

# Scrapie Infectivity and Proteinase K–Resistant Prion Protein in Sheep Placenta, Brain, Spleen, and Lymph Node: Implications for Transmission and Antemortem Diagnosis

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Probable transmission of bovine spongiform encephalopathy to humans has focused intense interest on all of the transmissible spongiform encephalopathies (TSEs) and how they spread. In all TSEs, an abnormal disease-associated, proteinase K–resistant protein referred to as PrP-res or PrP<sup>Sc</sup> accumulates in brain. In some species, PrP-res accumulates in other tissues as well. Sheep placenta, brain, spleen, and lymph node were analyzed in detail for PrP-res and infectivity. Both were detected in all brain and spleen samples and in placenta and lymph nodes of 80% of the scrapie-infected sheep. A perfect correlation was observed between infectivity and PrP-res detection. These results substantiate the probability that placenta plays an important role in natural transmission of scrapie, suggest that analysis of placenta for PrP-res could be the basis for an antemortem test for sheep scrapie, and show that PrP-res, scrapie infectivity, and scrapie disease are closely associated.

Sheep scrapie has been present in the world for >250 years and as such is the oldest recognized member of a group of diseases now collectively known as the transmissible spongiform encephalopathies (TSEs). Over the past several decades, other TSEs have been discovered and now include bovine spongiform encephalopathy (BSE), mink encephalopathy, wasting disease of deer and elk, and feline spongiform encephalopathy of animals [1] as well as the human diseases Kuru, Creutzfeldt-Jacob disease (CJD), Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia, and the newest member, new variant CJD [2, 3]. Experimental evidence suggests that new variant CJD represents BSE in humans [4, 5]. It is postulated that cattle, in turn, may have acquired BSE by consuming meat and bone meal supplements derived from cattle infected with an unrecognized endemic bovine-adapted TSE or alternatively from supplements containing sheep scrapie agent [1]. As a result of these probable interspecies transmissions, TSE in general but particularly sheep scrapie and BSE have attracted the concern of the scientific community and general public.

In all TSEs, a characteristic proteinase K–resistant protein referred to as PrP-res or PrP<sup>Sc</sup> accumulates in brain of affected individuals. In some species, such as sheep, PrP-res also accu-

mulates in lymphoid tissue, including spleen, lymph node, and tonsil, and a close association has been shown between PrP-res detection in these tissues and the presence of scrapie disease [6–8]. As a result of this association, detection of PrP-res in tissue sections by immunohistochemistry [8–10] or in tissue homogenates by immunoblot [7, 11–13] are now the methods of choice for diagnosis of sheep scrapie and sometimes for diagnosis or confirmation of TSEs in other species as well.

Transmission of TSE under natural conditions appears to involve a variety of mechanisms. For example, some CJD cases are iatrogenic because of inadvertent transfer of infected tissue from an infected person to another, while other forms of CJD appear to be mediated by genetic factors. The majority of CJD cases, however, are sporadic [14]. Sporadic and inherited forms of TSE may involve transmission of an infectious agent, but because human TSE incubation periods are often measured in decades, it is impossible to define the sources and circumstances of exposure. Sheep scrapie, however, is clearly infectious, but precise mechanisms of spread are not fully understood. Infectious agent has not been detected in sheep urine, feces, saliva, serum, blood, milk, or colostrum [15–17]. However, sheep dosed orally with suspensions of fetal membranes obtained from scrapie-positive ewes killed during advanced pregnancy developed scrapie [18], and there are reports of scrapie transmission to mice from placenta also recovered from scrapie-infected ewes in advanced pregnancy [19]. So far, fetal membranes and placenta are the only tissues ordinarily associated with living sheep and naturally shed to the environment that have yielded infectious agent.

Here we analyzed both naturally expelled sheep placenta and placenta recovered from scrapie-positive ewes killed in

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Received 16 March 1998; revised 14 May 1998.

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**The Journal of Infectious Diseases** 1998;178:949–53  
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advanced pregnancy for infectivity and PrP-res. Brain, spleen, and lymph nodes from the ewes were also analyzed.

## Materials and Methods

**Preparation of tissue.** Tissues were obtained from 10 scrapie-positive and 8 normal Suffolk sheep. Scrapie-positive sheep were selected on the basis of clinical signs of scrapie, scrapie-consistent microscopic changes in brain, and/or detection of PrP-res in brain. Normal ewes were derived from flocks with no known exposure to scrapie and exhibited no clinical or microscopic evidence of disease. Pieces (1–2 g) of previously frozen spleen, lymph node, or placenta were dissociated by forcing the tissue through a sterile fine-mesh stainless steel screen with the plunger from a 10-mL plastic syringe to obtain a 20% suspension in 0.01 M Tris-HCl (pH 7.5) and 0.005 M MgCl<sub>2</sub>. When available, the cotyledonary area of fetal placenta was used. Aliquots (100–300 mg) of brain were dissociated in the same buffer with either a dounce homogenizer or disposable tube and pestle. Aliquots (1.0 mL) of these suspensions were frozen for subsequent bioassay. Deoxyribonuclease was added to the remaining suspensions (200 µg/g of original weight of tissue). After 1 h of incubation at 37°C, an equal volume of 20% sarkosyl in 0.01 M Tris-HCl (pH 7.5) was added, and incubation was continued at room temperature for 1 h. Suspensions were centrifuged at 10,000 g for 30 min at 10°C, and the supernatant was centrifuged at 215,000 g for 2.5 h at 10°C. Pellets were resuspended by sonication in 1.0 mL of distilled H<sub>2</sub>O. Proteinase K (20 µg) was added, and the suspension was incubated at 37°C for 30 min, after which 50 µL of 0.1 M phenylmethylsulfonyl fluoride was added, and incubation was continued for 15 min at 4°C. Suspensions were then centrifuged at 215,000 g for 1 h at 10°C. The pellet was resuspended in SDS-PAGE electrophoresis sample buffer, and immunoblot analysis was done as previously described using antisera R27 [7].

**Bioassay of sheep tissues for infectivity.** Frozen aliquots of the initial 20% tissue suspensions were thawed, vortexed for 2 min, and then sonicated for 2 min before dilution to 1% in physiologic buffered balanced salt solution containing 2% fetal calf serum. Aliquots (50 µL) were inoculated intracerebrally into Rocky Mountain Laboratory Swiss mice (12 mice/group). Mice were observed at least weekly for clinical evidence of scrapie (ataxia and progressive somnolence). Brains from 1 or 2 mice from each assay group in which scrapie was diagnosed on the basis of clinical appearance were analyzed for PrP-res by immunoblot to confirm the diagnosis.

## Results

In the present experiments, we sought to detect scrapie infectivity and PrP-res in sheep placenta, brain, spleen, and lymph node and determine the relationship between infectivity and PrP-res in each of these tissues. Tissues from 10 naturally infected and 8 uninfected Suffolk sheep were analyzed. Neither infectivity nor PrP-res was detected in the uninfected animals (data not shown). PrP-res was detected in 4 placentas shed naturally from ewes that were clinically normal at parturition but died of scrapie 126, 477, 174, and 470 days later (sheep

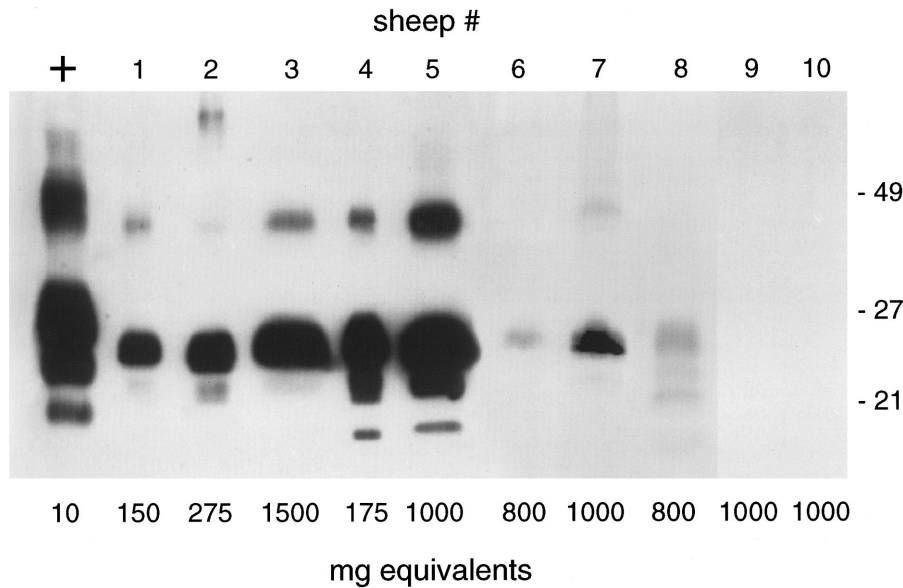
1, 2, 6, and 8, respectively, in figure 1). PrP-res was not detected in 2 other placentas shed 252 and 109 days, respectively, before sacrifice of the ewes (figure 1, sheep 9 and 10). In contrast, 4 placentas recovered at necropsy from pregnant ewes with terminal scrapie were PrP-res-positive (figure 1, sheep 3–5 and 7). A second placenta was recovered from sheep 2, 250 days before her death, and a second was recovered from sheep 8 at necropsy. Each of these placentas was PrP-res-negative (data not shown).

Visualization of PrP-res from placenta, by immunoblot, involved analysis of 150- to 1500-mg equivalents (wet weight) of tissue (figure 1). Some placentas (figure 1, nos. 1–5) would have scored as positive with much less tissue than was used on the immunoblot, whereas others (figure 1, placentas 6–8) would have been negative if less than the already large amount of tissue had been used. In contrast, visualization of PrP-res from brain generally required 1- to 20-mg equivalents of tissue, whereas spleen and lymph node required 200- to 1000-mg equivalents (data not shown). PrP-res banding patterns on immunoblots of sheep brain, spleen, and lymph node were identical to those shown before [7].

The predominant PrP-res protein species from placenta had a molecular mass of ~25–26 kDa, in contrast to brain, in which the predominant PrP-res species had a molecular mass of ~27–30 kDa (figure 1). PrP-res derived from spleen or lymph nodes gave molecular masses characteristic of brain [7]. Other less intense PrP-res bands were also smaller than their counterparts from brain (figure 1).

To prove that the protein bands visualized in placenta were PrP-res, we immunoblotted placenta from some samples (lanes + and 1–6 in figure 1) as before, but we preabsorbed the primary rabbit antibody with the synthetic PrP peptide antigen used to develop the specific antisera before addition to the blot. Protein bands that subsequently disappear are PrP-res-specific. Thus, the protein bands visualized in placenta are PrP-res (figure 2).

To determine if scrapie infectivity was present in sheep placenta, brain, spleen, and lymph node, we inoculated suspensions of these tissues from the 10 scrapie-positive sheep as well as brain from the 8 normal sheep into Rocky Mountain Laboratory Swiss mice. Mice were observed at least weekly for clinical evidence of scrapie. Scrapie infectivity was not detected in brain from the normal sheep (data not shown). Scrapie infectivity was detected in brain and spleen of all 10 scrapie-positive sheep and in lymph node and/or placenta of 8 of the 10 scrapie-positive sheep (figure 3). Four tissue samples, 2 from placenta and 2 from lymph node, did not cause disease in mice (figure 3). The same 4 samples gave no PrP-res signal on immunoblot, whereas the 36 samples positive for scrapie agent were also PrP-res-positive (immunoblots of brain, spleen, and lymph node; not shown). Thus, in the tissues analyzed for both PrP-res and infectivity, there was a perfect correlation between the presence of PrP-res in a given tissue and presence of detectable scrapie agent. Bioassay of the second placenta from sheep 2 and 8 was not done. The interval to



**Figure 1.** Immunoblot showing presence or absence of PrP-res in sheep brain or placenta. Lanes: +, brain derived from 1 scrapie-infected sheep; 1–10, placenta from 10 scrapie-positive sheep. Sheep placenta from 8 additional normal sheep gave same negative pattern as sheep 9 and 10 (data not shown). Placentas from sheep 1, 2, 6, and 8–10 were shed naturally, whereas placenta from sheep 3–5 and 7 were obtained at necropsy from scrapie-positive ewes in advanced pregnancy. Mg equivalents of tissue added to each lane are shown at bottom. Immunoblot was developed with antiserum R27. Indicated molecular masses were determined by comparing migration of individual protein bands with that of prestained molecular mass standards (not shown).

death in assay mice was always shortest and the percentage of assay mice dead of scrapie highest for brain. Intervals to death for assay mice inoculated with spleen, lymph node, or placenta were generally similar to each other except for the 4 tissue samples that gave no detectable infectivity (figure 3).

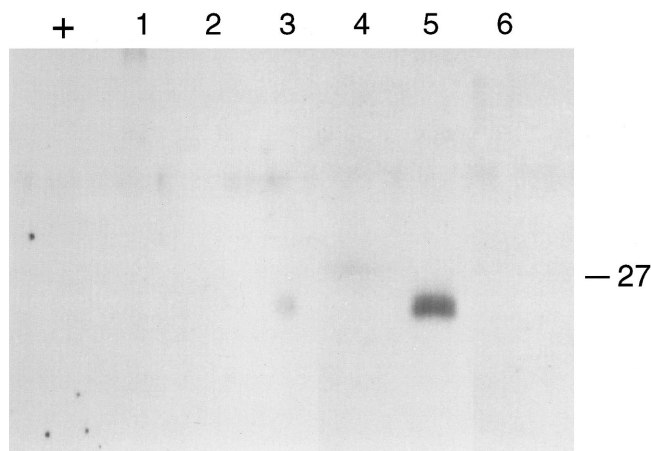
**Discussion**

Detection of scrapie infectivity in placenta from 8 of 10 scrapie-positive sheep substantiates the probability that pla-

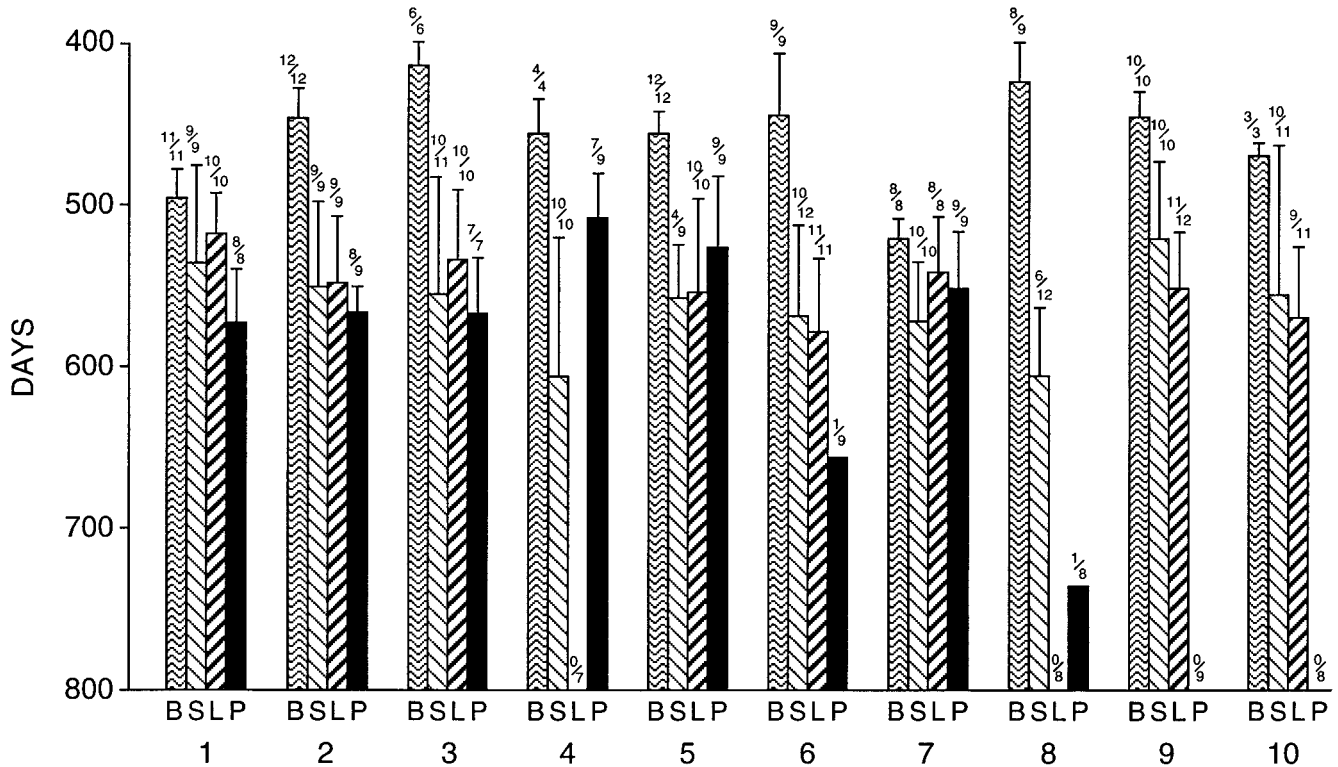
centa plays a predominant role in transmission of scrapie under natural conditions. Several aspects of sheep herd management support the possibility. When lambing, sheep are often commingled with one another in the same confined areas year after year. Because scrapie agent is extraordinarily resistant to inactivation [2, 20], it is likely that scrapie agent derived from infected placenta would accumulate in lambing areas over a period of years, giving ewes and lambs a high probability of contact with infectious material. Transmission between ewes or from ewe to lamb could also occur in a more direct fashion during and shortly after parturition. For example, occasionally ewes that are lambing at the same time will eat the placenta of other ewes, and ewes could become infected in this way. Also, the udder and perineum of ewes often becomes contaminated by placental discharge at parturition. Lambs could become infected directly by suckling the udder and perineum of their own mother or other ewes in the flock. All of these factors are consistent with the finding that the infection percentage is highest in sheep born to infected mothers in infected environments [21]. Sheep might also be exposed to scrapie agent under natural situations when they graze pasture or rangeland contaminated by tissues of sheep that have died and decayed.

Prion protein polymorphisms are associated with clinical scrapie in Suffolk sheep [22]. The scrapie-positive suffolk ewes in this study died of scrapie at 33–64 months of age, suggesting that they represented susceptible genotypes. Future studies should examine the relationship between infectivity and PrP-res detection in sheep preselected to represent all relevant genotypes and should include both clinical and nonclinical sheep.

Long incubation periods in bioassay mice inoculated with sheep placenta and the general requirement for substantial amounts of placental equivalents to visualize PrP-res on immunoblots of placenta suggest low levels of scrapie agent. However, it is well-documented that interspecies transmission of scrapie is inefficient [23–25]. It is therefore probable that the



**Figure 2.** Specificity of protein bands shown in figure 1. Aliquots of samples shown in figure 1, lanes + and 1–6, were immunoblotted as before, except anti-PrP serum R27 was preabsorbed with synthetic peptide used to develop R27 antiserum [7] (24 µg in 30 mL of 5% nonfat dried milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature before addition to filter. Protein bands that disappear are PrP-res. Band remaining in sheep 5 lane is either non-PrP protein or indicates that amount of peptide used in absorption was not sufficient to bind all of PrP-res in sample. Latter possibility is likely because sheep 5 band in figure 1 is intense.



**Figure 3.** Bioassay for presence or absence of scrapie agent in sheep tissues. Bar graphs show interval (days) from inoculation to death or sacrifice of scrapie-positive mice in each group. Nos. above graphs depict no. of mice that developed scrapie/no. at risk. No scrapie infectivity was found in any normal sheep analyzed (not shown). Although 12 mice were in each group initially, intercurrent nonscrapie deaths occurred in many groups before mice were at risk and were excluded from totals. Mice used for assay were inoculated intracerebrally with 50  $\mu$ L of 1% suspensions of tissues indicated. B = brain, L = lymph node, S = spleen, P = placenta. Individual sheep are numbered 1–10 along horizontal axis and correspond directly to sheep 1–10 in figure 1.

mouse bioassay system used to detect sheep-derived scrapie agent underestimates the amount of infectivity available for sheep. Thus, detection of even apparently small amounts of agent could represent a significant amount of infectivity and risk to sheep under natural conditions. Previous studies seem to support this conclusion. When fetal membranes derived from scrapie-infected ewes killed in advanced pregnancy were fed to other sheep, most of them developed scrapie [18]. Studies in which mice were inoculated with placenta gave inconsistent results. Infectivity but not PrP-res was recovered from placenta of 2 of 3 scrapie-infected ewes in one study [19] and in 0 of 2 in another study [16]. In the study reported here, 4 of the 5 placentas recovered at necropsy and 4 of 7 shed naturally were infectivity- and PrP-res-positive. Thus, infection of placenta may be even more prevalent than previous data would suggest. Precisely how soon scrapie agent begins to accumulate in placenta following infection is not known. Two of the naturally shed placentas (sheep 2 and 8) were derived from ewes that did not die of scrapie until 474 and 470 days later, suggesting that infectious agent may begin to accumulate in placenta early after infection of the ewe. Surprisingly, additional placentas obtained from ewe 2, 250 days before her death, and from ewe 8 at necropsy were PrP-res-negative even though earlier

placentas from these sheep were PrP-res- and infectivity-positive. Thus, infection of placenta in a given pregnancy was not necessarily associated with infection of placenta in subsequent pregnancies. Why early placentas from these 2 ewes were PrP-res-positive whereas later ones were negative will require more knowledge of mechanisms of agent transport in infected animals.

Detection of PrP-res in sheep placenta also has diagnostic significance. Although it is technically feasible to diagnose sheep scrapie in the living animal by surgically obtaining and analyzing lymph node, spleen, or tonsil [6–8], such an approach is not practical under most circumstances. Diagnosis is generally dependent on analysis of brain recovered at necropsy. However, finding more than half of the naturally shed placentas positive for PrP-res suggests that analysis of placenta could be the basis for an antemortem “flock” test for scrapie. By testing placenta from several sheep within a flock, it is likely that flocks in which scrapie is present would be identified. Trained persons could then monitor the flock for clinical evidence of disease or suggest further analysis. A significant advantage of testing placenta over existing techniques is that sheep need not be killed to obtain suitable tissue to test, and no specialized training is required of those collecting the placentas. Persons

raising sheep, who are unlikely to be familiar with the clinical signs and manifestations of scrapie, could themselves collect, freeze, and submit placentas for analysis.

Although we reported a very close association between PrP-res and scrapie disease, the association between PrP-res and infectious scrapie agent in sheep tissues has been presumed but never proven [7]. Of the 40 tissue samples analyzed in this study for both PrP-res and infectivity, 36 were positive for both PrP-res and infectious scrapie agent, whereas 4 were negative for both. Two PrP-res-negative placenta samples were not bioassayed. Therefore, there were no instances in which one test was positive and the other negative. Detection of PrP-res was strongly correlated with both scrapie disease and the presence of scrapie agent.

PrP-res bands derived from sheep placenta had lower molecular masses than did corresponding bands from brain. Because differences in PrP-res size and glycosylation patterns have been used to differentiate scrapie strains [26–28], we wondered if placenta might harbor a unique scrapie agent. To investigate this possibility, we analyzed PrP-res banding patterns in brains of mice that were inoculated with sheep placenta. The larger PrP-res species typical of brain were observed rather than those characteristic of placenta. Therefore, the differences in PrP-res size and glycosylation patterns in placenta and brain probably reflect different PrP-res processing in the different tissues [29]. Alternatively, proteolytic activity in placenta could differ from that in brain, resulting in truncation of the PrP-res molecule at different sites, resulting in the molecular mass differences. To firmly determine the factors responsible for PrP-res and glycosylation differences in placenta and brain as well to determine if a unique placenta-associated scrapie strain exists will require additional study.

#### Acknowledgments

We thank Darwin Ernst for expert technical assistance, Bob Evans and Gary Hettrick for graphic arts assistance, and Irene Cook Rodriguez for helping with the preparation of the manuscript.

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