



Intracerebral Transmission of Scrapie to Cattle

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To determine if sheep scrapie agent(s) in the United States would induce a disease in cattle resembling bovine spongiform encephalopathy, 18 newborn calves were inoculated intracerebrally with a pooled suspension of brain from 9 sheep with scrapie. Half of the calves were euthanatized 1 year after inoculation. All calves kept longer than 1 year became severely lethargic and demonstrated clinical signs of motor neuron dysfunction that were manifest as progressive stiffness, posterior paresis, general weakness, and permanent recumbency. The incubation period was 14–18 months, and the clinical course was 1–5 months. The brain from each calf was examined for lesions and for protease-resistant prion protein. Lesions were subtle, but a disease-specific isoform of the prion protein was present in the brain of all calves. Neither signs nor lesions were characteristic of those for bovine spongiform encephalopathy.

Scrapie in sheep is the prototype of a group of naturally occurring diseases of humans and animals that is characterized by progressive neurodegeneration resulting in spongiform encephalopathy [1, 2]. Other members of the group are Creutzfeldt-Jakob disease, Gerstmann-Straussler-Sheinker syndrome, kuru and fatal familial insomnia of humans [1, 3, 4], scrapie of goats [5], chronic wasting disease of captive mule deer and elk [6, 7], transmissible mink encephalopathy [8], and bovine spongiform encephalopathy (BSE) [9], the most recently identified member of the animal group.

Scrapie is an ancient disease of sheep that was first described in detail by Comber in the United Kingdom in 1772 [10]. Earlier clinical descriptions indicate that the disease probably was present in Europe as early as 1700 [10]. The disease currently is endemic in most countries of the world, with only Australia and New Zealand recognized as free of

scrapie by most countries [11]. Scrapie was first diagnosed in the United States in 1947 in a flock of Suffolk sheep in Michigan [11, 12]. Other diagnoses of scrapie were made within a few years in flocks in Ohio, Illinois, and California. All cases were traced to sheep imported from the United Kingdom via Canada. The disease gradually spread and now has been diagnosed in 43 states. Most of the cases have been reported in farm flocks east of the Missouri River. From 1952 until 1983, the disease was kept in check by an eradication plan requiring the slaughter of all sheep in a flock in which the disease was diagnosed. This included the source flock after 1957. In 1983, because of the recognition of the influence of host genetics upon the disease [13–15], the regulation was changed to require slaughter only of affected sheep and their female bloodlines. Since then, the reported incidence of scrapie cases has gradually increased [11].

The mode of natural transmission of scrapie is uncertain and has both infectious and hereditary components. Lateral transmission has been demonstrated but is slow and infrequent [16, 17]. Experimental transmission by intracerebral, oral, and subcutaneous routes has been demonstrated [18]. Intracerebral transmission by injection of a suspension of brain from sheep with scrapie has been demonstrated in sheep [19], goats [20], mice [21], mink [22], and squirrel monkeys [23]. The disease was also transmitted intracerebrally to rats and hamsters after the agent was adapted to goats and mice, respectively [24, 25]. Oral transmission has been shown in goats [26], mice [21], and monkeys [23].

BSE was first diagnosed and characterized in the United Kingdom in 1986 [9]. In many respects, the disease resembles scrapie, and epidemiologic evidence indicates the infectious agent is transmitted from sheep to cattle through feeding of rendered by-product from infected sheep [27]. Spongiform neurodegeneration in several other species may be of the same origin as BSE [28]. The other species affected

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are nyala (*Tragelaphus angasi*) [29], gemsbok (*Oryx gazella*) [29], eland (*Taurotragus oryx*) [30], Arabian oryx (*Oryx leucomyx*) [31], greater kudu (*Tragelaphus strepsiceros*) [31], domestic cat (*Felis domesticus*) [32], cheetah (*Acinonyx jubatus*) [33], and puma (*Felis concolor*) [34].

The etiology of scrapie and related spongiform encephalopathies is complex and controversial. Bacteria and unconventional viruses have been implicated, but efforts to identify foreign nucleic acid in the brains of affected animals have failed [2, 35, 36]. Familial forms of the human disease are known as are genetic influences that control length of the incubation periods in sheep and mice [37-39]. Current evidence indicates that the diseases are caused by protease-resistant isoforms of a normal cellular protein that slowly accumulates in neurons, causing cell damage and death [35, 40]. Also, evidence has been presented that the isoform, called scrapie prion protein or resistant prion protein (PrP-res), is formed after translation by neurons along an endocytic pathway or at the plasma membrane by conformational changes in the normal protein, called cellular prion protein or sensitive prion protein (PrP-sen) [41-43]. In contrast to PrP-sen, which is degraded by protease, PrP-res is highly resistant to protease and is believed by some to accumulate in neurons following internalization and truncation by lysosomal enzymes [41-43]. Other evidence indicates that only part of the PrP-sen is degraded by lysosomal proteases and that the intact protein cycles between the cell surface and an endocytic compartment [44]. The suggestion was made that PrP-res may be formed along this cyclic pathway. Formation of PrP-res is influenced by genetic factors and perhaps by exogenous PrP-res acting as a template for production of additional PrP-res. Both factors are believed to be linked to the prion gene but neither have any influence on the rate of formation of PrP-res, which is believed to be solely dependent upon the rate of formation of PrP-sen [45].

The increased incidence of scrapie reported in the United States and the epidemiologic evidence that BSE in the United Kingdom was transmitted from sheep to cattle by feeding rendered by-products has heightened concern in the livestock and related industries in the United States. Therefore, this project was undertaken to determine if the US scrapie agent(s) could be transmitted to cattle and cause BSE. To do this, young calves were inoculated intracerebrally with brain tissue from sheep affected with scrapie. Half of the calves were euthanatized 1 year after inoculation, and tissues were examined for lesions and for PrP-res. Calves that later developed signs of neurologic disease and 2 control calves were euthanatized and tissues were examined for lesions and for PrP-res.

Materials and Methods

Inocula. Inoculum for challenge calves (scrapie sheep brain) was prepared from the brains of 9 sheep with signs and lesions of

scrapie. The sheep were from five flocks in Illinois, Iowa, South Carolina, and Wisconsin and were of Suffolk ($n = 7$) and Hampshire ($n = 2$) breeds. The brain from each sheep was aseptically removed from the calvarium, and half of the brain stem was ground with a mortar and pestle in saline containing 100 $\mu\text{g}/\text{mL}$ of gentamicin, to make a final concentration (wt/vol) of 10% brain. Each suspension was filtered through sterile gauze and examined on bovine blood agar for sterility after incubation at 37°C for 48 h. Sterile suspensions were pooled for inoculation of calves. Inoculum for control calves (normal sheep brain) was similarly prepared from the brain of a clinically normal Columbia breed sheep from a flock with no history of scrapie.

Cattle. Twenty-six male jersey ($n = 19$) and holstein-friesian ($n = 7$) calves were purchased at age 1 or 2 days from five suppliers and assigned to challenge and control groups. Challenge calves ($n = 18$) were housed in a BL-2 isolation facility and fed commercial milk replacer at the manufacturer's recommended rate, followed by pelleted growth and maintenance rations. Control calves ($n = 8$) were housed together in an open shed and fed milk replacer followed by growth ration and alfalfa hay. Calves had access to clean water and were maintained according to current American Association for Accreditation of Laboratory Animal Care guidelines. They were castrated and dehorned at 1-3 months of age. To prevent cross contamination of calves, personnel were required to wear protective clothing while in the challenge facility and to shower when leaving the facility.

Experimental procedure. Calves <1 week old (14 jerseys, 4 holsteins), were inoculated intracerebrally with 1 mL of scrapie sheep brain inoculum according to the method described by Dawson et al. [46]. The procedure consisted of sedating the calf with xylazine (50 $\mu\text{g}/\text{kg}$), making a 1- to 2-cm midline incision in the skin at the junction of the parietal and frontal bones, drilling a 1-mm hole through the calvarium, inserting a 22-gauge 9-cm needle into the midbrain, and injecting the inoculum as the needle was withdrawn. The skin incision was closed with a single suture. Five control calves (3 jerseys, 2 holsteins) were inoculated intracerebrally with normal sheep brain inoculum; 3 control calves (2 jerseys, 1 holstein) were not inoculated.

One year after challenge, 8 calves from the challenge group (1 jersey died of causes unrelated to scrapie) and 2 calves (1 intracerebrally inoculated and 1 not inoculated) from the control group were euthanatized with pentobarbital. Samples of brain, spleen, and a mesenteric lymph node near the ileocecal valve were examined for lesions by routine histopathologic methods and for PrP-res by immunohistochemical [47] and immunoblotting [48, 49] techniques.

Two immunoblotting techniques were used for 6 brains; their sensitivities were compared and the most sensitive test was used to examine the rest of the tissues. Differences in test sensitivities were attributed to the amount of brain tissue utilized for each test. For immunoblotting, samples were taken from the medulla oblongata and occasionally from other parts of the brain.

For histopathologic examination, tissues from six levels of the brain stem (obex, middle medulla oblongata, pons, midbrain, thalamus, and corpus striatum) and the cerebral and cerebellar cortices as well as spleen, mesenteric lymph node, and other major organs were fixed for ≥ 3 weeks in buffered 10% formalin.

processed by routine paraffin embedding procedures, sectioned at 4- μ m intervals, and stained with hematoxylin-eosin (H&E).

Brain sections were also stained for glial fibrillary acidic protein (GFAP) using monoclonal antibody (no. 360; Chemicon International, Temecula, CA), and sections of brain, spleen, and mesenteric lymph node were stained for PrP-res [47]. For the latter, tissues were held in PLP (0.01 M periodate, 0.1 M lysine, 0.125% paraformaldehyde) fixative for 7-14 days, trimmed, and held in 70% ethanol for 3-5 days, then further dehydrated and embedded by standard methods. Paraffin sections were mounted on positively charged glass slides (ProbeOn Plus; Fisher Scientific, Pittsburgh) and heated at 80°C for 10 min. Staining was done with an automated capillary action system (Code-On Slide Stainer; Fisher Scientific). Pairs of slides were positioned with the tissue sections in apposition so that reagents could be applied to both slides simultaneously. Detergents, as specified below, were added to aqueous reagents to maximize the rate of capillary flow. The solvent used for deparaffinization was prepared by mixing xylene with a dewaxing agent (Hemo-De; Fisher Scientific) at a ratio of 1:3. The first application of solvent was for 1 min at room temperature and was followed by two successive 5-min applications at 45°C. Slides were then immersed twice in solvent mixed 1:1 with 100% ethanol, followed by three treatments with ethanol alone. The slides were moved from the automatic stainer to a chemical fume hood for a 30-min exposure to 99% formic acid (Sigma Chemical, St. Louis). After the formic acid was removed, slides were returned to the stainer and rinsed eight times in distilled water than contained 0.25% Brij 35 (Biomed, Foster City, CA) and 0.1% Tween 20 (Bio-Rad Laboratories, Richmond, CA). Each water rinse was for 1 min. Slides were then rinsed 8 times in 95% ethanol, followed by a rinse in buffer (Automation Buffer; Biomed).

Next, a blocking solution, consisting of 0.45- μ m-filtered normal goat serum (Vector Laboratories, Burlingame, CA) diluted 1:5 in Automation buffer, was applied for 20 min at room temperature. This was followed by incubation in primary antibody (polyclonal rabbit anti-mouse-PrP-res antibody supplied by R. Rubenstein, Institute for Basic Research in Developmental Disabilities, Staten Island, NY), diluted 1:200 in blocking solution, overnight at 4°C. The slides were rinsed three times in Automation buffer and held in the buffer at 37°C for 30 min. Biotinylated anti-rabbit IgG (Vector Laboratories), diluted 1:200 in blocking solution, was applied at 37°C for 30 min. After a rinse in Automation buffer, slides were exposed for 20 min at room temperature to avidin-alkaline phosphatase complex (Ultra-Probe basic kit; Biomed), followed by three rinses in 0.1 M TRIS-HCl buffer, pH 8.2, with 0.25% Tween 20. This same buffer was used to prepare the substrate (alkaline phosphatase substrate kit I with Vector Red; Vector Laboratories) with the addition of levamisole as directed (Vector Laboratories). The substrate was centrifuged at 1000 g for 5 min, then applied at room temperature in three cycles of 5, 5, and 10 min, without intervening washes. After two rinses in distilled water, slides were stained for 4 min in Mayer's hematoxylin with 0.25% Brij 35 and 0.1% Tween 20, followed by two water rinses, a rinse in Automation buffer to "blue" the hematoxylin, and two more water rinses. Tissues were then dehydrated through the same

alcohol and solvent solutions used for hydration. A xylene-compatible permanent mounting medium (Permount; Fisher Scientific) was used for application of coverslips.

The remaining calves (9 challenge, 6 control) were held for further observation. When clinical signs resulting in permanent recumbency were seen, calves were euthanatized for humane reasons by intravenous injection of pentobarbital. Two calves died suddenly before recumbency was permanent. Calves with clinical signs of disease were examined similarly to those euthanatized 1 year after challenge, except three levels of spinal cord (cervical, thoracic, and lumbar) were also included in immunohistochemical and histopathologic procedures. In addition to the experimental calves, brains from 2 slaughtered steers of unknown history were examined histopathologically and immunohistochemically.

Results

There was no acute adverse effect from the intracerebral inoculation procedure in either challenge or control calves. Neurologic disease occurred in calves that were held >1 year after inoculation with scrapie sheep brain inoculum but not in calves that were euthanatized 1 year after inoculation or in control calves. The incubation period was 14-18 months, and the course of the disease was 1-5 months in calves that developed clinical signs of disease. Length of incubation and course of the disease were not mutually related.

Signs were insidious in onset and progressive until the calves died or were euthanatized. The first signs were lethargy and stiffness of the legs and back. Stiffness was usually associated with kyphosis, incoordination, posterior weakness and, in some calves, a slow incoordinated pace-like gait. Clonic spasms of muscle bundles in the rear legs were seen in 1 calf early in the course of the disease, some calves had transient recurring lameness of 24-48 h duration, and paddling while in lateral recumbency was seen in 1 calf late in the course of the disease. There was decreased appetite with some loss of weight in most calves. Lethargy was severe in all calves and was accompanied by a decrease of tactile and auditory sensory perceptions. Strabismus with bilateral exophthalmia occurred in 5 of the 9 calves.

Brain lesions were equivocal and inconsistent. No lesions were found in calves examined 1 year after inoculation or in control calves. Microscopic examination of H&E- and GFAP-stained sections of the brain from affected calves revealed only minor changes. Although some vacuolation of neutrophils and shrinkage and central chromatolysis of neurons was seen in the brain stem and spinal cord of some affected calves, this did not differ greatly from that seen in the unaffected control calves. One calf had small vacuoles in two neurons in the medulla oblongata and very granular chromatin in some shrunken neurons of the brain stem and spinal cord (figures 1 and 2). Large clear vacuoles were seen in a few neurons of the red nucleus but not in other areas of the brain in both challenge and control calves. Staining for



Figure 1. Medulla oblongata from clinically affected calf inoculated intracerebrally with scrapie agent. Note small vacuoles in shrunken neuron. Hematoxylin-eosin stain. Bar = 40 μ m.

GFAP showed that fibrous astrocytes were common in the central white matter of the cerebrum and cerebellum, especially near the junction with gray matter, but not substantially different from controls. Lesions were not present in other tissues.

PrP-res was present in the brains of all calves that were inoculated intracerebrally with scrapie sheep brain. Except for 2 calves that were examined 1 year after inoculation,



Figure 2. Granular staining of chromatin in neurons of lumbar cord from clinically affected calf after intracerebral inoculation with brain from sheep with scrapie. Hematoxylin-eosin stain. Bar = 32 μ m.



Figure 3. Pons from calf with clinical signs of disease stained for protease-resistant prion protein. Staining is confined to perikaryon and processes of neurons. Note granularity of staining, possibly representing accumulated prion protein in lysosomes. Bar = 143 μ m. Inset: same area at higher magnification. Bar = 38 μ m.

PrP-res was detected by both immunohistochemical and immunoblotting techniques (figures 3-5). In those 2 calves, PrP-res was detected by only one of the two techniques. The immunohistochemical technique showed that PrP-res was widely distributed in the brain stem and spinal cord within the perikaryon and processes of neurons but not in extraneuronal spaces. Subjective estimates indicated that the quantity of PrP-res was 10 or more times greater in calves with clinical disease than in calves euthanatized before clinical signs appeared. PrP-res was not present in the spleen or mesenteric lymph nodes of calves examined by immunohistochemical or immunoblotting techniques.

Discussion

We conclude that American sources of sheep scrapie are transmissible to cattle by direct intracerebral inoculation but that the disease induced is not identical to BSE as seen in the United Kingdom. While there were similarities in clinical

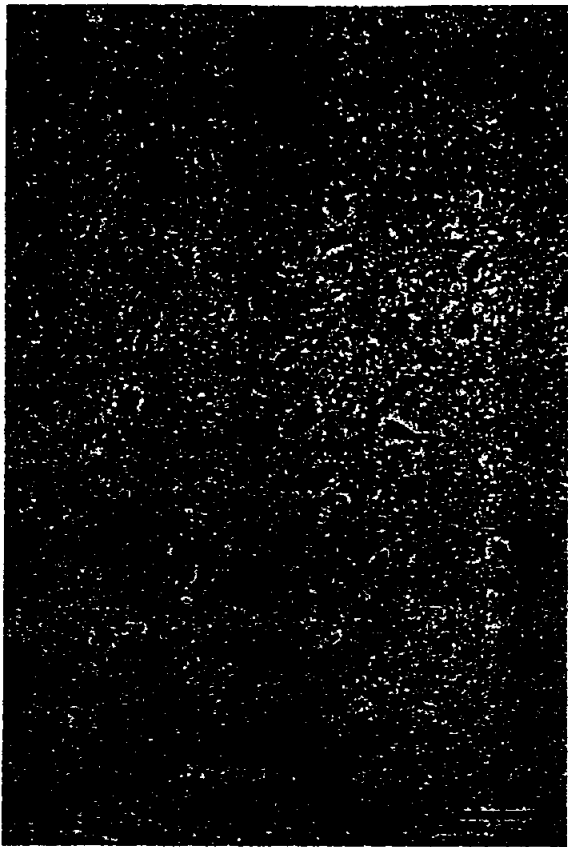


Figure 4. Pons from clinically normal control calf stained for protease-resistant prion protein. Bar = 92 μ m.

signs between this experimental disease and BSE, there was no evidence of aggressiveness, hyperexcitability, hyperesthesia (tactile or auditory), or hypermetria of limbs as has been reported for BSE [9]. Neither were there extensive neurologic lesions, which are primary for BSE, such as severe vacuolation of neurons and neuropil or neuronal necrosis and gliosis. Although some vacuolation of neuropil, chromatolysis in neurons, and gliosis were seen in the brains of some affected calves, these were indistinguishable from those of controls. Vacuolated neurons in the red nucleus of both challenged and normal calves were considered normal for the bovines as previously described [50].

PrP-res was found in all challenged calves regardless of clinical signs, and the amount of PrP-res positively related to the length of the incubation. The PrP-res was located by immunohistochemistry only in the body and processes of neurons, which is in contrast to the distribution in sheep where PrP-res is common in perivascular locations and abundant in gray matter neuropil [47]. Similarly, the distribution of PrP-res in cattle affected with BSE has been shown by immunohistochemistry to be primarily in extraneuronal spaces [51].

We also conclude from these studies that scrapie in cattle

might not be recognized by routine histopathologic examination of the brain and suggest that detection of PrP-res by immunohistochemistry or immunoblotting is necessary to make a definitive diagnosis. Thus, undiagnosed scrapie infection could contribute to the "downer-cow" syndrome and could be responsible for some outbreaks of transmissible mink encephalopathy as proposed by Burger and Hartsough [8] and Marsh and Hartsough [52]. Spongiform or prion diseases having aberrant neurologic lesions have been known in sheep and mink since 1976 [53, 54], and familial dementias of humans have been reported in which PrP-res was demonstrated immunohistochemically in the brain but lesions were not typical of spongiform disease [4, 55]. The route of inoculation or the amount of inoculum may have influenced the nature of the illness and types of lesions that were induced in our calves. The scrapie agent in other hosts can induce variable signs and lesions depending on the strain of the agent, species and strain of the host, route of inoculation, and number of passages in the host [53, 56, 57]. Experimental studies in sheep and mice have shown that the greatest variation in signs and lesions are dependent upon strain of the agent and genotype of the host [56, 58, 59]. Variations in signs and lesion profiles have been reported for other spongiform disease agents when introduced into foreign hosts [58, 60]. Multiple sources of sheep affected with scrapie and two breeds of cattle from several sources were used in the current study in an effort to avoid a single strain of either agent or host. Preliminary results from mouse inoculations indicate multiple

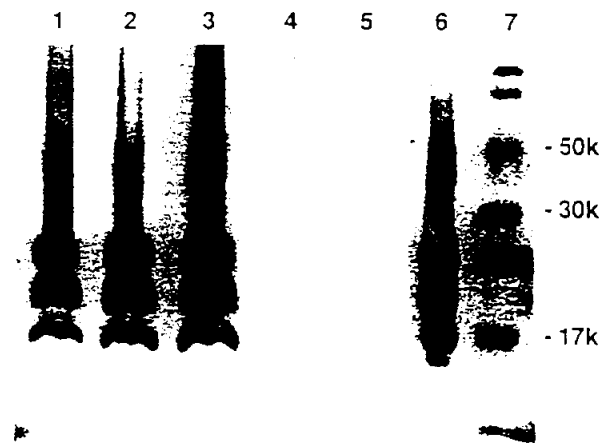


Figure 5. Immunoblot analysis of brain tissue for protease-resistant prion protein (PrP-res). Brain from calves inoculated with pooled homogenate of scrapie-affected sheep (lanes 1-3), inoculated with homogenate of normal sheep brain (lane 4), or not inoculated (lane 5). Lane 6 contains PrP-res derived from brain of scrapie-affected sheep. Lane 7 contains molecular weight standards from which approximate size of PrP-res bands can be estimated. Lanes 1-6 each contain 20 μ g equivalents of brain (wet weight before processing). Techniques of purification and band specificity have been described [48].

strains of the agent were present in the pooled inoculum (unpublished data). It is possible that additional passages through cattle would alter the character of the disease, resulting in signs and lesions that are more like those described for BSE. Additional studies will be required to answer these questions.

Transmission of the sheep scrapie agent to cattle was attempted in 1979 by using intracerebral, intramuscular, subcutaneous, and oral routes of inoculation of 5, 8- to 11-month-old cattle with a homologous mixture of brain from 1 affected sheep [61, 62]. One of the 5 cattle developed neurologic signs 48 months after inoculation. Signs were disorientation, incoordination, a stiff-legged stilted gait, progressive difficulty in rising, and finally in terminal recumbency. The clinical course was 2.5 months. Two of 5 cattle similarly inoculated with brain tissue from a goat with scrapie exhibited similar signs 27 and 36 months after inoculation. Clinical courses were 43 and 44 days. Brain lesions of mild gliosis and vacuolation and mouse inoculation data were insufficient to confirm a diagnosis of scrapie. This work remained controversial until recent examination of the brains detected PrP-res in all 3 cattle with neurologic disease but in none of the unaffected cattle [62]. Results of these studies are similar to ours and underscore the necessity of methods other than histopathology to diagnose scrapie infection in cattle. We believe that immunologic techniques for detecting PrP-res currently provide the most sensitive and reliable way to make a definitive diagnosis.

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