Transmissible spongiform encephalopathies: The identification of a new disease phenotype after experimental interspecies transmission

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that affect both humans and animals. TSEs, or prion diseases, are associated with the accumulation of the misfolded prion protein (PrP^{Sc}) in central and peripheral nervous system, and lymphoid tissues. Accumulation of PrP^{Sc} results from the misfolding of the normal, cellular prion protein (PrP^{C}) to the disease-associated form (PrP^{Sc}). TSEs have strain variations that can affect host susceptibility, incubation period, and disease phenotype, including PrP^{Sc} accumulation intensity and patterning. These strain variations also influence interspecies transmission. It has been demonstrated that white-tailed deer are susceptible to the scrapie agent (the TSE of sheep). However, sheep are not very susceptible to the CWD agent. The purpose of this thesis was to determine the susceptibility of sheep to the scrapie agent derived from white-tailed deer (WTD-scrapie).

Suffolk sheep of various genotypes were challenged oronasally with the WTD-scrapie agent from either deer cerebrum or brainstem at the level of the obex. We found that sheep with a valine (V) at codon 136 (VV136) of the prion protein (*PRNP*) had a shorter incubation period than sheep with an alanine (A) at codon 136 (AA136). This result is in contrast to the original sheep scrapie agent that had a faster incubation period in AA136 sheep. We observed differences in PrP^{Sc} accumulation intensity and spongiform change between sheep of different genotypes challenged with the same inoculum source and sheep of the same genotype challenged with different inocula. We also demonstrated that inocula prepared from sheep challenged with the WTD scrapie agent resulted in a reduced incubation period in transgenic mice expressing ovine

PRNP when compared to the original sheep scrapie isolate. The results presented in this thesis demonstrate that sheep are susceptible to the scrapie agent derived from WTD and the phenotype of disease is different than the original sheep scrapie inoculum source.

CHAPTER 1. GENERAL INTRODUCTION

Thesis organization

This thesis includes one manuscript relevant to this degree that will be submitted for publication in a peer-reviewed journal. Prior to the manuscript, I will present the significance of this research topic and provide a review of the literature. The manuscript will be followed by an overview discussion of the findings, prospective for future work, and general conclusions.

Introduction

Transmissible spongiform encephalopathies (TSEs; prion diseases) are fatal neurodegenerative diseases. Disease results from the misfolding of the normal, cellular prion protein (PrP^{C}) to the diseased-associated form (PrP^{Sc}) (Prusiner, Science1991). TSEs are known to affect cattle (bovine spongiform encephalopathy), humans (Creutzfeldt-Jakob disease), deer (chronic wasting disease), and sheep (scrapie). Prion diseases are typically diagnosed post-mortem by histopathology, western blot analysis, and enzyme immunoassay. In addition to these techniques, we also can study the spongiform change and incubation period to determine a disease's phenotype. TSE agents have strain variations that can influence disease phenotype. Differences can be detected in the immunoreactivity pattern, the molecular mass of the protein, the lesion profile, and the incubation period. These strain variations can have an affect on the susceptibility of animals to disease with prion protein polymorphisms as well as potential interspecies transmission (Sigurdson, Nat Met 2007).

Sheep are variably susceptible to scrapie depending on their prion protein gene (*PRNP*) sequence. Sheep have amino acid polymorphisms that affect susceptibility to

the scrapie agent at 3 major codons in the *PRNP.* These polymorphisms consist of: valine (V) or arginine (R) at codon 136, histidine (H) or arginine (R) at codon 154, and glutamine (Q) or arginine (R) at codon 171. Sheep susceptible to scrapie is associated with having alleles $V_{136}R_{154}Q_{171}$ and $A_{136}R_{154}Q_{171}$, whereas sheep with alleles $A_{136}R_{154}R_{171}$ are known to be resistant to scrapie (Goldmann, J Gen Virol 1994). The most influential codon is 171, where QQ171 is associated with susceptibility and RR171 is resistant (Greenlee, ILAR 2015). This work demonstrates that differences in the *PRNP* gene can have an effect on susceptibility, disease phenotype, and the incubation period.

Sheep and deer can share the same geographical region so it is important to understand the potential for interspecies transmission between these species. Experimental studies have demonstrated that sheep are not very susceptible to the CWD agent. Sheep intracranially inoculated with white-tailed deer CWD (4 out of 15; Greenlee unpublished) or mule deer CWD (2 out of 10, respectively; Hamir, JVDI 2006) demonstrated a low attack rate. Oronasal inoculation of CWD to sheep has to date been unsuccessful (study currently at 50 MPI). In contrast, experimental studies have demonstrated that white-tailed deer are highly susceptible to the scrapie agent. Deer intracranially or oronasally inoculated with the scrapie agent had a 100% attack rate (Greenlee, Vet Res 2011; Greenlee submitted). This work uses Suffolk sheep of various genotypes and transgenic mice expressing ovine *PRNP* to determine the susceptibility of sheep to the scrapie agent derived from white-tailed deer.

Literature Review

Prions

In 1991, Stanley Prusiner proposed the prion hypothesis that stated that a protein is necessary and is also the source of prion diseases (Prusiner, Science1991). He was later awarded the Nobel Prize in 1997 for his work (Prusiner, Proc Natl Acad Sci 1998). Prusiner coined the termed “prion” to describe the proteinaceous infectious particles that caused scrapie and Creutzfeldt-Jakob disease. Prion proteins are composed of a normal, cellular version of the protein (PrP^{C}), that is highly conserved and is protease-sensitive. However, the disease-associated form of the protein, PrP^{Sc} is protease resistant.

Hamsters have been used to study prion diseases (Chandler, Vet Res Sci 1972; Kimberlin, J Infect Dis 1975). Stanley Prusiner used a hamster model to do much of his early work involving scrapie, which helped him identify and classify the prion protein (Prusiner, Prog Clin Biol Res 1980; Prusiner, Biochemistry 1980; Prusiner, Proc Natl Acad Sci 1981). Protein purification performed on scrapie-diseased hamsters revealed the molecular mass of the prion protein (Prusiner, 1998). Infectivity analysis using sucrose gradients on the scrapie-infected hamster tissue, found that the infectious agent was localized near the bottom of the enriched fraction (Bolton, Science 1982). Further analysis of this agent through radioiodination and gel electrophoresis, revealed that the prion protein (PrP) band had a molecular size of 27,000 to 30,000 daltons (27-30 kDa), giving it the reference name of PrP 27-30 (Prusiner, Biochem 1982). Using a cDNA library and Southern blot analysis it was demonstrated that PrP 27-30 was encoded by a chromosomal gene (Oesch, Cell 1985). It was also demonstrated that the

chromosomal gene that encodes the PrP 27-30 fragment also encodes the cellular form of PrP (PrP^C; Basler, Cell 1986). The unchanged levels of PrP messenger RNA (mRNA) throughout the course of disease led to the identification of PrP^C and to the notion that conversion of PrP^C to PrP^{Sc} is a post-translational process (Prusiner, 1991).

Physiological Role of PrP^C

The physiological role of PrP^C is thought to be involved in many processes. Mice that are devoid of the prion protein have been generated to help in understanding the role of PrP^C. These mice are generated by disruptive modifications in the open reading frame (ORF) of the prion protein (Weissman, Br Med Bull 2003; Bueler, Nature 1992; Manson, Mol Neurobiol 1994). PrP knockout ($PrP^{-/-}$) mice do not display any abnormal behaviors, phenotypic deficits, and showed normal development and reproduction, suggesting that the prion protein is not an essential protein (Manson, 1994; Jackson, PLoS One 2014; Nuvolone, J Exp Med 2016). PrP^C has been suggested to play a part in peripheral nerve myelination, and in neuroprotection against neurotoxic stimuli (Watts, Acta Neuropathol 2017). All $PrP^{-/-}$ mice generated have an age-related onset of chronic demyelinating polyneuropathy (CDP) in their peripheral nerves (Bremer, Nat Neurosci 2010; Nishida, Lab Investig 1999). Interestingly, it was also demonstrated that this phenotype is restored by re-introduction of PrP^C expression (Bremer, Nat Neurosci 2010) confirming the role of PrP^C in the myelination of peripheral nerves.

It has also been demonstrated that PrP^C can have a neuroprotective role. In some cases, when PrP^C is expressed the cell is resistant to apoptosis (Diarra-Mehrpour, Caner Res 2004). Other research has demonstrated that PrP^C can act against apoptotic

stimuli in various cell lines (Bounhar, J Biol Chem 2001; Kim, Brain Res Mol 2004; Kuwahara, Nature 1999; Roucou, Cell Death Differ 2005; Yu, PLoS One 2012).

In addition, PrP knockout studies in mice have demonstrated that PrP^{C} is required for the propagation of prion disease (Moreno, Prions Met 2017; Bueler, Cell 1993; Prusiner, Proc Natl Acad Sci 1993; Sailer, Cell 1994). $PrP^{-/-}$ mice challenged with scrapie failed to develop disease (Bueler, Cell 1993). There is also evidence that the amount of PrP^{C} present in the brain has an influence on disease phenotype (incubation period, immunoreactivity intensity and patterns, molecular mass of the prion protein, and spongiform change). In the early stages of disease, high amounts of PrP^{Sc} accumulate in the brain independent of the amount of PrP^{C} present, which has been demonstrated in transgenic mouse lines that express different levels of PrP^{C} (Bueler, Mol Med 1994; Sandberg, Nature 2011; Watts, Acta Neuropathol 2017). However, once the maximal amounts of PrP^{Sc} have accumulated, the phenotype of the disease is proportional to the amount of PrP^{C} present in the brain (Sandberg, Nature 2011; Sandberg, Nat Commun 2014).

Transmissible Spongiform Encephalopathies (TSEs)

TSEs are typically spread through infectious forms of the prion but there also are inherited or sporadic forms. Human prion diseases consist of: sporadic CJD (sCJD), variant CJD (vCJD), familial Creutzfeldt-Jakob disease (fCJD), fatal familial insomnia (FFI), Gerstmann–Sträussler–Scheinker syndrome (GGS), and kuru. The sporadic, or irregular form of disease is most common among human CJD case with a rate of about 85-90% of total CJD cases (Geschwind, Continuum 2015). sCJD is typically classified by the molecular mass profile of the abnormal isoform of the prion protein that differs

from vCJD (Kobayashi, Acta Neuropathol Comm 2013). vCJD was the result of ingestion of cattle infected with bovine spongiform encephalopathy (BSE) (Ironside, Folia Neuropathol 2012). Familial CJD is the inherited form of human prion disease, and are associated with having mutations in the prion protein gene (Lee, BMC Med Genomics 2014).

Animal TSEs include: bovine spongiform encephalopathy (BSE) in cattle (Wells, Vet Rec1987), chronic wasting disease (CWD) in deer and elk (Williams, Vet Pathol 2005), scrapie in sheep and goats (Cullie, 1939; Fast and Groshup 2013) and transmissible mink encephalopathy (TME) in mink (Ekroade, 1973). These TSEs are most commonly transmitted through the ingestion of the infectious form of the protein from contaminated feedstuff. In addition, CWD also can be also be transmitted within the environment when exposed to a carcass, blood, or excreta from a diseased animal like in the cases of chronic wasting disease (Williams, Vet Pathol 2005).

The “species barrier” influences efficiency of interspecies transmission of TSEs and is partially dependent on the prion protein (*PRNP*) gene sequence (Hagiwara, J Biochem 2013). Efficient transmission between species can be associated with a weak species barrier, whereas inefficient transmission between species can be correlated to a strong species barrier. Strength of the species barrier can be correlated to the similarities or differences between the *PRNP* gene sequence of the donor PrP^{Sc} and host PrP^{C}, in addition to the conformation properties of the β_2-α_2 loop of the prion protein (Sigurdson, J Clin Invest 2010). Similar β_2-α_2 loops were correlated with efficient transmission, while β_2-α_2 loops that were not similar did not lead to transmission (Sigurdson, J Clin Invest 2010; Bett, FASEB 2012). In a comparison study, mice that

express the wild-type (WT) prion protein developed disease when challenged with scrapie or BSE, but mice that express a prion protein that produces a rigid loop (RL) did not develop disease (Sigurdson, J Clin Invest 2010).

PRNP also has an influence on intraspecies susceptibility, incubation period, and disease phenotype (Lloyd, Top Curr Chem 2011). Each TSE strain can have variations that affect disease phenotype. These strain variations can be studied on the basis of incubation period (Bruce, BrMed Bull 1993), histopathology (Moore, vet path 2016) and vacuolation profiling (Bruce, BrMed Bull 2003). When experimentally transmitted to mice, TSE agents can have different incubation periods that help identify multiple strains from the same source (Morales, BBA 2007; Westaway, Cell 1987; Bruce, Techniques in Prion Res 2004). Histopathology was used in cases of natural scrapie in sheep with the same genotype to discern between multiple strains of scrapie due to the immunoreactivity patterns of PrP^{Sc} that were present (Gonzalez, J Gen Virol 2010). The severity and location of the spongiform change can be determined by vacuolation profiling and can be used to discriminate between different strains of scrapie. This technique has been used to distinguish disease phenotypes in cases of natural scrapie in sheep of the same genotype (Ligios, J Comp Pathol 2002).

TSE Detection

TSEs are typically diagnosed postmortem by the detection of PrP^{Sc} in the central nervous system (Spiropoulos, Neuropath 2007) and in some species, the lymphoid system (Jeffrey, J Pathol 2006). Currently, immunohistochemistry, western blot, and enzyme immunoassays are used to detect PrP^{Sc} in tissues. Antemortem diagnosis is

accomplished in some species, like sheep, goats, and deer by taking rectal mucosal biopsy samples for histological examination (Spraker, J Vet Diagn Invest 2009).

Immunohistochemistry

Immunohistochemistry (IHC) uses antibodies against the prion protein to detect PrP^{Sc}. The abnormal form of the prion protein can accumulate in different regions of the brain and in different cell types, resulting in a variety of immunolabeling patterns. Immunolabeling types consist of: granular, which can be found in the neuropil (network of neuronal axons, dendrites, and neuroglial that forms most the CNS grey matter) and presents as small particulate PrP^{Sc} deposits; aggregates, which also can be found in the neuropil but is a dense deposition of PrP^{Sc} and has a focal point that can vary in size; intraneuronal type can be found within the cytoplasm of larger neurons and appears as having small granular-like PrP^{Sc} deposits; stellate patterning, which is associated with glial cells within the neuropil and appears as radiating deposits of PrP^{Sc} (Corda, Vet Res 2012). These patterns of PrP^{Sc} in different brain regions can be profiled and help distinguish between different strains of the same TSE (like scrapie; Moore, Vet Pathol 2016) or between different TSEs (BSE compared to scrapie; Bencsik, PLoS One 2011).

Western Blot and Enzyme Immunoassay

Since PrP^{Sc} is protease resistant, a western blot can be done utilizing proteinase K digestion prior to electrophoresis. Proteinase K removes the PrP^{C} from the sample, leaving only PrP^{Sc}. This will determine if a sample is PrP^{Sc} positive and the molecular mass of the protein (Konold, BMC Vet Res, 2006). The PrP molecule has two possible glycosylation sites at residues 180 and 196 that are variably occupied and can be di-, mono-, or unglycosylated (Piro, J Virol 2009; Endo, Biochemistry 1989; Locht, Proc Natl

Acad Sci 1986). TSE strains may have different molecular masses that allow us to discern the TSE origin (Martin, BMC Vet Res 2009). In addition, multiple strains of BSE can be differentiated using western blot analysis. The two atypical forms of BSE have a molecular mass that differs from classical BSE (Greenlee, ILAR 2015). H-type (high-type) BSE has a higher molecular mass of the unglycosylated PrP^{Sc} band compared to that of classical BSE (Biacabe, EMBO 2004; Biacabe, Prion 2007). L-type (low-type) BSE has a lower molecular mass of the unglycosylated PrP^{Sc} compared to that of classical scrapie (Balkema-Buschmann, J Toxicol Environ Health 2011; Konold, BMC Vet Res 2012).

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) were initially developed as a rapid screening test for livestock due to the BSE epizootic events in the United Kingdom (Smith, Am J Vet Res 2014). EIA uses an antigen-capture plate to detect misfolded protein in brain, lymphoid, or spleen tissues. The plate consists of an immobilized PrP^{Sc}-specific ligand that allows for the binding of the PrP^{Sc} conformer when present. PrP^{Sc} can then be detected using PrP-specific antibodies (IDEXX, BSE-scrapie insert). The enzyme-linked immunoassay (ELISA) by BioRad was approved as a diagnostic tool for CWD in October 2002 (Bio Rad, 2003). A field validation study was then published in 2003 to assess the ELISA test for detecting CWD in retropharyngeal lymph nodes (RLN) or obex from deer (Hibler, J Vet Diagn Invest 2003). Recently, it also was demonstrated how ELISA can be used for detection of PrP^{Sc} in retropharyngeal lymph nodes of sheep with different genotypes (Kittelberger, N Z Vet J 2014). In addition, other tissues like the retina have been tested for the presence of PrP^{Sc} (Smith, Am J Vet Res 2014).

Vacuolation Profiling

Vacuolation profiling is another diagnostic tool for TSEs and can be correlated to the progression of PrP^{Sc} accumulation (Begara-McGorum, J Comp Path 2002). Spongiform change is caused by the vacuolation of neuronal cell bodies or the loss of neuronal projections in the white matter of the brain. Spongiform change can present with different patterns that can be characterized for a particular TSE (Gonzalez, J Comp Path 2002). This spongiform change can be assessed in specific neuroanatomical locations and semiquantiatively "scored" based on the severity of the vacuolation. These scores are then graphed by score vs. location to determine the lesion profile (Simmons, Vet Rec 1996) that can then be used to differentiate between TSE origins (e.g. BSE and scrapie) depending on the PrP^{Sc} vacuolation profile (Jeffrey, Acta Neuropathol 2011).

Scrapie

Scrapie was the first TSE identified and was described as early as 1732 (Irman, Virol 2011). The word scrapie is derived from Scottish origin and refers to the act of rubbing or scraping against objects (Plummer, Can J Comp Med Vet 1946). Sheep with scrapie may also show signs of biting the flank and hind legs, circling, failure to eat, and lameness (Brown, BMJ Brit Med J 1998). Susceptibility or resistance of sheep to scrapie is determined largely by polymorphisms at three codons in the *PRNP* sequence (136, 154, and 171). Polymorphisms consist of valine (V) or alanine (A) at codon 136, arginine (R) or histidine (H) at codon 154, and arginine (R) or glutamine (Q) at codon 171. Sheep susceptible by natural routes of exposure to scrapie are known to have the alleles $V_{136}R_{154}Q_{171}$ and $A_{136}R_{154}Q_{171}$ while sheep with alleles $A_{136}R_{154}R_{171}$ are very

resistant and $A_{136}H_{154}Q_{171}$ are somewhat resistant to scrapie (Baylis, Curr Mol Med 2004). The genotype of sheep is known to have an affect on PrP^{Sc} immunolabeling. In cases of sheep with natural scrapie, sheep of the ARQ/ARQ genotype had more glial and intraglial-type accumulation whereas sheep of the VRQ/VRQ genotype had more granular and coalescing, or aggregate-like accumulation (Spiropoulos, Neuropath 2007). Polymorphisms in the *PRNP* gene also have an affect on interspecies transmission of disease. Sheep with at least one valine (V) at codon 136 were more susceptible to BSE (VV136, 1 out of 1; AV136, 1 out of 1) compared to sheep with two copies of alanine (A) at codon 136 that were less likely (AA136, 1 out of 3) to develop disease when challenged with BSE (Goldmann, J Gen Virol 1994).

Scrapie was one of the first TSEs to be experimentally transmitted to other species (Cutlip, JID 1994). Scrapie has been successfully transmitted to cattle (Hamir, JVDI 2011), white-tailed deer (Greenlee, Vet Res 2011), elk (Hamir, JVDI 2004), raccoons (Hamir, Vet Rec 2003), pigs (Greenlee, Food Safety Comm 2016), and mice (Chandler, Lancet 1961). Although mice are not a natural host of scrapie, early transmission studies have lead to the identification of strain variations that have since been studied in depth (Bruce, Mol Neurobiol 1994; Beck, Vet Res 2012).

Scrapie Strains

There are multiple scrapie strains that have been thoroughly investigated by experimental studies. The strains come from vast geographical regions including the United States (No.13-7, x124), United Kingdom (SSBP/1), and Norway (Nor98).

US No.13-7

The US No.13-7 scrapie strain is an isolate that was made from a pool of 13 scrapie-affected Suffolk sheep from 7 source flocks, all sheep having the genotype ARQ/ARQ (Hamir, JVDI 2005). The No.13-7 isolate was used to intracranially challenge sheep of the ARQ/ARQ genotype for four serial passages. The strain stabilized after this fourth passaged with a mean incubation time of 10.7 months-post inoculation (MPI) (Hamir, Infect Dis 2009). This isolate was also transmitted experimentally in genetically susceptible sheep by three different routes: peritoneal, conjunctival, and nasal (Hamir, Vet Pathol 2008).

This strain also has been used to intranasally challenge sheep of various genotypes (AA136, AV136, and VV136). Sheep of the AA136 genotype had an average incubation period (IP) of 20.1 months; sheep of the AV136 genotype had an IP of 22.8 months; and sheep of the VV136 had an IP of 26.7 months (Moore, Vet Pathol 2016). PrP^{Sc} accumulation was widespread throughout the central nervous system (CNS), enteric nervous system, and in the lymphoreticular system (Hamir, Infect Dis 2009; Moore, Vet Pathol 2016). These results demonstrate that the No.13-7 scrapie has a faster incubation period in sheep of the AA136 genotype.

x-124 scrapie

A more recently identified US scrapie strain is known as x124 scrapie. This strain was found after sheep of various genotypes were challenged with an inoculum made from a pool of seven sheep with different genotypes; 5 sheep had the genotype ARQ/ARQ, 1 sheep had the genotype ARQ/VRQ, and one sheep had the genotype VRQ/VRQ (Bulgin, AJVR 2006). Sheep with the genotype AV136 were the first to

develop clinical disease and had an incubation period of 9 to 11 months, whereas the first sheep with the AA136 genotype develop clinical signs at 27 MPI, no sheep of the VV136 genotype were challenged (Bulgin, AJVR 2006). They suggested this faster incubation time was influenced by the presence of valine (V) at codon 136.

When a second passage of x124 was intracerebrally inoculated in sheep of various genotypes, sheep of the VV136 or AV136 genotype were the first to develop disease at an average of 4.3 and 5.6 MPI, respectively, compared to sheep of the AA136 genotype that developed clinical disease at a mean of 14.3 MPI (Hamir, Vet Pathol 2009). When sheep were challenged oronasally with x124, the sheep of the VV136 genotype were the first to develop clinical disease at 6.9 MPI, sheep of the AV136 genotype had an incubation period of 11.9 MPI, and AA136 sheep did not develop disease or have evidence of PrP^{Sc} accumulation (Moore, Vet Pathol 2016).

SSBP/1

The SSBP/1 (Scrapie Sheep Brain Pool number 1) was derived from the Neuropathogenesis Unit in the UK and has been used to experimentally challenge Cheviot sheep since the 1960s (Hunter, Br Med Bull 2003). Sheep with the VV136 genotype had the fastest incubation period of 160 days when challenged subcutaneously with SSBP/1; sheep that are heterozygotes at codon 136 (VRQ/ARQ or VRQ/ARR) had an incubation period of 260 and 360 days respectively; and sheep homozygous for alanine at 136 (AA136) were resistant to disease (Houston, J Gen Virol 2002; Goldmann, J Gen Virol 1991). SSBP/1 PrP^{Sc} can be found throughout the CNS in sheep challenged with SSBP/1 (Houston, PLoS One 2015) and the lymphoreticular system (Gossner, Vet Microbiol 2011).

Atypical (Nor98) Scrapie

Atypical scrapie was first described in Norway in 1998 (Benestad, Vet Rec 2003). This strain was denoted atypical because the disease phenotype differed from that of classical scrapie. Atypical scrapie presented with different clinical features, PrP^{Sc} distribution, and immunoreactivity patterns, the genotype of sheep affected, and epidemiology (Benestad, Vet Res 2008). Atypical scrapie also presents with a different molecular mass profile compared to that of classical scrapie. Atypical scrapie has a 5-band profile with a prominent band at 12 kDa, whereas classical scrapie has a 3-band profile with the unglycosylated band at 19-21 kDa (Hayashi, Biochem Biophys Res Commun 2005; Hope, J Gen Virol 1999; Sommerville, J Gen Virol 1990). When 7 field cases of atypical scrapie were analyzed in sheep of various genotypes, five of the seven sheep had PrP^{Sc} accumulation in the brain, but did not have evidence of PrP^{Sc} accumulation in the lymphoreticular system (Andreoletti, PLoS Pathog 2011). This differs from classical scrapie that has PrP^{Sc} throughout the brain and lymphoreticular system and would make early detection of atypical scrapie more challenging.

Chronic Wasting Disease

Chronic wasting disease (CWD) is a TSE of cervids (deer and elk) that was identified in Colorado and Wyoming in 1967 (Haley, Annu Rev 2015). Symptoms of CWD include: progressive weight loss, locomotive changes, head tremors, altered head placement, and ataxia (Gilch, Top Curr Chem 2011). The CWD agent is efficiently transmitted horizontally, when naïve deer come into contact with an affected deer's excreta (Williams, Vet Pathol 2005; Moore, Emerg Infect Dis 2016). The CWD agent has been found in urine, blood, saliva, feces, and velvet (Saunders, Emerg Infect Dis

2012; Mathiason, Science 2006; Miller, Emerg Infect Dis 2004; Mathiason, PLoS One 2009). Zoonotic transmission of CWD to humans has been an increasing concern since BSE is transmissible to humans. However, studies using transgenic mice expressing the human *PRNP* sequence support that CWD is not transmissible to humans since the mice did not develop disease or present with TSE features even after 756 days (Sigurdson, Vet Res 2008; Kong, J Neurosci 2005).

Another concern is the possible transmission of CWD to sheep as cervids and sheep can share the same grazing lands. Experimental transmission of mule deer CWD to sheep by intracranial route had a low attack rate (2 out of 10 positive for disease; (Hamir, JVDI 2006). This study suggested that susceptibility of sheep to CWD might be dependent on sheep genotype, as one of the sheep that developed clinical signs had the genotype AV136 (Hamir, 2006). The other PrP^{Sc} positive sheep (AA136; 72 MPI) had evidence of PrP^{Sc} by IHC and WB but did not show signs of clinical disease and was euthanized at the end of the study (Hamir, 2006). White-tailed deer- and elk-derived CWD, was not transmissible to transgenic mice expressing ovine *PRNP* (Tg338; VRQ) (Madsen-Bouterse, J Gen Virol 2016). These studies suggest that transmission of CWD to sheep is unlikely, but could be possible depending on the source of the agent and the host sheep genotype.

Mouse Models

Experimental transmission of scrapie to mice was reported as early as 1963 (Chandler, Res Vet Sci 1963). Wild-type mice are the standard to identify and characterize TSE strains. The incubation period is a primary characterization tool for studying TSE strains. For a given TSE strain in a genetically similar non-transgenic

mouse strain, the incubation period has a small standard error and can be highly repeatable. However, the incubation period can be influenced by genetic factors in different mouse models (Bruce, BrMedBull 2003). Two alleles of the PrP gene have been identified in mice (denoted $prnp^a$ and $prnp^b$) and represent the prion protein that is encoded by amino acids that differ at codons 108 and 189 (Westaway, Cell 1987). The incubation period in a $prnp^a$ versus a $prnp^b$ mouse can differ by hundreds of days and allows for the identification of different TSE strains (Bruce, BrMedBull 2003; Dickinson, Mol Gen Genet 1971; Bruce, J Gen Virol 1991; Dickinson, J Comp Pathol 1968).

Transgenic mouse models that express the prion protein gene from many different species is an essential part of studying TSE disease. These transgenic mice do not express the mouse prion protein but instead are generated by knocking out the PrP or by insertion of the *PRNP* gene from a species of interest (Diack, Prog Mol Biol Transl Sci 2017). The first transgenic mouse over-expressed the hamster PRNP gene by random insertion into the murine genome (Scott, Cell 1989). Since then transgenic mice have been generated that express the bovine (TgBov XV; Buschmann and Groschup, J Infect Dis 2005), ovine (Tg338; Laude, C R Biologies 2002), cervid (Tg12; Kong, J Neurosci 2005), and human (Tg40; Kong, J Neurosci 2005) *PRNP* genes. These models have been used for the characterization of disease phenotypes (Thackray, J Gen Virol 2011). Transgenic mouse models also help with the understanding of the diversity of prion strains and their potential host range.

Strain Variation

TSEs are known to have strain variations that can influence disease phenotype and susceptibility to disease, based on the genotype of the animal. Strains can differ in

incubation period, vacuolation profile, PrP^{Sc} accumulation, and PrP^{Sc} glycosylation profile (Solforosi, Prion 2013). Prion strain factors can be identified when the prion isolate is passed to a new host species (Wemheuer, Front. Aging Neurosci 2017). Strain variation first became apparent when sheep scrapie was: 1) transmitted to goats and 2) resulted in two different disease phenotypes (Pattison, J Comp Pathol 1961). Since then, mouse models have been used extensively to study strain variation. Strain typing can be done after disease characteristics have stabilized, which requires multiple passages of the agent in mice (Bruce, BrMedBull 1993). Stabilized strains in mice portray specific lesion profiles and PrP^{Sc} immunoreactivity patterns. Lesion profiles can differ in severity and the brain region that is affected, while PrP^{Sc} accumulation can target specific neurons, or differ in the type of accumulation (granular or aggregates) (Bruce, BrMedBull 2003). After stabilization and characterization, the strain is identified based on the incubation period, lesion profile, and PrP^{Sc} immunolabeling properties (Bruce, BrMedBull 2003).

Interspecies Transmission

Interspecies transmission allows us to the study the potential host range for any TSE. Studying interspecies transmission is typically accomplished by experimental intracranial (IC) or oral inoculation. Intracranial inoculation can be used to determine if the agent has any potential to transmit disease to the new host species. However, to better characterize the disease pathogenesis the more natural, oral route can be used (Greenlee, ILAR 2015). The study of interspecies transmission has been accomplished using large animal as well as transgenic mouse models.

Sheep have been orally challenged with the bovine spongiform encephalopathy agent in lambs at 24 hours of age that produced high attack rates (7 out of 10 at a dose of 1.0 g) and at 2-3 weeks of age (12 out of 12 at a dose of 1.0 g; Hunter, J Virol 2012). Sheep have also been challenged with the BSE agent intracerebrally (Foster, Vet Rec 1993) or intravenously (Houston, Lancet 2000). Sheep have also been challenged with the mule deer CWD agent by the intracranial route, and had a low attack rate (2 out of 10, as discussed previously) (Hamir, J Vet Diagn Invest 2006). In addition, sheep have been challenged intracerebrally with the transmissible mink encephalopathy agent and had a low attack rate of (5 out of 20; Hadlow, Can J Vet Res 1987).

Transgenic mouse models can also be used to study interspecies transmission. Mouse studies typically take significantly less time than large animal studies and are a good indication of whether a large animal study would be necessary. For example, the sheep scrapie isolate SSBP/1 in experimental studies, readily transmits to transgenic mice expressing the deer prion protein but does not produce disease in transgenic mice expressing the elk prion protein (Angers, PNAS 2014). Due to the findings in mice from this paper, the authors subsequently conducted an experiment by transmitting sheep scrapie to deer.

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CHAPTER 2. DISEASE PHENOTYPE OF SCRAPIE IN SHEEP IS CHANGED UPON EXPERIMENTAL PASSAGE THROUGH WHITE-TAILED DEER

A paper in preparation for Veterinary Research

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Abstract

Transmissible spongiform encephalopathy (TSE) agents have strain variations that influence disease phenotype and may affect the potential for interspecies transmission. Since deer and sheep may use the same grazing land, it is important to understand the potential for transmission of TSEs between these species. A US scrapie isolate (No.13-7) had a 100% attack rate in white-tailed deer (WTD) after oronasal challenge. The purpose of this study was to determine if sheep are susceptible to oronasal challenge with the scrapie agent from WTD. Suffolk lambs of various *PRNP* genotypes were challenged by the oronasal route with a 10% brain homogenate from scrapie-affected WTD. Upon development of clinical signs, sheep were euthanized and necropsied. Tissues were tested for PrP^{Sc} by enzyme immunoassay (EIA), western blot (WB), and immunohistochemistry (IHC). The first sheep to develop clinical signs at approximately 29 months post-inoculation (MPI) had the VRQ/VRQ genotype. One of the two sheep of the ARQ/ARQ genotype also developed clinical signs at 48 MPI. This is in contrast to the original No.13-7 inoculum that has a faster incubation period in sheep with the ARQ/ARQ genotype (20 MPI). A more rapid incubation period in VV136 rather than AA136 sheep indicates a phenotype change and potentially the generation of new scrapie strain properties. This work raises the potential concern that scrapie

infected deer could serve as a confounding factor to scrapie eradication programs as scrapie from deer is transmissible to sheep by the oronasal route.

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative diseases that are the result of misfolding of the prion protein from the normal cellular form (PrP^{C}) to the disease-associated form (PrP^{Sc}) [1]. TSE's affect sheep (scrapie), deer (chronic wasting disease; CWD), cattle (bovine spongiform encephalopathy), and humans (eg. Creutzfeldt-Jakob disease). TSEs can be identified and differentiated by characterizing the PrP^{Sc} accumulation in various brain regions using immunohistochemistry, western blot, or bioassay in transgenic mouse models. There are strain variations between TSE agents that influence disease phenotype (e.g. incubation time, location of PrP^{Sc} deposition, etc.) and may affect susceptibility of animals with different prion protein gene (*PRNP*) sequences [2].

Susceptibility of sheep to the scrapie agent is associated with polymorphisms in *PRNP* at 3 codons: 136 valine (V), 154 arginine (R), and 171 glutamine (Q) (VRQ). The strongest resistance of sheep to scrapie is associated with codons 136 alanine (A), 154 arginine (R), and 171 arginine (R) (ARR haplotype) [3]. The most influential codon being 171, where QQ171 sheep are susceptible and RR171 sheep are resistant [4]. Disease phenotype can be influenced by genotype of the sheep donor, and the species or tissue from which the TSE is derived [5, 6].

Since it is possible for sheep and deer to share the same grazing lands, it is important to understand the potential for interspecies transmission of the scrapie and CWD agents. Suffolk sheep intracranially inoculated with the CWD agent from white-

tailed deer had an attack rate of 26.66% (4 out of 15) (unpublished). To date, attempts to oronasally transmit CWD to sheep has been unsuccessful (study currently at 50 MPI). However, white-tailed deer had a 100% attack rate when intracranially or oronasally inoculated with a US scrapie isolate (No.13-7) [7; Greenlee submitted]. The purpose of this study was to determine the susceptibility of sheep to oronasal challenge with the scrapie agent derived from inoculum made from white-tailed deer cerebrum or brainstem at the level of the obex.

Methods and Materials

All animal experiments described were reviewed and approved by the National Animal Disease Center's Institutional Animal Care and Use Committee (protocol number 2777 [sheep] and 2730 [mouse bioassay]) and were carried out in strict accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington DC) and the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (Federation of Animal Science Societies, Champaign, IL).

Suffolk lambs from the National Animal Disease Center's (NADC) scrapie-free flock of *PRNP* genotype $VV_{136}RR_{154}QQ_{171}$ (VV136) (n=4), $AV_{136}RR_{154}QR_{171}$ (n=2), and $AA_{136}RR_{154}QQ_{171}$ (AA136) (n=4) were challenged by the oronasal route at approximately 5 months of age with a 10% brain homogenate from No.13-7 scrapie-affected WTD cerebrum (n=5) or brainstem at the level of the obex (n=5). Sheep were observed daily throughout the duration of the experiment. Clinical signs included: poor body coordination, slow to rise, biting in the flank area, weight loss, and hunched

posture. Upon development of intercurrent disease or neurologic signs consistent with TSE, or at the end of the experiment, sheep were euthanized and necropsied.

The inocula were composed of brain homogenate from a white-tailed deer (#22) that was challenged oronasally with the scrapie isolate No.13-7 (Greenlee et al., submitted). The deer received scrapie inoculum (No.13-7) that had been passed four times in sheep of the ARQ/ARQ genotype [8]. A 10% w/v brain homogenate from either the deer cerebrum or brainstem at the level of the obex was made in phosphate buffered saline (PBS) with 100 µg/mL gentamicin. The high and low migration patterns of the two inocula sources were confirmed by western blot. The relative amounts of PrP^{Sc} of the two inocula sources were also quantified by enzyme immunoassay (EIA) (see below). EIA's were done on samples from both brain regions at dilutions of 1:1, 1:25, 1:50, and 1:100. Optical density (OD) values are listed in table 2. One mL of 10% (wt./vol.) homogenate made from either the deer cerebrum or brainstem at the level of the obex was oronasally inoculated into the left nostril of lambs as previously described [9].

A complete necropsy was performed on all sheep. Two tissue samples were taken, one to be frozen and a second to be put in 10% buffered formalin to be paraffin embedded and sectioned at 5 µm for staining with hematoxylin and eosin (HE) or with anti-prion protein antibodies. Tissue samples collected consisted of sections of brain (olfactory cortex, brain at the level of the optic chiasm, hippocampus, thalamus, midbrain, pons, cerebellum, and brainstem at the level of the obex), eye, glands (pituitary, salivary, adrenal), spinal cord (cervical, thoracic, lumbar), dorsal root ganglion, nerves (optic, sciatic, trigeminal), striated muscle (tongue, masseter,

diaphragm, heart), liver, lung, trachea, kidney, spleen, pancreas, skin, urinary bladder, intestines (ileum, jejunum, cecum), forestomachs, lymph nodes (retropharyngeal, mesenteric, prescapular, popliteal), tonsils (palatine and nasopharyngeal), thymus, thyroid, and rectal mucosa [9].

Paraffin embedded sheep tissues were sectioned and stained for PrP^{Sc} by an automated method using a cocktail of two anti-prion protein monoclonal antibodies, F99/97.6.1 [10] and F89/160.1.5 [11] at a concentration of 5 µg/ml as previously described [9]. The morphology of the PrP^{Sc} deposits was defined as previous described [12]. The stellate pattern is characterized by glial-type nuclei with radiating branches of immunoreactivity. The term “aggregates” was used instead of “coalescing” and is defined as amyloid-like deposits that result in the merging of coarse particulate PrP^{Sc} deposits [13]. The linear pattern is observed in the neuropil and has thread-like PrP^{Sc} deposits in a linear form [12]. The term “granular” was used instead of the term “fine punctate” [12]. Granular patterning is the small, diffuse PrP^{Sc} deposits typically found in the neuropil [13,14]. After processing, images were captured using a Nikon DS camera on a Nikon Eclipse 55*i* microscope.

Frozen samples taken at necropsy were used for immunodetection of the abnormal prion protein by western blot (Prionics AG, Switzerland). Brainstem samples at the level of the obex were homogenized at a final concentration of 20% w/v in phosphate buffered saline (PBS). Homogenized samples were diluted 1:2 in deionized (DI) water prior to running on SDS-PAGE gel. The western blot was performed as previously described [7]. Immunodetection was done using an anti-prion protein

monoclonal antibody, P4 (R-Biopharm AG, Darmstadt, Germany) that targets the ovine prion protein at amino acids 89-104 at a final concentration of 1:10,000.

The BSE-Scrapie Antigen Test Kit, EIA (IDEXX, Westbrook, ME) was used on a 20% homogenate in PBS of the brainstem at the level of the obex, cerebellum, and neocortex for the sheep of the current study. The test kit was also used on a 10% homogenate of the inocula used to challenge the sheep were assayed for PrP^{Sc} (Table 1). The inocula (1% homogenate) used for the mouse bioassay studies were also tested for the relative amount of PrP^{Sc} present (Table 2). Each sample of the 10% sheep inoculum, 1% mouse inoculum or the 20% homogenate of the three brain regions listed above (Table 3) were first tested for PrP^{Sc} without being diluted (1:1). Samples were then diluted 1:25, 1:50, and 1:100 in PBS to determine the relative amounts of PrP^{Sc}. Diluted samples were then assayed on the antigen capture plate with the provided controls using the suggested short protocol with slight modifications. Each 100 µL sample received 25 µL of working plate diluent prior to being added to the plate. The capture plate then incubated for an hour and a half with agitation. After washing, the small ruminant brain conjugate concentrate (SRB-CC) was used as described and incubated for one hour without agitation. The antigen capture plate was then read on a SpectraMax 190 (Molecular Devices, Sunnyvale, CA) with an optical density of 450nm and a reference wavelength of 650nm. The negative sample cut-off value was determined by adding 0.180 to the negative control sample provided in the kit, as described in the protocol. Samples were deemed positive if their OD value were greater than the cut-off value. Since a maximum OD reading of 4.0 using EIA can result from

increasing levels of misfolded protein, we ran the various dilutions to determine the relative amount of PrP^{Sc} present in each sample.

Vacuolation profiles were generated by scoring the defined brain regions listed in Table 4 using hematoxylin and eosin stained slides. Grey matter scores were based on a published method: 0 = no vacuolation, 1 = occasional vacuole, 2 = several vacuoles, evenly distributed, 3 = moderate to many vacuoles evenly distributed, and 4 = severe vacuolation with possible coalescence[15]. Each score for the perspective area was graphed for the corresponding animal, with the exception of sheep 4 that received a score of 0 for each area. An average score for each area for all the sheep was calculated and graphed. The graph was made using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA).

Figure 5 provides a summary of EIA and neuropathological (vacuolation and IHC) results. Each circle represents a group of n = 1 (VRQ/ARR) or n = 2 (VRQ/VRQ and ARQ/ARQ) sheep that were inoculated with homogenate prepared from a particular part of the brain (cerebrum or obex at the level of the brainstem). Within a circle, each quadrant contains results from an individual brain region: upper left: obex; upper right, cerebellum grey matter; lower right: cerebellum white matter; lower left, neocortex. Within a quadrant, each colored sector represents the results of a test: purple, severity of vacuolation; green EIA optical density; and pink, distribution of PrP^{Sc}. The shade of the color indicates the magnitude of the test result. Severity of vacuolation: light purple, average vacuolation score = 1; medium purple = 1-2; dark purple = 2-3. EIA OD value: very light green, OD = 0.300-.500; light green, OD = .500-1.00; medium green, OD = 1.00-2.00; dark green, OD = 2.00-4.00. Immunohistochemistry, surface area with PrP^{Sc}

accumulation: very light pink, PrP^{Sc} accumulation covers <10% of the neuroanatomical area of interest; light pink, 10-30%; medium pink, 40-60%; dark pink, 60-100%.

Brain material was bioassayed in transgenic mice expressing the ovine *PRNP* Tg338 (ovine, PrP^{VRQ}) [16]. Four groups of mice were inoculated with brain homogenate prepared from the obex at the level of the brainstem from sheep 2 and 6 of the current study, a sheep inoculated with No.13-7, and a second strain of scrapie from the same genotype of sheep, x124 [9] (see Figure 6). EIA's were done on the inoculum sources to determine the relative amounts of PrP^{Sc} as described above. OD values for each inoculum source are listed in Table 2. Mice (n = 17 for No.13-7, n = 20 for WTD scrapie sheep 2, n = 18 for WTD scrapie sheep 6, and n = 20 for x124) were inoculated by the intracranial route using 20 µL of a 1% (wt./vol.) brain homogenate made from sheep. Animal care staff monitored the mice daily for development of clinical signs. Mice were humanely euthanized when clinical signs of poor coordination, ataxia, difficulty moving, unable to move, and poor grooming with urine stained fur became apparent. Brains from the mice were prepared for EIA by making a 20% homogenate in PBS and performed as described above. Attack rates were determined by taking the number of mice with a positive EIA result divided by the total number of mice. Incubation periods were determined by taking the average incubation period of all positive mice per group. Survival analysis for Figure 5 was done using GraphPad Prism 7 (GraphPad Software Inc.).

Results

To determine susceptibility, Suffolk sheep were oronasally challenged with the scrapie agent derived from white-tailed deer. We used inoculum made from either

cerebrum or brainstem at the level of the obex from deer that developed scrapie after oronasal inoculation with the No.13-7 scrapie agent from sheep. Western blots on brain samples from scrapie-affected deer resulted in two different molecular profiles. Samples from cerebrum had scrapie-like western blot pattern that had a lower molecular mass of the unglycosylated band, whereas the samples derived from the obex had a CWD-like pattern that had a higher molecular mass of the unglycosylated band (unpublished observation).

The No.13-7 scrapie agent has different incubation periods in sheep of the AA136 genotype compared to sheep of the VV136 genotype; all affected sheep had the genotype QQ171 [9]. Subsequently, sheep of three different genotypes were inoculated for the current study (VV136, AA136, $AV_{136}RR_{154}QR_{171}$). Animal results are summarized in Table 5. Three of the five sheep inoculated with the WTD scrapie agent from deer cerebrum developed clinical signs of scrapie. The first two sheep to develop clinical signs at 28 and 31 MPI had the VV136 genotype. One sheep with the AA136 genotype developed disease at 48 MPI. The second sheep of the AA136 genotype (56 MPI) was negative by EIA, IHC, and WB. One sheep inoculated with the WTD scrapie agent from deer obex (genotype VV136) developed clinical signs at 65 MPI. When compared to WTD scrapie-inoculated sheep in the current study, sheep challenged with original No.13-7 agent had a faster incubation period in sheep of the AA136 genotype (20 MPI) than sheep of the VV136 genotype (27 MPI) [9].

In the present study, sheep with evidence of PrP^{Sc} accumulation in the brain also had PrP^{Sc} accumulation in lymphoid tissues, retina, and peripheral nervous tissue when

challenged with the scrapie agent from either the cerebrum or brainstem at the level of the obex from white-tailed deer, (Table 5).

To determine if the relative amounts of PrP^{Sc} in the inoculum had an influence on the incubation period and attack rate of inoculated sheep, each inoculum was tested by EIA. At equivalent dilutions, samples of deer cerebrum (shorter incubation period) had lower optical density values compared to samples of deer brainstem at the level of the obex (Table 1). Thus, the shorter incubation period in sheep inoculated with deer cerebrum was not due to more PrP^{Sc} in the inoculum.

The two inoculum sources were selected based on the differences in western blot pattern observed in deer: deer cerebrum had a scrapie-like pattern whereas the deer obex at the level of the brainstem had a higher, CWD-like pattern. Brain samples from sheep determined positive by immunohistochemistry (sheep 1-3 and 6) also were positive by western blot (Figure 1). Samples from sheep negative using immunohistochemistry (sheep 4, 5, and 7-10) were also negative on western blot (data not shown). More work will need to be done to determine the molecular mass of sheep challenged with the WTD scrapie agent (Figure 1).

The *PRNP* genotype of the recipient sheep affected incubation period after challenge with the WTD scrapie agent. Potential differences in PrP^{Sc} distribution in sheep of different genotypes, that received different inocula, was assessed using immunohistochemistry with a monoclonal antibody against PrP. Sheep 1 and 2 (genotype VV136) that were inoculated with WTD scrapie from cerebrum had intense, widespread immunoreactivity throughout the obex, cerebellum, and neocortex (Figure 2 A-F). By comparison, sheep 3 (AA136) that also was inoculated with the scrapie agent

from deer cerebrum had less intense, but widespread PrP^{Sc} immunoreactivity (Figure 2 G-I). Sheep 4 (AA136) was negative via IHC (Figure 2 J-L). When compared to sheep inoculated with WTD scrapie agent from deer cerebrum, sheep 6 (VV136) inoculated with the WTD scrapie agent from deer obex had less PrP^{Sc} immunoreactivity throughout the brain (Figure 2 M-O). Also, in contrast to VV136 sheep inoculated with the WTD scrapie agent from deer cerebrum, the majority of the PrP^{Sc} in the cerebellum of the VV136 sheep inoculated with the WTD scrapie agent from deer obex was in the molecular layer and no immunoreactivity was detected in the white matter. These results demonstrate that there are differences in PrP^{Sc} immunoreactivity between sheep genotypes when challenged with the same inoculum and amongst sheep of the same genotype when challenged with different inocula.

To determine if the relative amounts of PrP^{Sc} were consistent with immunohistochemical results we used EIA to quantify PrP^{Sc} in the brainstem at the level of the obex, cerebellum, and neocortex (Figure 3). Optical density values from the obex at the dilution 1:50 were compared. Optical density values of cerebellum and neocortex for a 1:1 dilution were compared. EIA results for each dilution and brain region are listed in Table 3. Positive sheep challenged with the WTD scrapie agent from deer cerebrum (sheep 1, 2, and 3) had high optical density values correlating to more PrP^{Sc} present in the obex and cerebellum. Sheep 3 had a lower score for neocortex that was consistent with the immunoreactivity in Figure 2-I. Sheep 4 (AA136) tested negative in all brain regions. Sheep 6 (VV136) challenged with the WTD scrapie agent from deer obex had lower OD values for PrP^{Sc} in all brain regions when compared to sheep challenged with the WTD scrapie agent from deer cerebrum. These results indicate that the differences

in sheep genotype and inoculum source of the three sheep brain regions tested for PrP^{Sc} can be quantified by relative amounts of PrP^{Sc} using EIA and are consistent with IHC immunoreactivity in Figure 2.

Since spongiform change is a hallmark feature of prion disease, we used vacuolation lesion profiling to compare the severity and distribution of disease-associated spongiform change in the brains of sheep of the current study. Table 4 shows the areas scored on a scale of 1-4 as previously described [15] and the results are plotted in Figure 4. Differences in vacuolation scores were found between sheep of different genotypes, for example sheep 1 (VV136) and 3 (AA136) that were both inoculated with the WTD scrapie agent from deer cerebrum. A difference also was found between sheep 1 and 6 that were sheep of the same genotype (VV136) but challenged with inocula derived from different brain regions of the scrapie-affected deer. No spongiform change was observed in sheep 4.

Figure 5 summarizes the differences between sheep of different genotypes challenged with the same inoculum and sheep of the same genotype challenged with different inocula using three different assays: vacuolation profiling, EIA, and immunohistochemistry (see methods section for parameter details).

Previous studies demonstrate that sheep of the AA136 genotype have a faster incubation period when inoculated with the original No.13-7 isolate [9]. In contrast, when sheep were challenged with a second strain of scrapie, x124, sheep of the VV136 genotype (6.9 MPI) had a fast incubation period, and sheep of the AA136 genotype did not develop disease when challenged with x124 by the intranasal route [9]. The current study demonstrates that the WTD scrapie agent had a faster incubation period in VV136

sheep. To investigate the apparent change in phenotype, we used bioassay in transgenic mice (Tg338) that express ovine *PRNP* to compare attack rates and incubation period between the WTD scrapie inoculum and the original No.13-7 scrapie isolate. All scrapie strains had a 100% attack rate but with varying incubation periods. Mice inoculated with the WTD scrapie agent from sheep (inoculum from sheep 2 and 6, Table 2) had a much shorter incubation period (76 DPI; Figure 6) compared to those inoculated with the original No.13-7 scrapie isolate from either a VV136 (167 DPI; Figure 6) or an AA136 (266 DPI; not shown) sheep. Interestingly, mice inoculated with the WTD scrapie agent had a similar incubation period to the x124 isolate from sheep of the VV136 genotype (76 DPI; Figure 6). Survival analysis of ovinized mice inoculated with No.13-7 scrapie, WTD scrapie, and x124 demonstrates that the WTD scrapie agent is significantly shorter than the No.13-7 isolate (p-value <0.0001) and not different from the x124 isolate (p-value >0.9999).

Since the incubation period in sheep challenged with WTD scrapie or x124 was faster in VV136 sheep compared to AA136 sheep, we used immunohistochemistry to compare PrP^{Sc} accumulation in No.13-7 [9], WTD scrapie, or x124 [9] challenged VV136 sheep. All inocula had similar widespread accumulation in the obex (Figure 7 A, D, G). Differences in the intensity of immunoreactivity in the cerebellum and neocortex between inocula groups were notable. Sheep inoculated with No.13-7 had less extensive PrP^{Sc} immunoreactivity in the cerebellum compared to the cerebella of sheep inoculated with WTD scrapie or x124 (Figure 7 B, E, H). There was little immunoreactivity in the molecular layer of the cerebella of sheep inoculated with No.13-7, whereas sheep inoculated with either WTD scrapie or x124 had intense, widespread

immunoreactivity. There was less PrP^{Sc} deposition in the granular layer of the cerebella in No.13-7 challenged sheep compared to WTD scrapie or x124 challenged sheep. Stellate, small aggregates, linear, and granular labeling are present in neocortical layers I-III and V of WTD scrapie inoculated sheep and not present in No.13-7 inoculated sheep (Figure 7 C, F, I). Granular and linear labeling was not as prominent in layer IV of No.13-7 inoculated sheep compared to WTD scrapie or x124 inoculated sheep. WTD scrapie and x124 challenged sheep also had intense immunoreactivity of PrP^{Sc} in layer IV of the neocortex compared to No.13-7 inoculated sheep that had mild immunoreactivity in this layer. PrP^{Sc} deposition also was present in neocortical white matter of WTD scrapie or x124 sheep, whereas it was absent in the No.13-7 sheep (Figure 7). These results indicate that there are changes in immunoreactivity after the No.13-7 scrapie isolate has been passaged through deer. Furthermore, sheep challenged with WTD scrapie had similar PrP^{Sc} immunoreactivity patterns to sheep challenged with x124.

Discussion

A summary of the serial passage of the No.13-7 scrapie isolate to sheep in the current study is shown in Figure 8. The US No.13-7 scrapie isolate was passaged four times by the intracranial route in sheep of the AA136 genotype [8]. One sheep from this passage group was used to challenge white-tailed deer by the oronasal route (Greenlee submitted). Two brain regions from these deer (cerebrum and brainstem at the level of the obex) had different molecular weight profiles by western blot. In the current study, samples of brainstem and cerebrum from scrapie-affected deer were used to challenge Suffolk sheep by the oronasal route.

Sheep Are Susceptible to the Scrapie Agent Derived From White-tailed Deer

The first sheep to develop disease were challenged with the scrapie agent derived from deer cerebrum. In this challenge group, sheep of the VV136 genotype developed disease faster than sheep of the AA136 genotype. Only one of the five sheep challenged with the scrapie agent derived from deer brainstem at the level of the obex developed clinical signs and this sheep had the VV136 genotype. Overall, only one of the four sheep with the AA136 genotype developed disease. We previously reported that in sheep inoculated with the US No.13-7 scrapie isolate all sheep develop clinical signs. Sheep of the AA136 genotype develop disease faster than sheep of the VV136 genotype [9]. Therefore, passage of the US No.13-7 scrapie isolate through white-tailed deer results in a change in disease phenotype with sheep of the VV136 genotype developing disease faster than sheep of the AA136 genotype.

Sheep of the $AV_{136}QR_{171}$ genotype challenged with either cerebrum or brainstem at the level of the obex from deer failed to develop disease. These results are similar to findings of sheep with the $AV_{136}QR_{171}$ (0 out of 2) or $AA_{136}QR_{171}$ (0 out of 3) genotype challenged with BSE isolates [3]. It has also been reported that sheep of the $AA_{136}QR_{171}$ genotype do not develop disease or PrP^{Sc} accumulation when challenged by the oronasal route with the No. 13-7 scrapie isolate [17]. The findings combined are consistent with the presence of arginine (R) at this position (171) providing more resistance to disease [3].

We have shown that the transmission of the scrapie agent derived from white-tailed deer is possible by the oronasal route. In addition, passage of the US No.13-7

scrapie isolate through white-tailed deer results in a disease phenotype switch. The US No.13-7 scrapie isolate has a more rapid incubation period in sheep of the AA136 genotype prior to passage in deer. In contrast, the scrapie agent that results after passage in deer has a more rapid incubation period in sheep of the VV136 genotype.

The Phenotype Switch After Passage Through Deer Is Associated with New Sheep Genotype Susceptibilities

We observed a change in disease phenotype as a shortened incubation period in sheep of the VV136 genotype after the scrapie agent was passaged in deer. Sheep of the VV136 genotype developed disease faster than sheep of the AA136 genotype. A possible explanation for this phenotype change in sheep can be supported by previous work that reported on the susceptibility of sheep to mule deer CWD [18]. In that study, only 1 out of the 8 sheep of the AV136 genotype ($AA_{136}QR_{171}$ n = 4; $AA_{136}QQ_{171}$ n = 3; $AV_{136}QQ_{171}$ n = 1) challenged by the intracranial route with mule deer CWD developed clinical signs and detectable PrP^{Sc}. It was suggested that susceptibility of sheep to CWD might be partially dependent on valine (V) at codon 136. This is because the sheep that had evidence of PrP^{Sc} and clinical signs had the genotype AV136 and was the only one from the study with a valine (V) at codon 136 [18].

The Transmission of CWD To Sheep Is Less Efficient Than The Transmission of Scrapie to Deer

Sheep are not very susceptible to white-tailed deer CWD. To date, oronasal challenge with white-tailed deer CWD has been unsuccessful (current study at 50 MPI). CWD from either elk or mule deer into sheep was unsuccessful or had a low attack rate after intracerebral inoculation [18, 19]. In addition, there was no transmission of elk

CWD to sheep by the oronasal route [19]. Whereas, a low attack rate (2 out of 10) was found in sheep challenged IC with mule deer CWD [18]. These findings also are consistent with other reports that used transgenic ovinized mice. Tg338 mice (ovine *PRNP*; VRQ) did not develop disease when challenged with white-tailed deer CWD [20]. In addition, there was ineffective transmission of CWD to transgenic mice expressing ovine *PRNP* (Tg338) whether it was derived from elk, mule deer, or white-tailed deer [21]. Failure to develop CWD after oral exposure and low attack rates after IC inoculation supports the conclusion that sheep are not highly susceptible to CWD.

White-tailed deer are highly susceptible to the sheep scrapie agent. A 100% attack rate has been reported in white-tailed deer inoculated with No. 13-7 when intracranially [7] or oronasally (Greenlee submitted) inoculated. White-tailed deer also have a 100% attack rate when challenged by the intravenous route with the SSBP/1 scrapie isolate [22]. Conversely, there was no transmission of the sheep scrapie agent to cervidized mice when challenged with a naturally acquired strain of scrapie [20]. Interestingly, inoculum from sheep challenged by the intracranial route with elk CWD had efficient transmission to both ovinized and cervidized mice [20]. The findings combined suggest that transmission of the agents of prion disease between sheep and deer is one-way. That is, sheep have low susceptiblity to the CWD agent but deer are highly susceptible to the scrapie agent.

Differences in Patterns of Immunoreactivity were Notable Between Sheep Genotypes and Inoculum Sources

Sheep of the VV136 genotype inoculated with the scrapie agent from deer cerebrum had more PrP^{Sc} immunoreactivity in the cerebellum and neocortex compared

to sheep of the AA136 genotype. However, previous work in sheep challenged with the original No.13-7 isolate described much less extensive immunoreactivity in the neocortex of sheep with the VV136 genotype in comparison to sheep of the AA136 genotype [9]. This is consistent with immunoreactivity differences described in cases of naturally and experimentally challenged scrapie. PrP^{Sc} patterns demonstrated by immunohistochemistry were found to be different between sheep of the AA136 and VV136 genotypes in cases of natural scrapie [25-27]. Sheep with the AA136 genotype had predominately punctate patterning in the dorsal motor nucleus of the vagus nerve, whereas sheep of the VV136 genotype had predominately granular and coalescing patterning in the dorsal motor nucleus of the vagus nerve [25]. When comparing Welsh Mountain sheep of the VV136 genotype to Suffolk sheep of the AA136 genotype naturally infected with scrapie, sheep of the VV136 genotype had more vascular plaque PrP^{Sc} accumulation throughout the brain, whereas sheep of the AA136 genotype had more astrocyte-associated and neuropil PrP^{Sc} accumulation [26]. Differences in immunohistochemistry PrP^{Sc} patterns also were found in sheep orally challenged with scrapie. Sheep of the VV136 genotype had prominent neuronal and glial intracellular aggregates, whereas sheep of the AA136 genotype presented with more stellate and astrocyte-associated types and no glia-associated aggregates [27]. Consistent with these findings, the present study demonstrates that experimental transmission by the oronasal route can result in immunoreactivity differences between sheep genotypes.

Differences in the amount of PrP^{Sc} immunoreactivity were observed in sheep of the same genotype when challenged with different inocula sources. Sheep with the VV136 genotype challenged with the scrapie agent derived from deer cerebrum had

extensive PrP^{Sc} in the obex, cerebellum, and neocortex. In contrast, sheep (VV136) challenged with the scrapie agent from deer obex that had less immunoreactivity in these areas. We also noted differences in immunoreactivity in brain regions of sheep of the VV136 genotype when challenged with two different inocula, No.13-7 and x124 [9]. The differences in immunoreactivity between sheep genotypes and inocula source supports our conclusion that a change in phenotype occurs after the original No. 13-7 scrapie isolate was passaged through deer.

Passage of the No.13-7 Scrapie Agent Through Deer Results in Shorter Incubation Periods in Ovinized Mice

The ovinized mouse line, Tg338, is established for studying the biological behavior of scrapie and CWD isolates [20-21, 28-32]. Our most surprising result was the shortened incubation period of the No.13-7 isolate after passage through deer. This shorter incubation period was similar to another US scrapie isolate, x124, in ovinized mice. The x124 scrapie isolate has a rapid incubation time in sheep with the VV136 genotype [9, 33]. The shorter incubation period in ovinized mice inoculated with the WTD scrapie agent compared to the relatively long incubation period observed in mice inoculated with the No.13-7 sheep scrapie isolate provides further evidence of a phenotype switch from the No.13-7 scrapie isolate after passage through deer.

Passage of No.13-7 Through White-tailed Deer Results in a Phenotype Similar to x124

The bioassay findings led us to compare PrP^{Sc} immunoreactivity between VV136 sheep challenged with the No.13-7, WTD scrapie, or x124 agent. We found that sheep inoculated with WTD scrapie sheep had more PrP^{Sc} immunoreactivity in the obex,

cerebellum, and neocortex, which was in contrast to sheep inoculated with No.13-7. Similar immunoreactivity patterns were observed in the brains of sheep inoculated with WTD scrapie and x124. We previously described differences in PrP^{Sc} immunoreactivity patterns in VV136 sheep challenged with x124 or No.13-7 scrapie isolates [9].

These results indicate that passage of sheep scrapie through WTD leads to a change in disease phenotype. This phenotype switch is observed as different immunoreactivity levels and PrP^{Sc} accumulation patterns in sheep challenged with the WTD scrapie agent compared to sheep challenged with the original No.13-7 scrapie isolate. A change in disease phenotype is also supported by results of bioassay in transgenic mice expressing ovine *PRNP* that results in a shorted incubation period of the No.13-7 scrapie isolate after it was passaged in deer.

Conclusions

Sheep are susceptible to the scrapie agent derived from white-tailed deer when challenged by the oronasal route. Passage of the sheep-derived US No. 13-7 scrapie isolate through white-tailed deer results in a change in disease phenotype that is observed when the deer-passaged scrapie is inoculated back into sheep or ovinized mice. In sheep, the relationship between incubation period and genotype is reversed; the original No.13-7 scrapie isolate produces a shorter incubation period in AA136 sheep compared to VV136 sheep, while the deer-passaged scrapie isolate results in a shorter incubation period in VV136 sheep. In addition, passage of the No.13-7 isolate through deer results in a change in the pattern of PrP^{Sc} deposition in the brain such that the PrP^{Sc} patterns in VV136 sheep challenged with WTD scrapie look similar to x124. The results indicate that if there is contact between scrapie-infected deer and sheep,

there is the potential for reinfection of sheep with deer-derived scrapie. Furthermore, interspecies transmission of the scrapie agent can result in a phenotype switch that differs from the original inoculum and potentially promote the generation of new scrapie strain properties.

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Table 1. EIA optical densities of the WTD scrapie inocula

Dilution	Deer Cerebrum	Deer Obex
1:1	4	4
1:25	0.999	4
1:50	0.611	2.46
1:100	0.399	1.925

Samples of inoculum from either deer cerebrum or deer obex inocula samples were assayed on EIA in the following increasing dilutions to determine the relative amount of PrP^{Sc} in each sample.

Table 2. EIA optical densities of the mouse bioassay inocula

Dilution	No.13-7	Sheep 2	x124	Sheep 6
1:1	4	3.926	4	2.006
1:25	3.701	3.427	3.04	0.195
1:50	3.176	2.917	3.441	0.167
1:100	2.156	1.854	2.354	0.125

The inocula for mouse bioassay derived from sheep infected with No.13-7, WTD scrapie sheep 2, x124, and WTD scrapie sheep 6 used as inoculum source for mouse bioassay were tested via EIA in increasing dilutions to determine the relative amount of PrP^{Sc}.

Table 3. EIA optical densities of brain regions of sheep infected with the scrapie agent from WTD

Sheep Genotype: VVQQ									
	Sheep 1			**Sheep 2**			**Sheep 6**		
Dilution	Obex	Cerebellum	Neocortex	Obex	Cerebellum	Neocortex	Obex	Cerebellum	Neocortex
1:1	3.835	3.526	2.332	3.608	4	3.77	4	0.692	0.314
1:50	3.717	1.994	0.458	3.134	2.319	2.242	1.071	0.201	0.203
1:100	3.678	1.295	0.351	2.85	1.713	0.845	1.182	0.166	0.153
Sheep Genotype: AAQQ									
	Sheep 3			**Sheep 4**					
Dilution	Obex	Cerebellum	Neocortex	Obex	Cerebellum	Neocortex			
1:1	4	3.635	0.391	0.132	0.129	0.137			
1:50	3.278	1.048	0.204	0.133	0.115	0.147			
1:100	3.151	0.486	0.175	0.172	0.122	0.116			

Samples from the brainstem at the level of the obex, cerebellum, and neocortex of the animals tested for PrP^{Sc} immunoreactivity in Figure 2 were quantified using EIA in increasing dilutions to determine the relative amount of PrP^{Sc} present in each brain region.

Table 4. Brain regions scored for spongiform change

Neuroanatomical area	Area Code
Nucleus of the solitary tract	1
Nucleus of the spinal tract of V	2
Hypoglossal nucleus	3
Vestibular nuclear complex	4
Cochlear nucleus	5
Cerebellar vermis	6
Central grey matter	7
Rostral colliculus	8
Medial geniculate nucleus	9
Hypothalamus	10
Nucleus dorsomedialis thalami	11
Nucleus ventralis lateralis thalami	12
Frontal cortex	13
Septal nuclei	14
Caudate Nucleus	15
Putamen	16
Claustrum	17

Each brain region has a corresponding area code. Perspective score for each animal is shown in Figure 4.

Table 5. PrP^{Sc} distribution in sheep inoculated with the scrapie agent derived from white-tailed deer

Sheep Number	Genotype Codon 136	Inoculum	Incubation Period (MPI)	Clinical Signs	LRS head	LRS other	Rectal	Retina	PNS
1	VV	Cerebrum	31	+	+	+	+	+	+
2	VV	Cerebrum	28	+	+	+	+	+	+
3	AA	Cerebrum	48	+	+	+	+	-	+
4	AA	Cerebrum	56	-	-	-	-	-	-
5	AV	Cerebrum	72	-	-	-	-	-	-
6	VV	Obex	65	+	+	-	+	+	+
7	VV	Obex	72	-	-	-	-	-	-
8	AA	Obex	72	-	-	-	-	-	-
9	AA	Obex	72	-	-	-	-	-	-
10	AV	Obex	72	-	-	-	-	-	-

Animal data, incubation period, presence of clinical signs at death, and PrP^{Sc} distribution for lymphoid tissues and non-brain nervous tissue.
Abbreviations: Residue at codon 136 of the *PRNP* - V: valine, A; alanine. Months post-inoculation (MPI). LRS: lymphoreticular system. PNS: peripheral nervous system tissue, CNS: central nervous system.

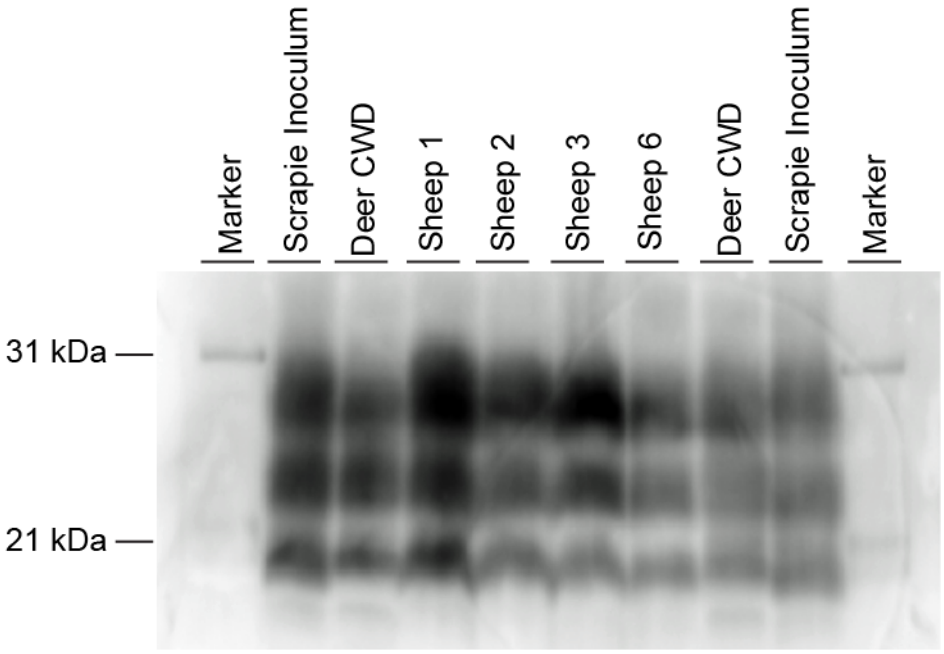

Figure 1. Western blot of brainstem samples from sheep with clinical signs after inoculation with the WTD scrapie agent. Western blot comparison of sheep samples inoculated with WTD scrapie to the original No.13-7 scrapie inoculum and deer CWD. Monoclonal antibody P4 was used for the detection of PrP^{Sc}. Marker = molecular mass marker.

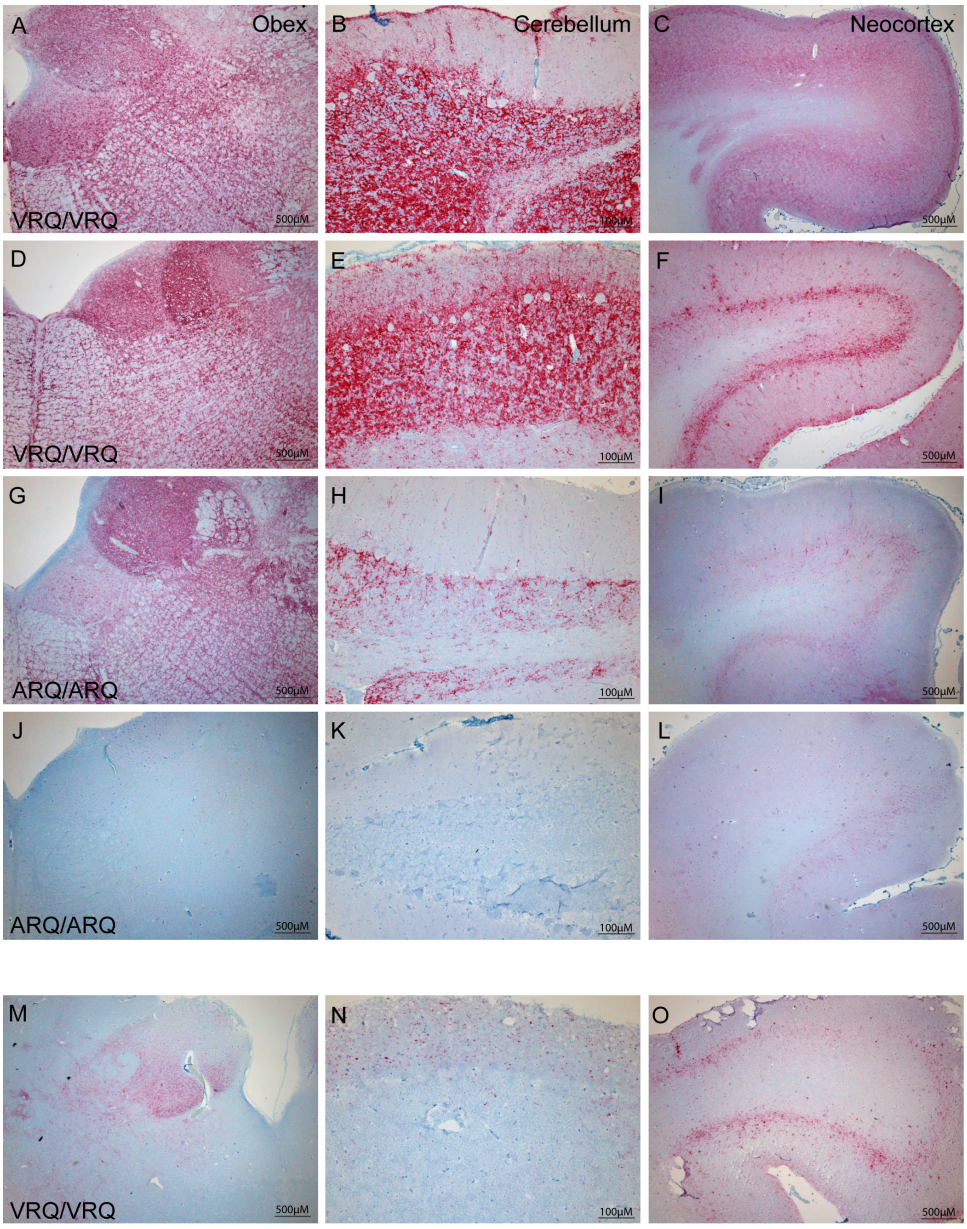

Figure 2. PrP^{Sc} deposition in brain regions varies with sheep *PRNP* genotype and source of inoculum. Sheep with immunoreactivity were euthanized at similar stages of clinical disease. Sheep 1 (A-C), sheep 2 (D-F), sheep 3 (G-I), sheep 4 (J-L), and sheep 6 (M-O). The two VRQ/VRQ sheep inoculated with the WTD scrapie agent from cerebrum (sheep 1 and 2) had intense widespread immunoreactivity in the brainstem at the level of the obex (A,D), cerebellum (B,E), and neocortex (C,F). An ARQ/ARQ sheep inoculated with the WTD scrapie agent from cerebrum (sheep 3) had less intense but widespread accumulation in the obex (G), cerebellum (H), and neocortex (I). Representative brain regions of ARQ/ARQ (sheep 4) were negative by EIA, WB, and IHC (J-L). A VRQ/VRQ sheep inoculated with the WTD scrapie agent from obex (sheep 6) had mild PrP^{Sc} accumulation in the obex (M), cerebellum (N), and neocortex (O).

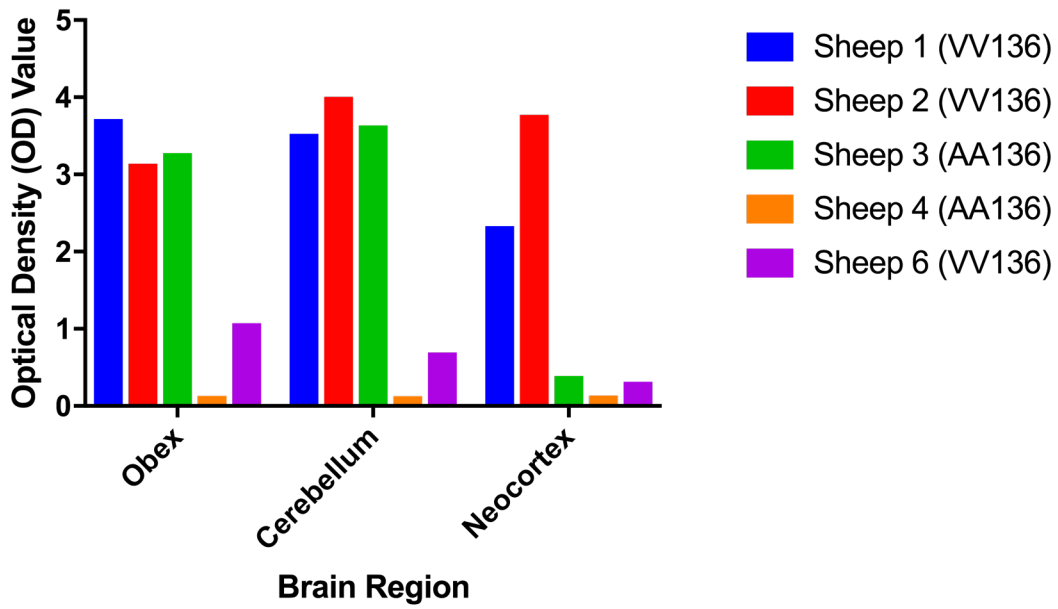

Figure 3. Quantitative analysis of PrP^{Sc} present in brainstem, cerebellum, and neocortex. Differences in the amount of PrP^{Sc} present in brain regions observed by IHC (Figure 2) can be assessed using EIA. The VRQ/VRQ sheep (sheep 1 and 2) have high OD values in all three sheep brain regions. The positive ARQ/ARQ sheep (sheep 3) had high OD values for obex and cerebellum but a lower score for the neocortex compared to VRQ sheep that received the same inoculum. The ARQ/ARQ sheep (sheep 4) without immunoreactivity by IHC also was negative by EIA in all three brain regions. A VRQ/VRQ sheep challenged with the scrapie agent from deer obex (sheep 6) was positive by EIA but with low OD values relative to sheep challenged with inoculum from deer cerebrum.

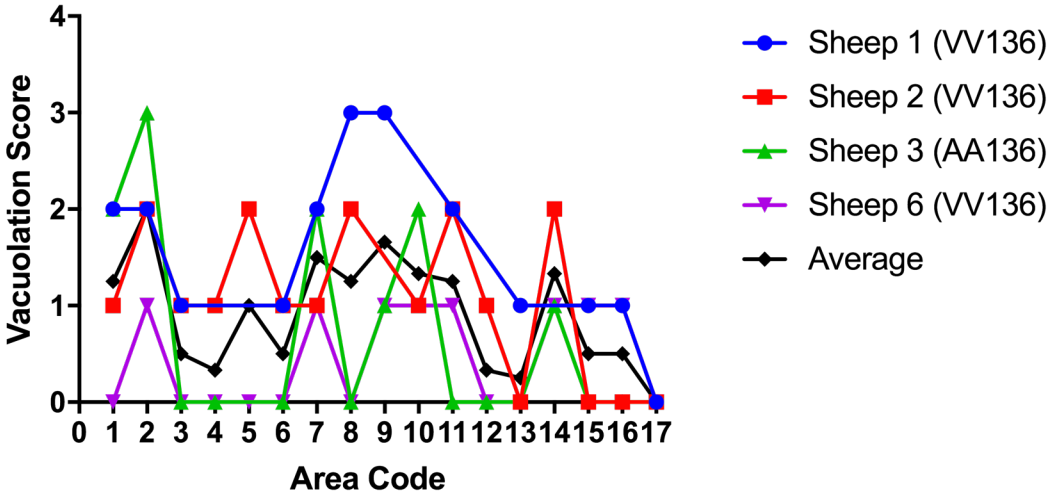

Figure 4. Gray matter vacuolation profiles for sheep inoculated with the WTD scrapie agent. Area codes for specific brain regions are defined in Table 4. Variations in vacuolation scores between sheep of different genotypes challenged with same inoculum and between sheep of the same genotype challenged with different inocula. Similar vacuolation scores were seen in VRQ/VRQ sheep that received WTD cerebrum (sheep 1 and 2). A ARQ/ARQ sheep (sheep 3) and the VRQ/VRQ sheep (sheep 6) inoculated with WTD obex had lower vacuolation scores compared to the VRQ/VRQ sheep challenged with WTD cerebrum. Differences were seen between sheep 1 and 3 and between sheep 1 and 6. Sheep 4 was left out of figure due to all areas receiving a score of 0 for vacuolation.

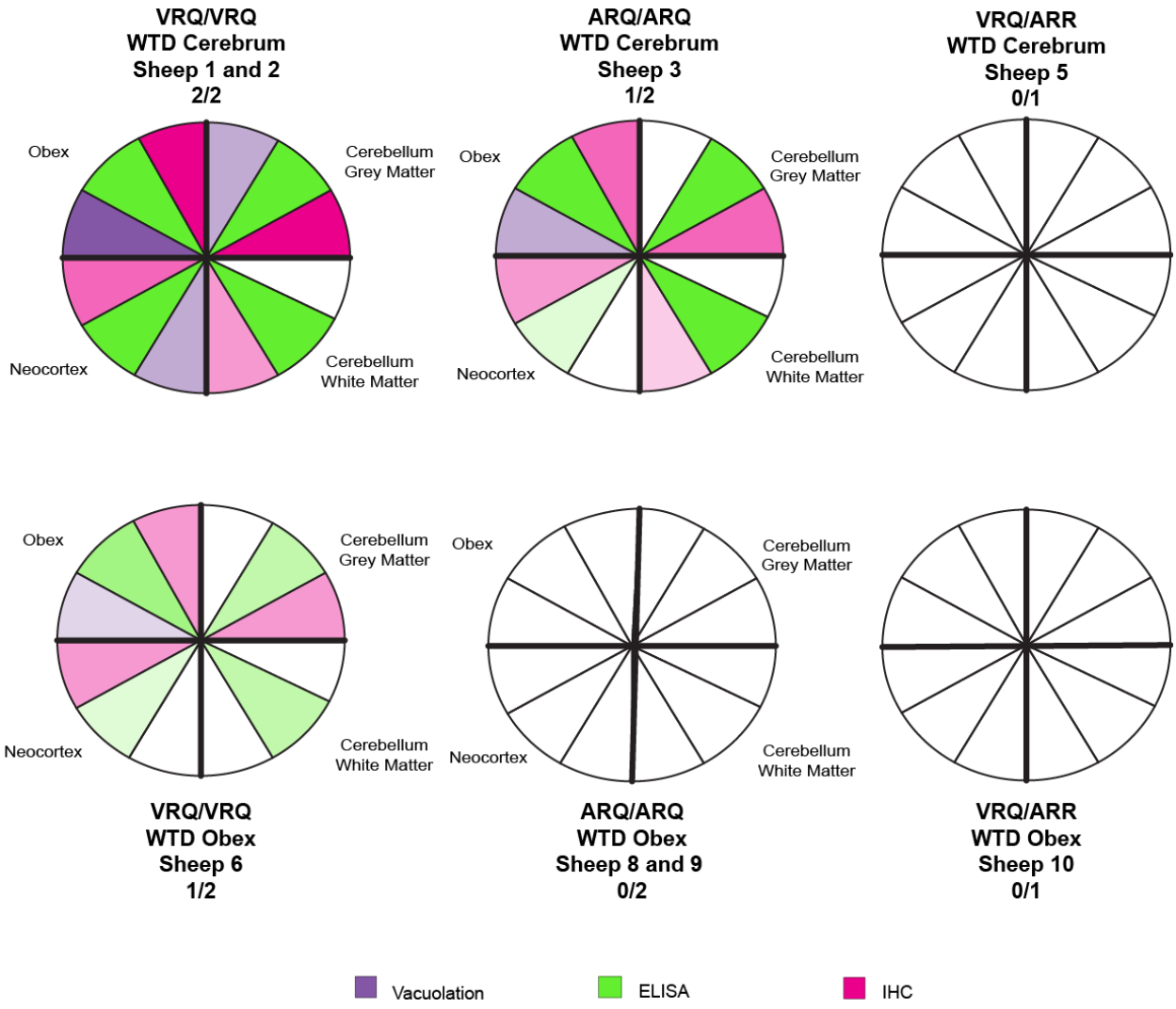

Figure 5. Association of vacuolation, EIA, and immunohistochemistry data with results by sheep genotype and inoculum source. This summary demonstrates that differences can be seen between sheep of different genotypes challenged with the same inoculum and sheep of the same genotype challenged with different inocula. Each quadrant represents a brain region tested (obex, cerebellum grey matter, cerebellum white matter, or neocortex). Each wedge in the quadrant is a different test (vacuolation, EIA, or IHC). The color of the wedge represents the severity of the results from each test, where lower scores received a lighter shade and higher scores received a darker shade. Legend line key: 1) sheep genotype 2) inocula source 3) sheep represented 4) attack rate. Sheep 4 and 7 were negative and not diagramed.

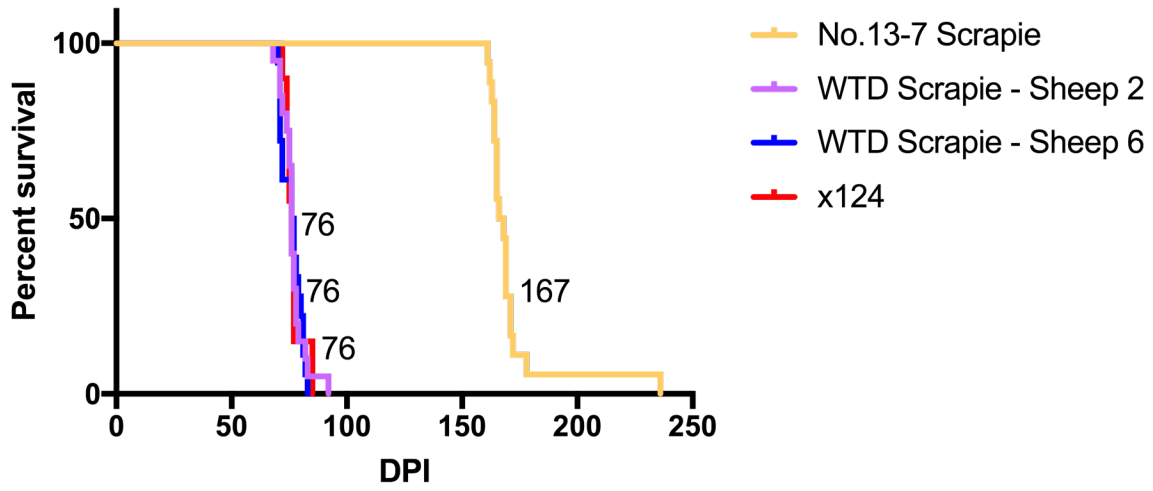

Figure 6. The incubation period of ovinized transgenic mice (Tg338) challenged with No.13-7 inoculum from sheep of the VV136 genotype was reduced after passage through deer. The incubation period of mice inoculated with WTD scrapie from sheep was shorter than the original No.13-7 inoculum and was similar to the incubation period of 76 days post-inoculation (DPI) to that of another strain of scrapie strain, x124. All inocula were from sheep with the VRQ/VRQ genotype.

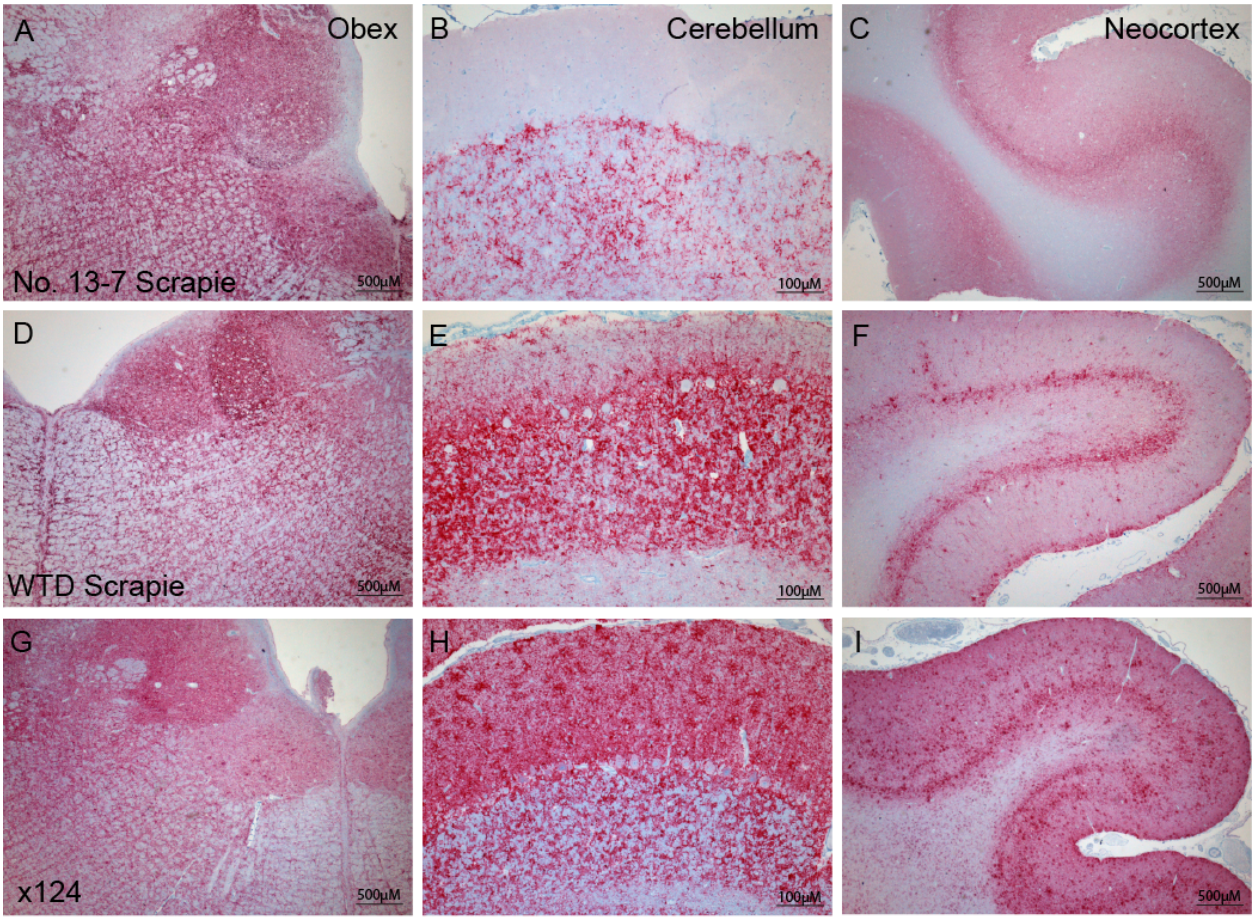

Figure 7. Comparison of PrP^{Sc} accumulation in various brain regions of VV136 sheep challenged with the scrapie agent from different sources. PrP^{Sc} distribution differences are noticeable in various brain regions of sheep inoculated with different scrapie isolates. The original No.13-7 scrapie inoculum in sheep is represented in figures A, B, C. WTD scrapie in sheep 2, has more PrP^{Sc} labeling in the cerebellum (E) and neocortex (F) compared to No.13-7 (B, C) and also similar, but less, labeling to x124 (Hamir, Vet Pathol 2009) (G, H, I).

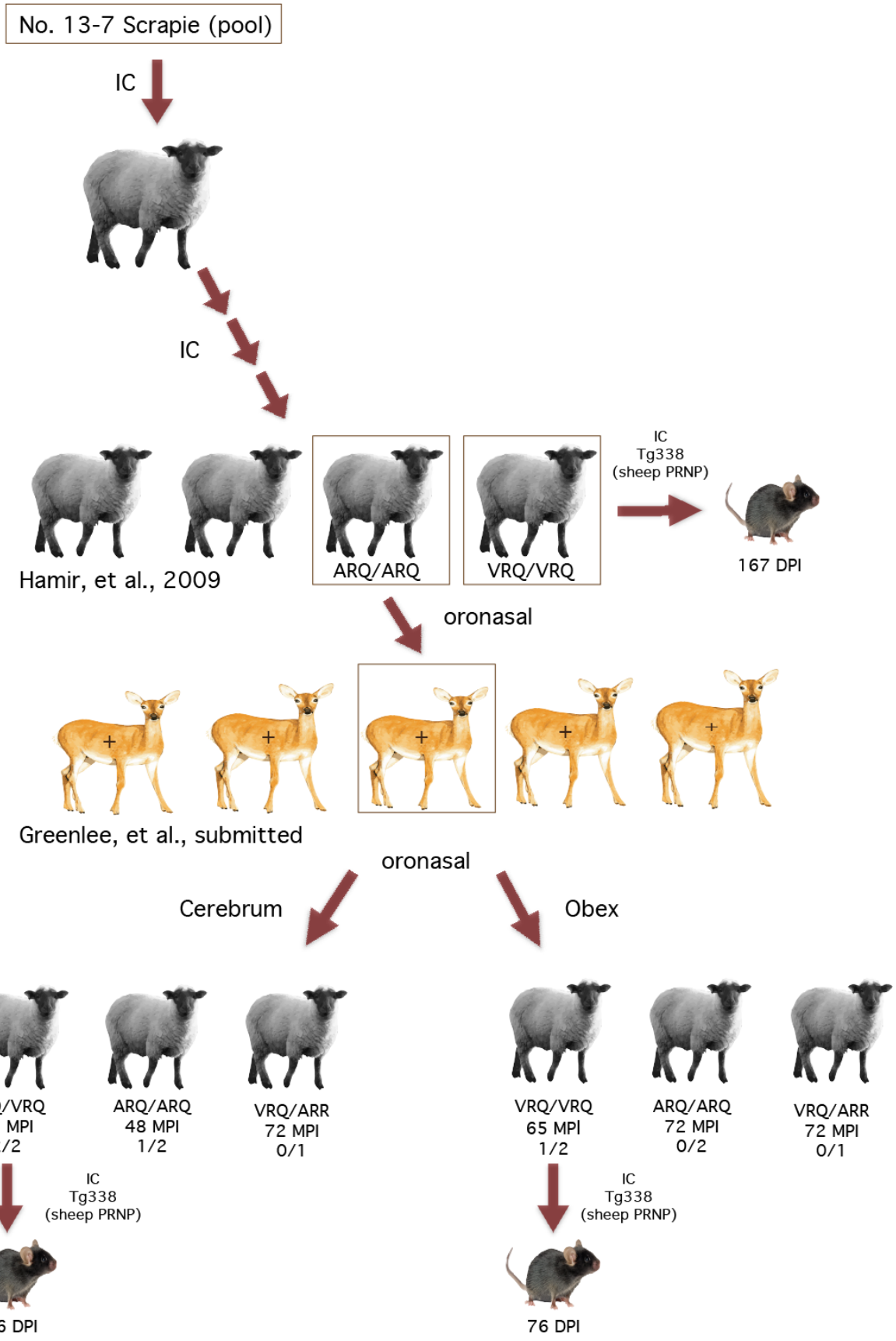
No. 13-7 Scrapie (pool)
IC
IC
Hamir, et al., 2009
ARQ/ARQ
VRQ/VRQ
IC
Tg338
(sheep PRNP)
167 DPI
oronasal
Greenlee, et al., submitted
oronasal
Cerebrum
Obex
VRQ/VRQ
29 MPI
2/2
ARQ/ARQ
48 MPI
1/2
VRQ/ARR
72 MPI
0/1
VRQ/VRQ
65 MPI
1/2
ARQ/ARQ
72 MPI
0/2
VRQ/ARR
72 MPI
0/1
IC
Tg338
(sheep PRNP)
IC
Tg338
(sheep PRNP)
76 DPI
76 DPI

Figure 8. Summary of serial passage of the original No.13-7 scrapie isolate. The original No.13-7 scrapie isolate was passaged four times by the intracranial route in sheep of the ARQ/ARQ genotype prior to its oronasal challenge in WTD. Inocula derived from cerebrum or obex sample from a deer with clinical signs was used to oronasally challenge sheep of various genotypes. The first two sheep to develop clinical signs (29 MPI) were challenged with the scrapie agent from deer cerebrum and had the VRQ/VRQ genotype. One ARQ/ARQ sheep challenged with deer cerebrum also developed disease but at 48 MPI. Sheep challenged with the scrapie agent from deer obex tested positive for PrP^{Sc} but with an extended incubation period of 65 MPI. Also shown is the inoculation route from WTD scrapie challenged sheep to Tg338 mice expressing ovine PrP. The mouse passage results can be found in Figure 5 of the current study.

CHAPTER 3. GENERAL CONCLUSIONS

Summary

The purpose of this work was to better understand the potential for interspecies transmission of TSEs between sheep and deer. I characterized the disease phenotype by looking at PrP^{Sc} accumulation intensities and pattern of deposition, incubation period, spongiform change, and the molecular mass of the misfolded prion protein. TSEs can result in different disease phenotypes depending on the recipient species and its *PRNP* polymorphisms. Here, I demonstrate how interspecies transmission can influence disease phenotype.

In chapter 2, I report results of work to determine if sheep were susceptible to oronasal challenge of the scrapie agent derived from white-tailed deer. It has been demonstrated that deer are readily susceptible to the agent of sheep scrapie, but sheep are not very susceptible to CWD. When white-tailed deer were challenged with the sheep scrapie agent, western blot analysis revealed two different molecular mass profiles from different brain regions. The cerebrum had a scrapie-like molecular mass, whereas the brainstem at the level of the obex had a CWD-like molecular mass. Because of these differences, sheep were challenged with brain homogenate made from either the cerebrum or the brainstem at the level of the obex. I determined that sheep are susceptible to the scrapie agent derived from white-tailed deer. I found that sheep of the VV136 genotype had a disease phenotype that differed from the original sheep scrapie isolate. When challenged with the original No.13-7 sheep-derived isolate, sheep of the AA136 genotype had the fastest incubation period. However, challenge of VV136 sheep with the deer-derived scrapie isolate resulted in a faster incubation period

than sheep of the AA136 genotype. Differences that further support a phenotype change includes PrP^{Sc} accumulation intensity and spongiform change between sheep of different genotypes challenged with the same inoculum and sheep of the same genotype challenged with different inocula. When sheep brain homogenate was bioassayed in transgenic mice expressing ovine *PRNP*, the WTD scrapie agent had a much shorter incubation period compared to the original sheep scrapie isolate.

I have identified that interspecies transmission of scrapie resulted in a disease phenotype switch that differs from the original source. The phenotype switch in sheep after passage through deer suggests interspecies transmission could generate new prion strain properties. This is of concern because scrapie-infected deer could pose as a risk for scrapie eradication programs. Since wild deer cannot be contained and can act as a host for the scrapie agent, it is important to characterize this potential new strain of scrapie that produces a new disease phenotype in sheep of different genotypes. Furthermore, scrapie-infected deer could carry infectivity back into geographical locations thought to be free from scrapie in sheep and goats.

Future Research

I have demonstrated that a phenotype switch occurred when sheep scrapie was passaged through deer and back to sheep. In order to characterize this new isolate, it is necessary to serially pass the WTD scrapie agent in sheep. A second passage of the WTD scrapie agent already has been done using inoculum made from a VV136 sheep and an AA136 sheep from the current work. Stabilization and characterization of this scrapie strain in sheep would allow us to be able to differentiate between a WTD

scrapie-infected sheep and a sheep infected with classical scrapie if there were to be an outbreak.

In order to classify this as a new strain of scrapie, it also will be necessary to pass the agent in strain typing mice. Briefly, this requires serial passage in three different strains of mice that would then make it possible to compare it to the original sheep scrapie isolate by comparison of incubation period and by lesion profiling. I would compare the incubation periods, PrP^{Sc} accumulation patterns and intensity, and spongiform change.

Passage of the WTD scrapie agent from sheep back to white-tailed deer would allow us to compare the disease phenotype of these deer to deer infected with CWD and to deer challenged with the original sheep scrapie agent (No.13-7). I would compare incubation periods, PrP^{Sc} immunoreactivity and molecular mass profiles. This work will further characterize the WTD scrapie agent. In addition, it would give us insight to multiple interspecies transmission events and the affect it would have on disease presentation.

Concluding Remarks

This thesis work addresses the concerns of the potential interspecies transmission of TSEs between sheep and deer. Using techniques to identify and differentiate between TSEs, we were able to demonstrate that a scrapie-infected deer could transmit the disease back to sheep. Furthermore, infected sheep presented with a disease with different characteristics than the original scrapie isolate. By demonstrating the differences between the original sheep scrapie isolate and the WTD scrapie agent, I

have provided insight into the ability of a TSE strain to change its properties after interspecies transmission.