of PrP<sub>res</sub>, representing the diglycosylated, monoglycosylated, and unglycosylated polypeptides. Brainstem from sheep No. 0021 had a weaker WB reaction compared with brainstem from sheep No. 0024 at the same milligram equivalent brain tissue (Fig. 11). When these samples were compared to a brainstem sample from a CWD-positive mule deer (Fig. 11) and a scrapie-positive sheep (data not shown), the molecular weight profiles were similar but not identical.

Discussion

Cross-species transmission studies provide useful information for identifying potential host ranges of known TSE agents. CWD is a TSE and, like all other TSEs, it is characterized by a long incubation period, which in deer is seldom less than 18 months.<sup>18,19</sup> In cervids, clinical CWD is characterized by emaciation, changes in behavior, and excessive salivation.<sup>18,19</sup> Although the latter was not observed in 1 sheep with clinical signs of experimental CWD, the sheep had reduced appetite and weight loss and showed neurologic signs that were similar to those of scrapie in sheep. Morphologic lesions in this sheep were severe and were present throughout the CNS; its tissues were strongly positive for PrP<sub>res</sub> by IHC and Western blot.

These findings indicate that some domestic sheep are susceptible to CWD by experimental intracerebral inoculation. However, the single sheep (No. 0024, Table 1) that developed clinical disease at 35 MPI had a rare genotype for this breed at PRNP codon 136 (AV; Table 1), indicating that the susceptibility of sheep to CWD may be partially controlled by the host’s genetic makeup, similar to that reported for scrapie. As revealed by IHC, the localization of PrP<sub>res</sub> accumulation in brain and tonsils (palatine and pharyngeal) and retina in the 2 CWD-positive sheep was similar to that seen in Suffolk sheep with scrapie.<sup>8</sup> Staining was seen, in variable degrees of intensity, in the sections of brain and spinal cord examined, with the exception of the hypothalamus of sheep No. 0021. Stain character was predominately particulate and diffuse or multifocally extensive in distribution, but it also appeared as small aggregates of particles in scattered foci. Staining of the inner and outer plexiform layers of the retina was characterized by diffusely (sheep No. 0024) or multifocally (sheep No. 0021) distributed particles. Staining in IHC-positive lymphoid organs was confined to the germinal centers of follicles and appeared as particulate or small aggregates of particles. However, unlike the sheep with clinical signs of disease (No. 0024 with AV at codon 136), in which all lymph nodes examined were positive, none of the lymph nodes were IHC-positive in sheep No. 0021. This difference and the prolonged incubation time for sheep No. 0021 are possibly related to the differences in the PRNP gene (Nos. 0024 and 0021 were AV and present in both inner and outer plexiform layers. Stained for PrP<sub>res</sub> labeling (red) by IHC (immunoalkaline phosphatase) and counterstained with HE. Bar = 70 μm.

Figure 9. Adrenal gland of sheep No. 0024 with clinical signs of disease. Diffuse PrP<sub>res</sub> labeling is present in medulla (m). Stained for PrP<sub>res</sub> labeling (red) by IHC (immunoalkaline phosphatase) and counterstained with HE. Bar = 70 μm.

Figure 10. Eye; retina (with artifactual separation) of sheep No. 0021 without clinical signs of disease. Mild multifocal PrP<sub>res</sub> labeling is present in both plexiform layers. Stained for PrP<sub>res</sub> labeling (red) by IHC (immunoalkaline phosphatase) and counterstained with HE. Bar = 70 μm.

Figure 11. Western blot analysis using monoclonal antibody 6H4 showing distinct profile of PrP<sub>res</sub> in CWD-infected sheep No. 0021 and No. 0024. A CWD-positive mule deer isolate (No. 12783) was used a positive control. No specific signal is seen in sheep No. 001, 002, 0007, 0011, 0013, 0016, and 0017, which were classified as WB negative. Molecular weight markers in kilodalton are indicated on the right side of the blot.
AA, respectively, at codon 136; Table 1). That is, AV at codon 136 is associated with increased susceptibility to scrapie.\(^7\)

When brainstem samples of CWD-infected sheep were analyzed for the presence of PrP\(^{res}\) using WB, only 2 sheep (Nos. 0021 and 0024) were positive. Comparison of the molecular profile of the sheep CWD samples with a brainstem sample from a CWD-positive mule deer (Fig. 11) and a scrapie-positive sheep (data not shown), revealed similar but not identical molecular profiles. Interestingly, the PrP\(^{res}\) polypeptides of the sheep scrapie control sample had a lower molecular weight compared to both the CWD sheep and mule deer brainstem samples. Brainstem from sheep No. 0021 did have a weaker WB reaction, compared with brainstem from sheep No. 0024 (Fig. 11). This indicates that sheep No. 0021, which was euthanized without clinical signs at 72 MPI, was subclinically infected with CWD. The amount of PrP\(^{res}\) present in the CNS of this sheep appeared to be notably less than in sheep No. 0024, which had neurologic signs of disease at 35 MPI.

In the present study, the possibility that the PrP\(^{res}\) seen in tissue sections represented residual CWD material from the inoculum was ruled out because of the multifocal distribution of PrP\(^{res}\) throughout the brain and the spinal cord of both sheep. Had the PrP\(^{res}\) represented residual inoculum, it would probably have been confined to the sites of deposition in the midbrain or cerebrum. Moreover, studies of sheep scrapie\(^9\) showed that intracerebrally inoculated brain material containing PrP\(^{res}\) was present for only a few days in sufficient quantity to be detectable immuno-histochemically.

The observation of neuroaxonal dystrophy in brainstems of 9 of 10 sheep is not surprising. It has been observed as a physiologic age-related change in older sheep\(^2\) and various other animals.\(^10\)

This study involved intracerebral inoculation of CWD\(^{nd}\) agent to sheep. This is an unnatural route and is only an oblique reflection of the potential for sheep to become infected under natural conditions of exposure. Based on the low attack rate of the current intracerebral inoculation (IC) study, it is likely that transmission of CWD to sheep by a more natural route, such as per os would likely require a much larger dose of inoculum and may be much more difficult to accomplish within the normal life span of the animal. On the other hand, experimental studies of CWD from other cervid species (elk and white-tailed deer) have not been documented in livestock. Preliminary studies (Hamir et al., unpublished data, 2006) of intracerebral inoculation of CWD from white-tailed deer into cattle suggests that this source is much more efficient at causing disease (as indicated by the attack rate) than CWD\(^{nd}\).

At this time a final assessment of relative risk for CWD transmission to sheep is not possible. However, results of this study show that the diagnostic confirmatory tests used for scrapie surveillance in the United States would also allow detection of CWD in sheep, should it occur in this country.

Thus far, among domestic animals, CWD\(^{nd}\) has been transmitted by the intracerebral route to a goat\(^18\) and cattle.\(^5,7\) The present findings demonstrate that it is also possible to transmit CWD\(^{nd}\) agent to sheep via the intracerebral route. However, the only sheep to develop clinical TSE within 35 MPI was genotypically AV at PRNP codon 136, suggesting that host genotype may play a notable part in successful transmission of the disease in this species. Although in Suffolk sheep the AV variant at codon 136 is very rare,\(^17\) selective breeding of Suffolk sheep with this codon has begun in the hope of testing this differential susceptibility hypothesis in a future study of CWD\(^{nd}\) transmission to sheep.

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a. Prionics-Check, Schlieren-Zurich, Switzerland.

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