Transmission of sheep scrapie to elk (Cervus elaphus nelsoni) by intracerebral inoculation: final outcome of the experiment


Abstract. This is a final report of an experimental transmission of sheep scrapie agent by intracerebral inoculation to Rocky Mountain elk (Cervus elaphus nelsoni). It documents results obtained in experimental (n = 6) and control (n = 2) elk. During the first 2 years postinoculation (PI), 3 animals died or were euthanized because of infection or injuries other than spongiform encephalopathy (SE). In years 3 and 4 PI, 3 other inoculated elk died after brief terminal neurological episodes. Necropsy of these animals revealed moderate weight loss but no other gross lesions. Microscopically, characteristic lesions of SE were seen throughout the brain and spinal cord, and the tissue was positive for proteinase K–resistant prion protein (PrP\textsuperscript{res}) by immunohistochemistry (IHC) and by Western blot. Scrapie-associated fibrils (SAF) were observed by negative-stain electron microscopy in the brain of elk with neurologic signs. PrP\textsuperscript{res} and SAF were not detected in the 3 inoculated elk necropsied during the first 2 years or in the 2 control animals. Retrospective analysis of the gene-encoding cervid PrP revealed a polymorphism at codon 132. The elk with SE were either homozygous (MM) or heterozygous (LM). These findings confirm that intracerebral inoculation of sheep scrapie agent results in SE with accumulations of PrP\textsuperscript{res} in the central nervous system of elk. Based on morphologic and IHC findings, the experimentally induced SE cannot be distinguished from chronic wasting disease of elk with currently available diagnostic techniques.

Experimental cross-species transmission of transmissible spongiform encephalopathy (TSE) agents provides valuable information for identification of potential host ranges of known TSEs.\textsuperscript{17} Scrapie is a naturally occurring TSE of sheep and goats and has been proposed as a possible source of chronic wasting disease (CWD) in cervids.\textsuperscript{22} Transmissible spongiform encephalopathy–infected animals have accumulations of an abnormal form of prion protein (PrP\textsuperscript{res}) in tissues of the CNS and lymphatic system. Detection of PrP\textsuperscript{res} in these tissues and characteristic histopathological changes in the brain are the basis of currently available diagnostic methods for TSEs.\textsuperscript{5}

In a previous publication, preliminary findings of experimental inoculation of sheep scrapie to elk through the intracerebral route were documented.\textsuperscript{27} This communication describes details of clinicopathological results of the study that was terminated in the fall of 2002, approximately 4 years after the study was initiated.

Experimental design, biocontainment protocol, selection of inoculum, inoculation procedure, sample collection, and diagnostic test methods have been published previously.\textsuperscript{27} Briefly, the experiment involved 8, 3- to 4-month-old male castrated elk calves. The calves were assigned to scrapie-inoculated (n = 6) and control (n = 2) groups.

A detailed necropsy was conducted within 1 hour after death or euthanasia of the animals. Tissue samples for histopathology were immersion fixed in 10% neutral buffered formalin for not less than 3 weeks before processing for histology. Tissues included representative sections of liver, kidney, spleen, skin, striated muscles (heart, tongue, diaphragm, masseter), thoracic aorta, thyroid gland, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), nerves (sciatic, optic, trigeminal), pituitary gland, Gasserian ganglion, brain (cerebral cortex, cerebellum, midbrain including anterior colliculi, brainstem including obex), spinal cord (cervical, thoracic, lumbar), and eye (retina). Tissues were embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) and by immunohistochemistry (IHC) for detection of PrP\textsuperscript{res}.

For immunohistochemistry (IHC), an automated technique was used. After deparaffinization and rehydration, tissue sections were autoclaved for 30 min in an antigen retrieval solution\textsuperscript{28} and stained with an indirect avidin–biotin system\textsuperscript{29} designed for an automated immunostainer.\textsuperscript{3} For all nonlymphoid tissues, the primary antibody was a cocktail of 2 monoclonal antibodies,\textsuperscript{30} F89/160.1.5\textsuperscript{14} and F99/97.6.1,\textsuperscript{18} each used at a concentration of 5 μg/ml, and incubation was carried out at 37 C for 32 minutes. For lymphoid tissues, only the 99/97.6.1 antibody was used, at a concentration of 10 μg/ml. The F89/160.1.5 antibody was not used because it can produce false-positive reactions in lymphoid tissues of deer.\textsuperscript{18} For all tissues, the secondary antibody was biotinylated anti-mouse,\textsuperscript{3} diluted 1:200, and incubated for 8 min at 37 C.

Frozen brainstem was used for immunodetection of PrP\textsuperscript{res} using a commercial Western blot (WB) method,\textsuperscript{31} and for

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identification of scrapie-associated fibrils (SAF). Rostral cerebrum was used to determine PrP genotypes by a previously described method. Within the first 2 years postinoculation (PI), 3 animals (Nos. 1–3; Table 1) died or were euthanized because of unrelated brain infection or self-sustained physical injuries.

At 25 months PI, elk No. 4 (Table 1) died after a brief neurological episode (falling and paddling with its legs while in lateral recumbency) that lasted for approximately 1 minute. Before this terminal episode, the elk had developed a slightly hostile attitude toward the animal caretakers. However, at that time it was presumed that the behavior was within the normal range of a wild animal in confinement. Ten and 21 months later (35 and 46 months PI, respectively; Table 1), elk Nos. 5 and 6 exhibited similar terminal neurological signs and died within 20 and 60 minutes, respectively, after becoming recumbent. Before the appearance of neurological signs, both elk had shown slightly reduced appetites for approximately 4 to 6 weeks and thus had lost some body weight. The uninoculated control elk (Nos. 7 and 8, Table 1) were euthanized at 47 months, and the carcasses were examined in a manner similar to that of the inoculated animals.

Significant gross lesions were not observed in any of the 3 inoculated animals with neurological signs (Nos. 4–6; Table 1). Microscopically, however, severe lesions characteristic of SE (vacuolation of neuronal perikarya and neuropil) were seen in the brain (Fig. 1) and spinal cord. Morphologically, the severity and distribution of spongiform changes were similar in all 3 elk. At affected sites there were also mild multifocal increases in glial cells, but neuronal degeneration was not a prominent feature in any of the sections.

In the brain, the SE lesions were most 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. 1). Examination of brainstem showed characteristic spongiform lesions in various nuclei, including the dorsal nucleus of the vagus nerve. However, spongiform change was much more severe and extensive in the pontine nucleus than in the nucleus of the dorsal vagus. Significant lesions were not present in any of the other examined tissues.

Immunohistochemistry revealed PrPSc in all areas of brain (cerebrum, cerebellum, brainstem) and spinal cord (cervical, thoracic, lumbar) from elk with SE (only the cervical cord was available from elk No. 5; Table 1). The immunostaining was diffusely distributed throughout gray matter neuropil in all parts of the central nervous system (CNS), including both granular and molecular layers of cerebellum (Fig. 2). The staining pattern was primarily punctate and granular, with some small aggregates and only a few accumulations large enough to be described as plaques. Some neurons contained large stained granules in the perikaryon, but more commonly the location of PrPSc was perineuronal, forming a ring around the cell.

Staining for PrPSc was also detected by IHC in 1/3 Gasserian (trigeminal) ganglia and 3/3 retina of elk with SE
Figure 1. Brain; cerebellum of elk No. 63. Spongiform encephalopathy characterized by variable-sized clear vacuoles is present in neuropil of the molecular layer and in Purkinje cells. HE. 200×.

Figure 2. Brain; cerebellum of elk No. 63. There is presence of PrP<sup>res</sup> in molecular (m) and granular (g) layers of the cerebellum. PrP<sup>res</sup> staining is not present in the cerebellar white matter (w). Stained for PrP<sup>res</sup> by IHC. 100×.
(Table 1; Figs. 3, 4). At both locations the staining was granular. At the former location it was present within isolated neurons (Fig. 3), whereas in the latter it was diffusely seen in the outer and inner plexiform layers with multifocal extension into the ganglion cell layer, where it was predominantly perineuronal (Fig. 4). Prion protein staining was not present in the area of optic discs or in optic nerves.

Brain of the 3 elk with SE (Nos. 4–6; Table 1) was positive for PrP\textsuperscript{res} by WB (Fig. 5) and for SAF by negative-staining electron microscopy (Table 1).

None of the 3 animals that died during the first 2-year PI period (Nos. 1–3; Table 1) had lesions of SE and none was positive for PrP\textsuperscript{res} by IHC or WB. Similar findings were observed in control elk (Table 1).

Analysis of brains for PrP genotype at codon 132 revealed that 5 elk were MM homozygous and 2 were LM heterozygous. Elk No. 46 was not genotyped (Table 1).

Transmissible spongiform encephalopathies in both domestic and wild ungulates are characterized by long incubation times. For example, scrapie in sheep and goats can take between 2 to 4 years duration for manifestation of clinical signs,\textsuperscript{4} and the incubation period of CWD in elk is at least 18 months.\textsuperscript{22} As in these TSEs, the incubation time of scrapie agent in elk Nos. 4–6 was quite long (25–46 months, Table 1). Although the transmission route used in this investigation does not mimic the natural route of infection (which in the wild would most likely be through ingestion), the study does demonstrate that elk are experimentally susceptible to the scrapie agent. It is speculated that experimental oral inoculation of elk with the scrapie agent would have required a higher infectious dose and may have resulted in longer incubation time for the disease to develop.

Clinical cases of CWD in elk are characterized by severe emaciation, changes in behavior, and excessive salivation.\textsuperscript{23} Although in this study, some abnormal behavior in one animal (hostile to animal handlers) and terminal neurologic signs in others were noticed, these were not considered predominant features of this experimental infection.

Although in this study spongiform changes were more severe in certain anatomic locations of the brain (cerebellum, thalamus, pontine nucleus), in general the pattern was similar to that reported in elk with CWD.\textsuperscript{24} In contrast, successful intracerebral transmission of the scrapie and CWD agents to cattle did not result in significant neurohistopathologic lesions.\textsuperscript{1–3,10} The finding, therefore, of extensive SE in elk inoculated with scrapie agent could reflect biological differences of the host species, strain or titer differences in the inocula used, or a similar pathogenicity of scrapie and CWD agents for elk. The latter alternative suggests that scrapie and CWD agents may be closely related. However, incubation periods of these agents in experimentally inoculated raccoons are significantly different (2 years for scrapie; no transmission after 4 years for CWD), suggesting substantial lack of homology. Strain typing of scrapie and CWD agents may resolve this question.

The pattern of PrP\textsuperscript{res} immunostaining in the brains of elk inoculated with scrapie agent was similar to that described previously in elk with naturally acquired CWD.\textsuperscript{5,16} Although large PrP\textsuperscript{res} plaques were uncommon and PrP\textsuperscript{res} accumulations around blood vessels were not observed. Also absent were the “florid plaques” considered characteristic of CWD in captive mule deer.\textsuperscript{5,12} Florid plaques, which consist of PrP\textsuperscript{res} accumulations surrounded by vacuoles, are considered pathognomonic for variant Creutzfeldt–Jakob Disease in humans.\textsuperscript{11,21}

Staining of PrP\textsuperscript{res} in the plexiform layers of retina has been described previously in scrapie-affected sheep.\textsuperscript{10,12} However, in elk retina the PrP\textsuperscript{res} also multifocally extended into the ganglion cell layer. Although PrP\textsuperscript{res} was detected by IHC in retina of the 3 clinical elk with SE, no staining was observed.
in corresponding optic nerves. Similarly, in sheep with natural scrapie, PrP staining has been reported in retina but not in optic nerves. Because the most likely route for prion deposition in retina would have been through the optic nerve, failure to observe PrPres in these nerves was surprising and suggests that the presence of PrPres in optic nerves is transient and may occur in the initial phase of the disease.

In elk with CWD there is usually PrPres in lymphoid organs. However, none of the elk inoculated with scrapie showed PrPres staining in lymphoid tissues (Table 1). This observation was not surprising. Because the elk were inoculated by the intracerebral route, the inoculum would have essentially been confined to the CNS.

Results of WBs using the commercial Prionics-Check® technique suggest that the scrapie agent had a similar molecular profile after intracerebral inoculation into elk. In a recent publication, a modified version of this technique was used to differentiate between natural scrapie cases and sheep experimentally infected with bovine spongiform encephalopathy. In this study, analysis for PrP genotype was done on brain samples obtained at necropsy. This investigation was done retrospectively because at the time the experiment was initiated in 1998, information regarding genetic susceptibility of elk to CWD and its association with PrP polymorphisms was not documented. In the preliminary report of this experiment, it was suggested that because only 2 inoculated elk had developed TSE (Nos. 4 and 5, Table 1) by the end of 3 years PI, the remaining animal might not be genetically susceptible to scrapie. Retrospective sequence analysis of the gene-encoding PrP showed that the last elk to develop SE was heterozygous (LM) at codon 132, whereas the first 2 elk were MM at that site (Table 1). Because previous research suggested that MM homozygosity may predispose elk to CWD, it is tempting to speculate that the longer incubation period observed in animal No. 6 reflects a genetic influence of codon 132 heterozygosity.

In conclusion, findings of this study show that the sheep scrapie agent can be transmitted to elk by intracerebral inoculation in 100% of inoculates that survive for 2 years PI. The resulting infection manifests as severe, widely distributed microscopic SE, accompanied by accumulations of PrPres in the CNS. With the aid of currently available diagnostic tests, the transmitted SE in these animals could not be differentiated by histopathology or IHC from lesions of CWD in elk.

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Sources and manufacturers

a. DAKO® Target Retrieval Solution, DAKO Corp., Carpinteria, CA.
b. Basic Alkaline Phosphatase Red Detection Kit, Ventana Medical Systems, Inc., Tucson, AZ.
c. NexES IHC module, Ventana Medical Systems, Inc., Tucson, AZ.
d. Dr. Katherine O’Rourke, USDA Agricultural Research Service, Animal Diseases Research Unit, Pullman, WA.
e. Biotinylated anti-mouse IgG (made in horse), Vector Laboratories, Burlingame, CA.
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References


**Effect of feeding green onions (Allium ascalonicum) to White Chinese geese (Threskiornis spinicollis)**

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Abstract. Sudden increase in mortality was observed in 2 different flocks of mature breeder geese fed green onions. At necropsy, birds had pale epicardium with random petechiation, sanguinous fluid accumulation in the pericardial sac, and mild swelling of the liver and spleen. Histologically, there was accumulation of hemosiderin in hepatocytes, Kupffer cells of the liver, macrophages, and renal tubules. There was also moderate to severe hepatic necrosis, vacuolation of hepatocytes, splenitis, and renal tubular nephrosis. To assess the effects of green onion ingestion, 2 feeding trials were carried out in 3 mature White Chinese geese. In the first trial, onions were thoroughly mixed with pellet maintenance ration. In the second trial, onions were offered in a separate trough from the pelleted diet. During the 21 days of experiments, the red blood cell count and hematocrit decreased, whereas the polychromasia and reticulocyte estimate increased. The blood changes were more marked in birds from the second feeding trial. Gross and histologic changes were similar in both trials. Mild swelling and severe darkening of the liver were the only significant findings at necropsy. Histologically, the liver looked similar to that seen from the field outbreak. The liver contained moderate amounts of hemosiderin in the hepatocytes and Kupffer cells, and had centrolobular necrosis and vacuolation of hepatocytes. This experimental study demonstrated that anemia and liver pathology could be caused by ingestion of onions. Furthermore, Heinz bodies are not a consistent finding in the blood of geese fed onions.

In areas where onions are grown commercially, it is common practice to use culled onions as a source of feed for livestock. The ingestion of onions has been associated with hemolytic anemia accompanied by the formation of Heinz bodies within the erythrocytes in cattle, cats, horses, dogs, and sheep,¹ most farmers use onions in strictly limited quantities. However, there are no reports describing the effects of onions in avian species. This brief communication describes the pathology found in 2 independent goose flocks associated with ingestion of green onions and the results from an experimental trial in which geese were fed this vegetable.

Sudden increase in mortality was observed in 2 different flocks of mature breeder geese. The first case occurred in November 2001, where 6% mortality was observed in a flock of 1,400 breeders of 11 different goose breeds. Most of the birds (95%) that died were White Chinese geese (Threskiornis spinicollis). The second case was in August 2002, and mortality as high as 8.4% was observed in a 2-day period in a flock of White Chinese geese (634 birds). In both cases, birds had been fed a pelleted maintenance ration (110–160 g/day/bird) plus free choice of green onions (Allium ascalonicum) for 7–10 days. In both cases, after onions were removed from the diet, mortality in the flock went back to normal levels.

A total of 12 adult geese (6 birds from each of the flocks) were submitted to the California Animal Health and Food

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