Short Communication

Oral inoculation of neonatal Suffolk sheep with the agent of classical scrapie results in PrP^{Sc} accumulation in sheep with the *PRNP* ARQ/ARQ but not the ARQ/ARR genotype

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ABSTRACT

Scrapie is a transmissible spongiform encephalopathy that can be transmitted amongst susceptible sheep. The prion protein gene (*PRNP*) profoundly influences the susceptibility of sheep to the scrapie agent. This study reports the failure to detect PrP^{Sc} in nervous or lymphoid tissues of Suffolk sheep of the *PRNP* ARQ/ARR genotype after oral inoculation with a U.S. scrapie isolate. Lambs were inoculated within the first 24 hours of birth with 1 ml of a 10% (wt./vol.) brain homogenate derived from a clinically affected ARQ/ARQ sheep. The inoculated sheep were observed daily throughout the experiment for clinical signs suggestive of scrapie until they were necropsied at 86 months post inoculation. Tissues were collected for examination by immunohistochemistry and enzyme immunoassay, but all failed to demonstrate evidence of scrapie infection. Neonatal sheep of the ARQ/ARQ genotype receiving the same inoculum developed scrapie within 24 months. Lambs of the ARQ/ARR genotype that received the same inoculum by intracranial inoculation develop scrapie with a prolonged incubation period and with abnormal prion present within the central nervous system, but not peripheral lymphoid tissues. Results of this study suggest that ARQ/ARR sheep are resistant to oral infection with the scrapie isolate used even during the neonatal period.

Keywords

Prion protein, PRNP, scrapie, sheep, transmissible spongiform encephalopathy

Transmissible spongiform encephalopathies (TSE) or prion diseases are fatal neurologic diseases of animals and humans. Susceptibility to scrapie, the natural TSE of sheep, appears to be age dependent (St Rose et al.). Scrapie may be transmitted from infected ewes to their scrapie-susceptible fetus during gestation (Foster et al.; Garza et al.; Spiropoulos et al.) or to susceptible lambs sharing the same environment during the perinatal period. Abnormal prion protein (PrP^{Sc}) accumulates at the fetal-maternal interface within the ovine placenta (Tuo et al.) of scrapie infected ewes that are carrying lambs of susceptible genotypes (Alverson et al.). Thus, exposure from placenta and placental fluids is thought to be one of the main routes of scrapie transmission, but milk has also been demonstrated to be a potential route of transmission (Konold et al.). In a previous study, we demonstrated that 4-month-old lambs orally inoculated with scrapie inoculum succumbed to the disease in an average of 32 months post-inoculation (MPI) (Hamir et al.). These lambs were inoculated with a large dose of inoculum (3 g of scrapie-infected brain tissue), yet only 5 of 9 recipients developed scrapie.

The susceptibility of sheep to scrapie is greatly influenced by host prion protein gene (*PRNP*). Amino-acid polymorphisms at codons 136, 154 and 171 are major determinants of relative susceptibility or resistance. The five common allelic variations, resulting from amino-acid substitutions are A₁₃₆R₁₅₄R₁₇₁, A₁₃₆R₁₅₄H₁₇₁, A₁₃₆H₁₅₄Q₁₇₁, A₁₃₆R₁₅₄Q₁₇₁ and V₁₃₆R₁₅₄Q₁₇₁ (Belt et al., 1995). The V₁₃₆ and Q₁₇₁ haplotypes are linked to scrapie susceptibility, especially so in the homozygous (VRQ/VRQ) state (Diaz et al., 2005; Hunter et al., 1997a; Hunter et al., 1997b), and the ARR/ARR genotype is strongly associated with resistance (Cosseddu et al., 2007; Diaz et al., 2005; Hunter et al., 1996). Therefore, PRNP polymorphisms found within a regional or national flock can influence

1 selective breeding recommendations to reduce the incidence of scrapie (Dawson et al.,
2 1998; Warner et al., 2006). Many scrapie eradication programs are based on using rams
3 of resistant genotypes: arginine (R) at codon 171. This approach enhances the resistance
4 to classical scrapie on the flock level. This breeding program can result in the birth of
5 ARQ/ARR lambs that are moderately resistant to scrapie. The purpose of this experiment
6 was to test the susceptibility of *PRNP* ARQ/ARR Suffolk sheep to a U.S. scrapie isolate
7 after oral exposure as neonates.

8 This experiment was carried out in accordance with the Guide for the Care and
9 Use of Laboratory Animals (Institute of Laboratory Animal Resources, National
10 Academy of Sciences, Washington, DC) and the Guide for the Care and Use of
11 Agricultural Animals in Research and Teaching (Federation of Animal Science Societies,
12 Champaign, IL). The protocol was approved by the Institutional Animal Care and Use
13 Committee at the National Animal Disease Center (protocol number: 3283). Results of
14 susceptible sheep (ARQ/ARQ) inoculated under the same protocol were previously
15 published.(Hamir et al.) Briefly, four ARQ/ARR (see genotyping method below) lambs
16 and their dams were obtained from the scrapie-free sheep flock at the National Animal
17 Disease Center. Lambs were orally inoculated within the first 24 hours with 1 ml of
18 inoculum (no. 13-7)(Hamir et al.) as a 10% brain homogenate (wt./vol.) prepared in PBS.
19 The inoculum was derived from clinically ill sheep with PrP^{Sc} demonstrated by
20 immunohistochemistry on brain sections, and the inoculum was confirmed positive for
21 PrP^{Sc} by western blot. The lambs remained with the ewes until they were separated at 4
22 months of age. At that time, they were housed outdoors and fed a pelleted maintenance
23 ration free from ruminant-derived products. Positive controls for this experiment included

age matched neonates of ARQ/ARQ genotype that received the same inoculum (Hamir et al.) and ARQ/ARQ and ARQ/ARR lambs that received the same inoculum but via the intracranial route (Greenlee et al.).

Predicted *PRNP* amino acid sequences were determined as described previously (Greenlee et al., 2012). Primers specific for the functional gene in sheep (forward primer Sheep-F2: 5'- GGA GTG ACG TGG GCC TCT GC -3' and reverse primer Sheep-R4: 5'- CTC CCT CCC CCA ACC TGG CA -3) amplified a 775-bp region beginning at codon 19 in the prion protein-coding region in sheep. All PCR reactions were as follows: 95°C for 5 min, followed by 30 cycles of denaturation (95°C, 20 sec), annealing (60°C, 20 sec) and extension (72°C, 60 sec) followed by an extension cycle (72°C, 7 min). Following filtration, PCR products were sequenced using primers Sheep-F2, Sheep-F3 (5'- ATG GAG GTG GCT GGG GCC AA -3'), Sheep-R3 (5'- TCC CCC TTG GTG GTG GTG GT -3 and Sheep-R4. Resulting sequences were analyzed using Geneious version 6, created by Biomatters. Available from (<http://www.geneious.com/>)

The sheep were observed twice daily for the development of clinical signs. They were examined at necropsy and two sets of tissue samples were collected. One set of tissues included representative sections of lymphoid tissues (spleen, pharyngeal and palatine tonsil, retropharyngeal and mesenteric lymph node, and ileal Peyer's patches), liver, kidney, skin, striated muscles (heart, tongue, diaphragm, masseter), thyroid gland, nasal turbinate, lung, intestines (ileum), adrenal gland, pituitary gland, trigeminal ganglion, brain (hemisections of cerebral cortex, cerebellum, superior colliculus and brainstem including obex) and eye (retina). These tissues were fixed in 10% buffered formalin, embedded in paraffin wax,

1 sectioned (4 µm or 3µm for lymphoid tissues), and stained with hematoxylin and eosin (HE)
2 for light microscopy. The second set of tissues was frozen.

3 Enzyme immunoassays (EIA) were performed on brainstem, retropharyngeal
4 lymph node, and tonsil as previously described (Smith and Greenlee) using a
5 commercially available kit (HerdChek BSE-Scrapie Ag Test Kit, EIA, IDEXX
6 Laboratories, Westbrook, ME).

7 All paraffin embedded tissues were stained by an automated immunohistochemical
8 (IHC) method for detection of PrP^{Sc} as described previously (Hamir et al., 2005) using a
9 cocktail of 2 primary monoclonal antibodies was used: F89/160.1.5 (O'Rourke et al.) and
10 F99/97.6.1 (O'Rourke et al.), each at a concentration of 5 µg/mL.

11 Sheep genotypes were confirmed to be ARQ/ARR with all sheep shown to be
12 homozygous at other potentially polymorphic sites: 112M, 127G, 137M, 138S, 141L,
13 151R, 154R, 157M, 176N, 180H, 189Q, 195T, 196T, 211R, 220Q, 223R. The orally
14 inoculated sheep were observed daily for the duration of the experiment without any
15 clinical signs suggestive of scrapie infection. Rectal biopsies were performed at
16 approximately 81 MPI, but they failed to demonstrate PrP^{Sc} immunoreactivity. Due to
17 recurring lameness in two of the sheep, the experiment was terminated at 86.5 MPI and
18 all of the sheep were euthanized. No gross lesions were noted at necropsy. Microscopic
19 examination of hematoxylin and eosin stained slides of multiple levels of cerebrum,
20 thalamus, hippocampus, colliculus, pons, and brainstem at the level of the obex (Fig. 1)
21 did not reveal any changes suggestive of spongiform encephalopathy nor did
22 immunohistochemistry reveal immunoreactivity for PrP^{Sc} in any neural or lymphoid
23 tissue examined (Fig. 2). Similarly, EIA performed on retropharyngeal lymph node,

1 palatine tonsil, and brainstem samples collected at the level of the obex failed to reveal
2 the presence of the misfolded protein associated with scrapie infection. All optical
3 density readings fell well below the negative threshold of 0.258 established according to
4 kit instructions. This is in contrast to positive control sheep that included orally
5 inoculated ARQ/ARQ neonates (Hamir et al.) or intracranially inoculated ARQ/ARR
6 lambs (Greenlee et al.) with evidence of PrP^{Sc} accumulation by IHC and western blot of
7 central nervous and lymphoid tissues (ARQ/ARQ oral) or central nervous tissues only
8 (ARQ/ARR intracranial).

9 The ARQ/ARR Suffolk sheep in the present study that were orally inoculated as
10 neonates were allowed to incubate for 86 months without any detectable evidence of
11 scrapie infection. Previous studies suggest that ARQ/ARR sheep are poorly susceptible to
12 the agent of scrapie by the oral route. Many attempts to transmit scrapie to ARQ/ARR
13 sheep by the oral route have failed (Espenes et al.; O'Rourke et al.). However, one recent
14 experimental study demonstrated clinical scrapie in orally inoculated ARQ/ARR Cheviot
15 sheep, but with a prolonged incubation period (approximately 74 months) relative to
16 more susceptible genotypes (Gonzalez et al.). The experimental protocol for the present
17 study was different than the successful experimental transmissions in that we inoculated
18 younger Suffolk lambs (within 24 hours of birth vs. 10-15 days old Cheviot lambs), but
19 with a smaller amount of inoculum (0.1 g vs. 1 g repeated on 5 consecutive days). Lack
20 of PrP^{Sc} immunoreactivity in any of the sheep inoculated for the present study may
21 indicate a difference in scrapie isolate characteristics from those used in successful oral
22 transmissions to ARQ/ARR sheep or breed related differences in the sheep used for these
23 experiments. It also is possible that the amount of inoculum given in this instance is

below the threshold to infect sheep of this genotype. While we cannot definitively prove that the amount of inoculum was adequate without performing additional studies, inoculation of susceptible sheep (ARQ/ARQ) with the same inoculum did result in 100% occurrence of clinical scrapie with a mean survival of 24 MPI (Hamir et al.) compared to 32 MPI when the same genotype of sheep was inoculated orally at 4 months of age with 1.5 g of the same infectious material on 2 consecutive days (Hamir et al.). Since ARQ/ARQ sheep developed scrapie more rapidly with less total inoculum in the neonatal challenge model when compared to our previous work in older lambs, we consider this model (as applied in the present study) an efficient way of testing scrapie susceptibility by the oral route. Since the challenge dose in this model is derived from the brain of clinically affected sheep, it represents a highly concentrated source of PrP^{Sc}. Failure of any of the sheep in this study to develop scrapie or evidence of PrP^{Sc} accumulation suggests a substantial resistance of ARQ/ARR sheep to infection with the U.S. scrapie isolate used (Hamir et al.). Interestingly, when ARQ/ARR sheep received the same inoculum used in the present study by the intracranial route, they developed scrapie, but after a prolonged (56 MPI on average) incubation and without evidence of PrP^{Sc} in lymphoid tissues (Greenlee et al.). Future studies will address the connection, if any, between ARQ/ARR sheep failing to accumulate PrP^{Sc} in the lymphoid tissues after intracranial inoculation and their resistance to scrapie after oral inoculation within the first 24 hours of life as demonstrated in the present study. Determining if scrapie isolate or sheep breed characteristics are responsible for differences between our results and those of successful oral transmissions to ARQ/ARR sheep in the UK (Gonzalez et al.) also would require additional studies beyond the scope of this manuscript.

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1 **Figures**

2 **Figure 1.** Lack of evidence of spongiform change in the brainstem of ARQ/ARR sheep
3 after oral inoculation as neonates. Spongiform change and neuronal degeneration is
4 evident in the parasympathetic nucleus of the vagus nerve of an ARQ/ARQ sheep
5 inoculated as a neonate (A) (Hamir et al.), but was not present in any of the ARQ/ARR
6 sheep of the present study (B). Hematoxylin and eosin. Original magnification is 100x.

7 **Figure 2.** Lack of PrP^{Sc} immunoreactivity in the brainstem or tonsil of ARQ/ARR sheep
8 after oral inoculation as neonates. Abundant PrP^{Sc} immunoreactivity (red) is present in
9 the brainstem at the level of obex (parasympathetic nucleus of the vagus nerve; A) and
10 palatine tonsil (C) of ARQ/ARQ sheep after oral inoculation as neonates (Hamir et al.),
11 but in ARQ/ARR sheep, brainstem at the level of the obex (parasympathetic nucleus of
12 the vagus nerve; B) and tonsil (D) are devoid of PrP^{Sc} immunoreactivity at 86 months
13 post inoculation. Immunoenzyme (Alkaline phosphatase) staining, monoclonal antibodies
14 F89/160.1.5 and F99/97.6.1, Fast Red chromagen, hematoxylin counterstain. Original
15 magnification is 100 x (A, B) or 40x (C, D).

Figure 1.

Figure 2.