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### **Preliminary findings on the experimental transmission of chronic wasting disease agent of mule deer to cattle**

Amir N. Hamir, Randall C. Cutlip, Janice M. Miller, Elizabeth S. Williams, Mick J. Stack, Michael W. Miller, Katherine I. O'Rourke, Melanie J. Chaplin

**Abstract.** To determine the transmissibility of chronic wasting disease (CWD) to cattle and to provide information about clinical course, lesions, and suitability of currently used diagnostic procedures for detection of CWD in cattle, 13 calves were inoculated intracerebrally with brain suspension from mule deer naturally affected with CWD. Between 24 and 27 months postinoculation, 3 animals became recumbent and were euthanized. Gross necropsies revealed emaciation in 2 animals and a large pulmonary abscess in the third. Brains were examined for protease-resistant prion protein (PrP<sup>res</sup>) by immunohistochemistry and Western blotting and for scrapie-associated fibrils (SAFs) by negative-stain electron microscopy. Microscopic lesions in the brain were subtle in 2 animals and absent in the third case. However, all 3 animals were positive for PrP<sup>res</sup> by immunohistochemistry and Western blot, and SAFs were detected in 2 of the animals. An uninoculated control animal euthanized during the same period did not have PrP<sup>res</sup> in its brain. These are preliminary observations from a currently in-progress experiment. Three years after the CWD challenge, the 10 remaining inoculated cattle are alive and apparently healthy. These preliminary findings demonstrate that diagnostic techniques currently used for bovine spongiform encephalopathy (BSE) surveillance would also detect CWD in cattle should it occur naturally.

Since about 1967, a scrapie-like transmissible spongiform encephalopathy (TSE), chronic wasting disease (CWD), has

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been identified in captive and free-ranging cervids in Colorado and Wyoming.<sup>15,17–20</sup> Chronic wasting disease has been documented in mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*).<sup>9,15,17–20</sup> The disease has been experimentally transmitted by intracerebral inoculation of brain from mule deer into a variety of wild and laboratory animal species.<sup>19</sup> Except for intracerebral transmission of CWD to one goat,<sup>19</sup> CWD has not been transmitted to other domestic animals.

The primary objectives of this study were to determine if

the CWD agent could be transmitted to cattle and to provide information about clinical course, lesions, and suitability of currently used TSE diagnostic procedures for detection of CWD in cattle.

Sixteen 4–6-month-old calves of mixed breed (primarily red and black Angus) were purchased from a herd of cattle outside the CWD endemic area and were assigned to inoculated ( $n = 13$ ) and control ( $n = 3$ ) groups. Inoculated calves were housed in a Biosafety Level 2 isolation barn (2 animals per pen) at the National Animal Disease Center, Ames, Iowa. They were fed pelleted growth and maintenance rations that contained no ruminant protein, and clean water was available *ad libitum*. Control calves were housed together in an open shed and fed pelleted growth ration and alfalfa hay. Personnel wore protective clothing while in the isolation facility and showered when leaving the facility.

The inoculum was prepared from a pool of 28 CWD-affected mule deer brains. The inoculum was positive for scrapie associated fibrils (SAFs) by negative-stain electron microscopy and for protease-resistant prion protein (PrP<sup>res</sup>) by Western blot.<sup>14</sup> In addition, this pool was infective for orally inoculated mule deer.<sup>14</sup> The inoculum was ground in a mechanical grinder, gentamicin was added at 100  $\mu\text{g}/\text{ml}$ , and the final concentration of 10% (wt/vol) was made with physiological buffered saline (PBS).

Calves were inoculated intracerebrally with 1 ml of the CWD brain inoculum as described previously.<sup>4</sup> Briefly, the calves 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 drilled 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. Three calves (controls) were not inoculated.

Animals were euthanized with pentobarbital and a complete necropsy was conducted on each of the carcasses. Representative samples of all major organs were immersion fixed in 10% neutral buffered formalin. For the purpose of this report, only observations on central nervous system tissue are documented. The brain was cut longitudinally, and one half of the brain was fixed in formalin for not less than 3 weeks and the remainder of the brain was frozen. The formalin-fixed brain was cut into 2–4-mm-wide coronal sections. Sections of various anatomic sites (10–15 sections per animal) of cerebrum, cerebellum, brainstem (including the obex), and spinal cord (cervical, thoracic, and lumbar) were processed for routine histopathology, embedded in paraffin wax, and sectioned at 5  $\mu\text{m}$ . The sections were stained with hematoxylin and eosin (HE) and by an immunohistochemical (IHC) method<sup>8</sup> for detection of PrP<sup>res</sup>, with or without formic acid pretreatment.<sup>1</sup> Three different primary antibodies were used, including a rabbit polyclonal antibody<sup>12</sup> and 2 monoclonal antibodies, F89/160.1.5 and F99/97.6.1.<sup>10,11</sup> The latter 2 antibodies would recognize PrP sequences conserved in most mammalian species in which natural TSEs have been reported.<sup>10</sup>

For immunodetection of PrP<sup>res</sup>, a Western blot method<sup>a</sup> was used on frozen brain (caudal medulla).<sup>13</sup> Briefly, the method consisted of homogenization of tissues in a buffer<sup>a</sup> using a homogenizer<sup>b</sup> with disposable probes at 20,000 rpm

for 1 minute to give a 10% suspension. Protease treatment was carried out with 2 U/ml proteinase K at 47 C for 45 minutes. The protease was inactivated using digestion stop.<sup>a</sup> Suspensions were diluted 1:1 in sodium dodecyl sulphate sample buffer<sup>a</sup> heated to 96 C for 5 minutes and subjected to electrophoresis on 12% precast gels<sup>c</sup> at 200 V for 30 minutes. Each sample was processed in duplicate, and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) at 150 V for 1 hour at +4 C in a transfer tank equipped with plate electrodes.<sup>d</sup> At this stage, proteins bound to the PVDF membrane were identified by staining with 0.25% (w/v) Ponceau S dissolved in 0.25% acetic acid. This was destained with tris buffered saline plus 0.5% Tween-20 (TBST) until the red color had disappeared. The membranes were then incubated in PVDF blocking buffer<sup>a</sup> for 1 hour at room temperature to block nonspecific protein binding sites. PrP<sup>res</sup> was detected by incubation with monoclonal antibody (mAb) 6H4,<sup>a</sup> diluted 1:5,000 in blocking buffer, for 12–18 hours at 4 C. The membranes were then washed 4  $\times$  7 minutes with TBST and incubated with goat antimouse IgG antibodies coupled to alkaline phosphatase (AP) diluted at 1:5,00 in blocking buffer. After another 4  $\times$  7 minute wash, the PVDF membrane was allowed to equilibrate for 5 minutes in luminescence buffer<sup>a</sup>; excess buffer was then drained, and 5 ml of a substrate<sup>e</sup> was added for 5 minutes. The PrP<sup>res</sup> was characterized by its partial protease resistance, i.e., the N-terminal 60–70 amino acids was cleaved while the rest of the protein remained intact. This led to a reduction in molecular mass from about 35 to 27–30 kDa, which was visualized by developing and capturing the chemoluminescent reaction using a multimaginer. Computer software<sup>d</sup> was used to scan the resultant images from the PVDF membrane to the computer. Animals were considered positive when the protein core of 27–30 kDa was still present after proteinase K enzyme treatment.

Scrapie-associated fibrils (SAFs) were detected in fresh brain (caudal medulla) using negative-stain electron microscopy.<sup>16</sup> Where only formalin-fixed brain was available, a modified SAF extraction technique was used.<sup>2</sup>

Twenty-two and 23 months after intracerebral inoculation, 2 animals (Calves 1745 and 1768; Table 1) gradually become inappetent and lost weight. At about the same time, one of these animals (Calf 1768) became apprehensive and circled aimlessly in its pen. The other Calf 1745) became listless but was excited by loud noises. These behavioral changes continued with little variation until death of animals 8 and 14 weeks later. The altered behavior was subtle and was most obvious to the animals' daily caretaker. Both animals were found recumbent (24 and 26 months postinoculation; Table 1) and were euthanized. Following these 2 deaths, a control animal (Calf 1732; Table 1) was euthanized to obtain tissues for histopathology and to test for the presence of PrP<sup>res</sup>. A third animal (Calf 1744; Table 1) developed lameness in one leg at approximately 27 months postinoculation. Because it had overgrown hooves, its feet were trimmed under general anesthesia. Recovery was uneventful and the animal's gait appeared to improve. However, a week later, it was found recumbent and was euthanized.

At necropsy, 2 animals were emaciated (Calves 1745 and 1768; Table 1), but other gross lesions were not evident. Calf

**Table 1.** Preliminary findings in 3 bovines experimentally inoculated with the agent of chronic wasting disease (CWD) and in a control uninoculated animal.\*

Ear tag no.	CWD inoculation	Date euthanized	Incubation period	Disease course	Clinical signs	Histopathology	IHC	SAF	WB
1745	i/c	8/18/99	22 mo	2 mo	+	+/-	+	-	+
1768	i/c	9/22/99	23 mo	3 mo	+	+/-	+	+	+
1732	Control	11/17/99	NA	NA	-	-	-	-	-
1744	i/c	1/29/00	27 mo	3 days	+/-†	-	+	+	+

\* IHC = immunohistochemistry for PrP<sup>res</sup>; SAF = scrapie-associated fibrils; NA = not applicable; WB = western blot (Prionics-Check<sup>®</sup>); + = lesions or antigen present; - = lesions or antigen absent; +/- = signs/lesions equivocal; i/c = intracerebral.

† This animal was euthanized after acute lameness and recumbency.

1744 (Table 1), which was in a good nutritional state, had a large (approximately 20 cm in diameter) pulmonary abscess in one of the diaphragmatic lobes. The abscess contained copious, thick, greenish, purulent material that was surrounded by a thick, fibrous capsule. Other lesions were not seen in this animal or in the control animal (Calf 1732; Table 1).

Results of neurohistopathological findings and the PrP<sup>res</sup> tests are summarized in Table 1. Microscopic examination of HE-stained brain and cervical cord sections revealed isolated vacuolated neurons, a few degenerate axons and mild astrocytosis in 2 CWD-inoculated cattle (Calves 1745 and 1768; Table 1). Axonal degeneration and vacuolated neurons were in the medulla oblongata at the level of the obex of animal 1768. The degenerate axons did not involve any particular nuclei but were some distance from the dorsal vagus nucleus (Fig. 1), whereas the vacuolated neurons were in the accessory cuneate nucleus (Fig. 2). The control animal (Calf 1732) had moderate numbers of vacuolated neurons in the red nucleus.

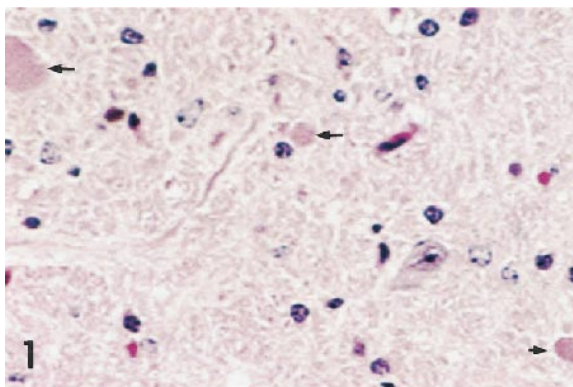
The PrP<sup>res</sup> was detected by IHC in the brains of all 3 inoculated cattle. The anatomical distribution and staining pattern was similar with all 3 antibodies, with or without formic acid treatment of sections. The anatomical distribution of PrP<sup>res</sup> was widespread, appearing in the cervical spinal cord and throughout the brain except in the cerebellar folia. The greatest amount of staining was present in the medulla oblongata and in the midbrain. The staining pattern

was multifocal (Fig. 3), and most of the reactivity was concentrated in or around astrocytes (Fig. 4). There was also some scattered particulate or granular staining in neuropil, and small plaques (up to 40  $\mu$ m in diameter) were occasionally observed (Fig. 5). Staining in neuronal cytoplasm was uncommon, and there was no perineuronal or perivascular staining. The PrP<sup>res</sup> was not present in tissue sections from brain of the control animal.

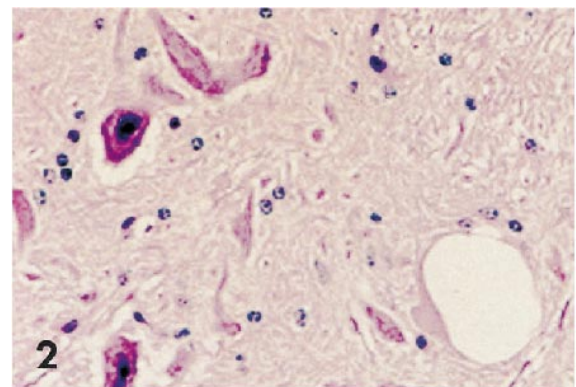
The PrP<sup>res</sup> was detected by Western immunoblotting in brain material from all 3 inoculated animals. A clear profile of the 3 glycosylated bands (diglycosylated, monoglycosylated, and unglycosylated) could be seen, although only a weak signal was present for animal 1744 (Fig. 6).

The SAFs were detected in formalin-fixed brain tissue extracts from animal 1744 (Fig. 7) and fresh tissue extracts from animal 1768 but not from the other inoculated animal or the control.

Chronic wasting disease is a transmissible spongiform encephalopathy and, like all other TSEs, it is characterized by a long incubation period, which in deer is seldom less than 18 months.<sup>19</sup> In cervids, clinical CWD is characterized by emaciation, changes in behavior, and excessive salivation.<sup>20</sup> Although the latter was not observed in the 3 CWD inoculates described in this study, 2 animals (Calves 1745 and 1768; Table 1) developed reduced appetite and had considerable weight loss approximately 2 years after inoculation and their carcasses were emaciated at necropsy. These animals also showed subtle behavioral changes that were more

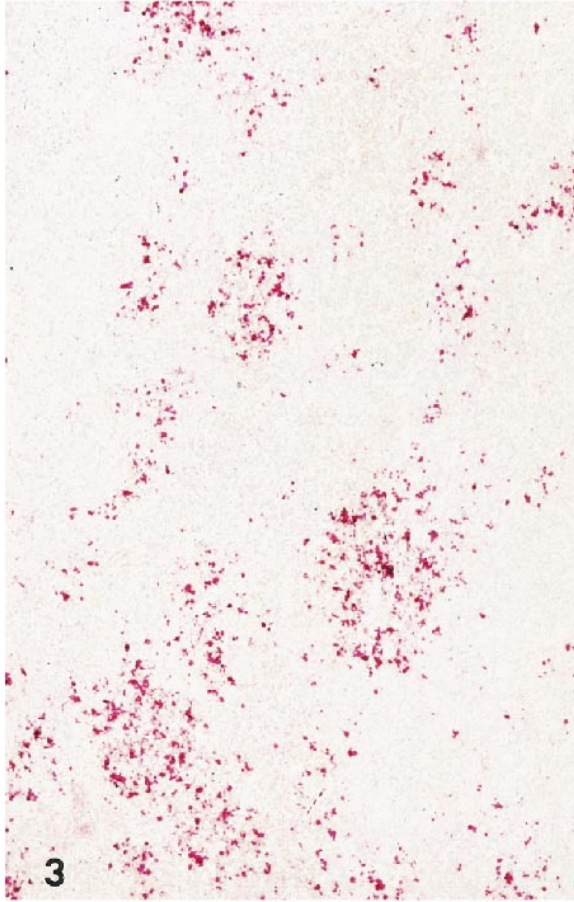


**Figure 1.** Brain; medulla oblongata of animal 1768. Several hyaline eosinophilic degenerate axons (arrows) are present near the nucleus of dorsal vagus (not showing the photomicrograph). HE 200 $\times$ .



**Figure 2.** Brain; medulla oblongata of calf 1768. A neuron with a single large vacuole is present in the accessory cuneate nucleus. HE 200 $\times$ .





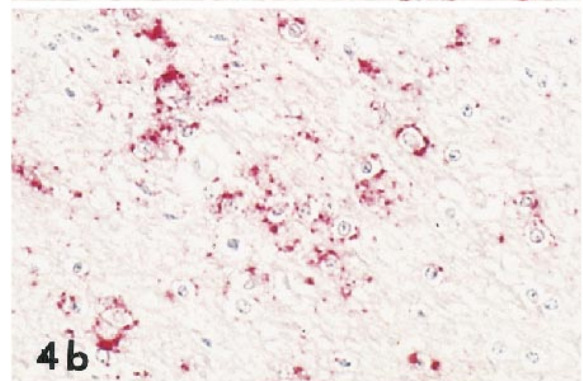
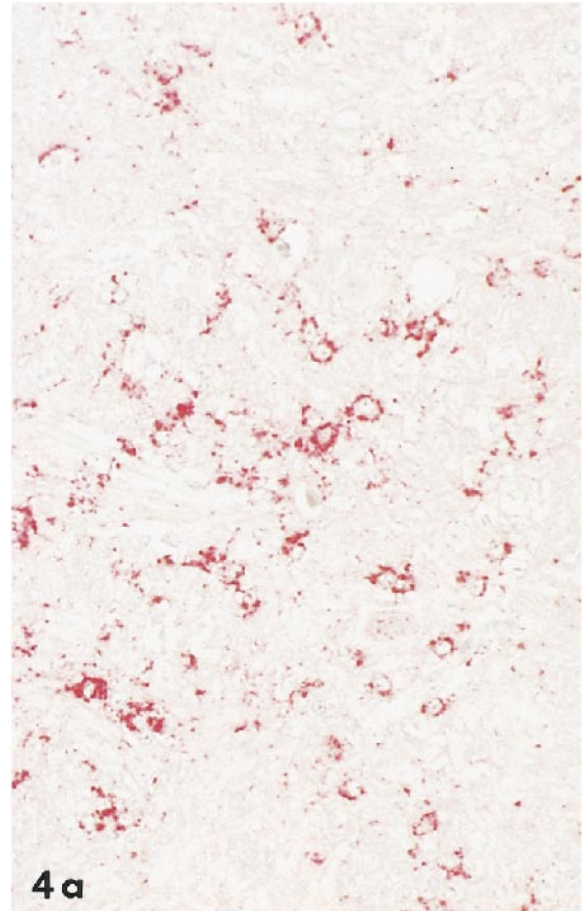
**Figure 3.** Brain; medulla oblongata of calf 1745. Multiple foci of PrP<sup>res</sup> staining (red) are present. Stained for PrP<sup>res</sup> by IHC. 30X.

obvious to animal caretakers who had frequent contact with the animals.

Morphological lesions in the current study were subtle in the first 2 affected animals and absent in the third animal. However, all 3 animals were positive for PrP<sup>res</sup> by IHC and Western blot, and in 2 of the animals, SAFs were detected. These findings indicate that some domestic cattle are susceptible to CWD by experimental intracerebral inoculation. However, only 3 of 13 animals have died to date, and the outcome of inoculation of the remaining cattle remains to be determined. In a previous experiment,<sup>4</sup> 100% of cattle inoculated intracerebrally with the US-scrapie agent died 14–18 months after inoculation.

It could be argued that the PrP<sup>res</sup> seen in tissue sections was residual CWD material from the inoculum. However, if this were true, then the PrP<sup>res</sup> would most likely have been observed as locally extensive areas of PrP<sup>res</sup> in the midbrain and cerebrum (site of inoculation). Instead, there was a multifocal distribution of PrP<sup>res</sup> throughout the brain (excluding cerebellar folia) and also in the cervical spinal cords of all 3 inoculated animals.

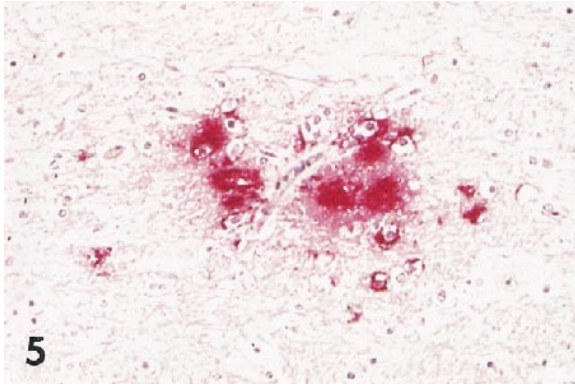
The localization of PrP<sup>res</sup> accumulation in brains of CWD-inoculated cattle was unusual because the primary target seemed to be astrocytes. The PrP<sup>res</sup> accumulation in astrocytes has been previously reported in a study of early scrapie



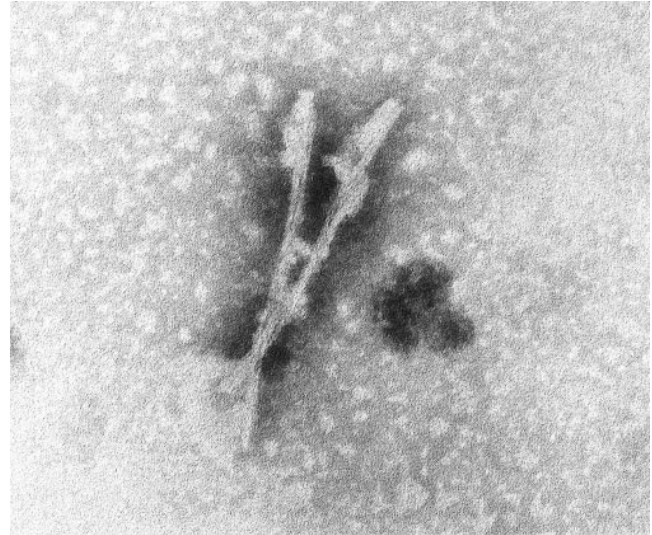
**Figure 4.** (a) Brain; medulla oblongata of calf 1745. (b) brain; cerebral cortex of calf 1744. The PrP<sup>res</sup> accumulations are located predominantly in or around astrocytes. Stained for PrP<sup>res</sup> by IHC. 200X.

pathogenesis in experimentally inoculated mice.<sup>5</sup> It should be noted that, because only 3 of 13 CWD-inoculated cattle have died thus far, the astrocytic PrP<sup>res</sup> may represent an early pathologic response to inoculation and a different IHC pattern could emerge later in the course of disease. This same caveat applies to the absence of clearly defined clinical signs or characteristic TSE histologic lesions in these 3 animals.

The astrocytic pattern of IHC staining CWD-inoculated cattle was distinctly different from the PrP<sup>res</sup> distribution ob-



**Figure 5.** Brain; thalamus of calf 1768. A cluster of several small PrP<sup>res</sup> plaques. Stained for PrP<sup>res</sup> by IHC. 125 $\times$ .

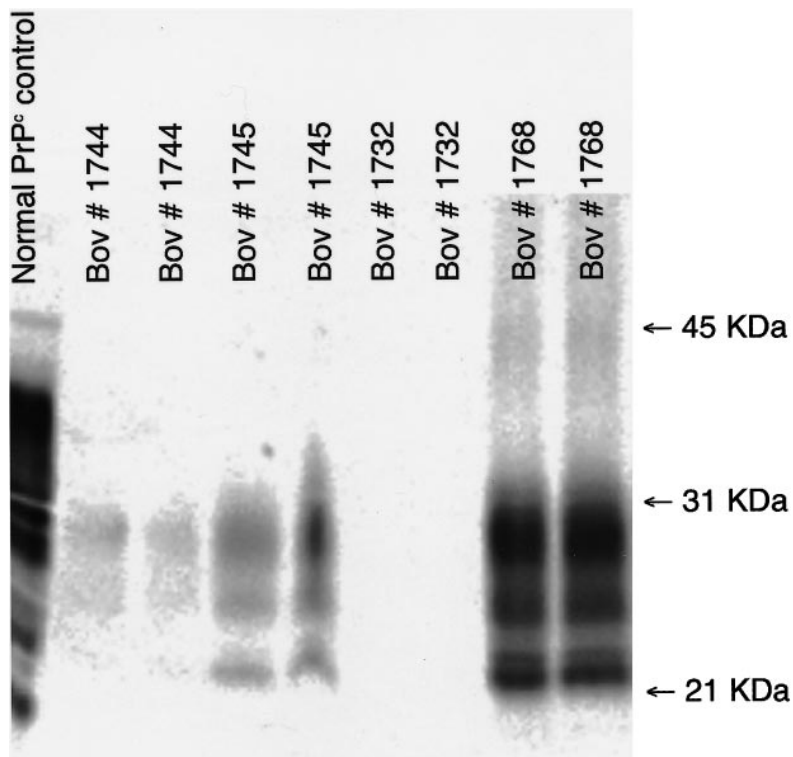


**Figure 7.** Negatively stained scrapie associated fibril (SAF) observed in formalin-fixed tissue extract (caudal medulla) from calf 1744. 156,000 $\times$ .

served in cattle inoculated intracerebrally with brain homogenate from scrapie-affected sheep.<sup>4</sup> In those animals, PrP<sup>res</sup> was almost totally restricted to neuronal cytoplasm, a staining pattern that persisted even after second passage in cattle.<sup>3</sup> It is intriguing that 2 such different PrP<sup>res</sup> staining patterns are produced in cattle by intracerebral inoculation of TSE agents, depending on the source of inoculum (scrapie or CWD). It should be noted that both of these staining patterns differ from the staining reactions described in animals with naturally acquired scrapie, BSE, or CWD.<sup>6-8</sup> In those diseases, the IHC reactivity is described as a diffuse particulate staining of gray matter neuropil, with occasional plaques

also being present in scrapie and CWD, especially in the latter.<sup>6-8</sup>

This study involved intracerebral inoculation of CWD agent to cattle. This is an unnatural route and as such has little bearing on the potential for cattle to become infected under natural conditions of exposure. It is likely that trans-



**Figure 6.** Western immunoblot showing distinct profile of PrP<sup>res</sup> (molecular weight 27–30 kD) in the 3 positive animals (Calves 1744, 1745, 1768). No signal is seen in calf 1732, classified as negative. A normal PrP<sup>c</sup> control (molecular weight 33–35 kD) is in the first lane. Molecular weight marker positions are indicated on the right-hand side of the image.



mission of CWD to cattle by a more natural route, such as *per os* as seen with scrapie in sheep, would be much more difficult or even impossible to accomplish. Such an experiment is currently in progress at the University of Wyoming, and approximately 3 years into the study, the exposed cattle remain healthy.

At the time of writing this article (approximately 3 years after inoculation), the 10 remaining intracerebrally inoculated cattle are alive and apparently healthy. They will be kept under observation for a further period of at least 4 years before the experiment is terminated.

Although a final assessment of relative risk for CWD transmission to cattle is not possible at this time, these preliminary results show that the diagnostic tests used for BSE surveillance in the USA would allow detection of CWD in cattle, should it occur.

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### Sources and manufacturers

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- b. Polytron homogenizer, OMNI GLH, OMNI International, Cambridge Ltd., Nuffield Road, Cambridge, UK.
- c. NuPage, Novex, Groningen, The Netherlands.
- d. Fluor-S MAX, Bio-Rad, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK.
- e. CDP-Star, Tropix Inc., PE Biosystems, Birchwood Science Park, Warrington, Cheshire, UK.

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