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PrP^{Sc} accumulation in fetal cotyledons of scrapie-resistant lambs is influenced by fetus location in the uterus

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Placentae from scrapie-infected ewes have been shown to accumulate PrP^{Sc} when the genotype of the fetus is of a susceptible genotype (VRQ/VRQ, ARQ/VRQ or ARQ/ARQ). Cotyledons from fetuses of genotypes ARR/ARR, ARQ/ARR and ARQ/VRR have previously been shown to be resistant to PrP^{Sc} accumulation. By using ewes from a naturally infected scrapie flock, cotyledons from fetuses of multiple births of different genotypes were examined. PrP^{Sc} was detected in fetal cotyledons of genotype ARQ/ARQ, but not in cotyledons from their dizygotic twin of genotype ARQ/ARR. This confirms earlier reports of single fetuses of these genotypes, but is the first description of such a finding in twin fetuses, one of each genotype. It is also demonstrated that cotyledons from sibling fetuses of genotypes ARQ/VRQ and ARQ/ARQ have different patterns of PrP^{Sc} accumulation depending on whether the dam is of genotype ARQ/ARQ or ARQ/VRQ. Lastly, it is shown that cotyledons from fetuses with resistant genotypes are weakly positive for PrP^{Sc} when they have shared the same pregnant uterine horn with a fetus of a susceptible genotype with cotyledons positive for the detection of PrP^{Sc}. Additionally, a PCR product for the *Sry* gene, a product specific to males, was found in cotyledons from female fetuses that had shared a uterine horn with a male fetus. This indicates that some sharing of fetal blood occurs between placentomes and fetuses residing in the same uterine horn, which can result in PrP^{Sc} accumulation in cotyledons with resistant genotypes.

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INTRODUCTION

Scrapie is a fatal, neurodegenerative disease of sheep and goats. It belongs to a group of diseases known as transmissible spongiform encephalopathies that are characterized by the accumulation of an abnormal isoform of a host-encoded protein, PrP^C. The abnormally folded, partially proteinase K-resistant isoform of PrP^C is designated PrP^{Sc} and accumulates in the central and peripheral nervous system and the lymphoreticular system (Ikegami *et al.*, 1991; Race *et al.*, 1998). The placenta has also been shown to accumulate PrP^{Sc} (Andréoletti *et al.*, 2002a; Caplazi *et al.*, 2004; Onodera *et al.*, 1993; Race *et al.*, 1998; Tuo *et al.*, 2001, 2002). Infectivity of the placenta was shown in an oral dosing experiment carried out in sheep and goats (Pattison *et al.*, 1972). PrP^{Sc} is thought to be transmitted from infected dams to susceptible lambs and cohort adult sheep during the perinatal period by exposure to infectious placental tissues and/or fluids, rather than *in utero* transmission (Andréoletti *et al.*, 2002b). Ovine susceptibility to scrapie is controlled by polymorphisms in the *Prnp* gene, encoding the PrP protein, at codons 136 (A or V), 154 (R or H) and 171 (R, Q or H) (Cloucard *et al.*, 1995; Hunter *et al.*, 1997b). Sheep with genotypes V¹³⁶R¹⁵⁴Q¹⁷¹/VRQ, ARQ/VRQ and ARQ/ARQ

are the most susceptible to scrapie, whilst ARR/ARR and ARQ/ARR sheep are considered to be resistant (Cloucard *et al.*, 1995; Hunter *et al.*, 1996, 1997b). Furthermore, in flocks where there is a high incidence of valine at codon 136 of the *Prnp* gene, sheep of genotype ARQ/ARQ are less susceptible or resistant to infection (Belt *et al.*, 1995; Evoniuk *et al.*, 2005; Goldmann *et al.*, 1991; Hunter *et al.*, 1991, 1994, 1997a; Laplanche *et al.*, 1993).

Previous studies have reported on the role of fetal genotype in the control of PrP^{Sc} accumulation in the placenta. Accumulation of PrP^{Sc} has been demonstrated in 171 QQ placentomes of scrapie-infected ewes, but not in placentomes of infected ewes pregnant with 171 QR fetuses (Tuo *et al.*, 2002). Furthermore, it has been shown that ARR/ARQ fetal cotyledons were negative for PrP^{Sc} accumulation when conceived by ARQ/VRQ dams (Andréoletti *et al.*, 2002b). Fetal cotyledons of genotype ARR/VRQ from ARQ/VRQ or ARR/VRQ dams were also shown to be negative for PrP^{Sc} accumulation. This study investigates how the spatial relationship of the fetuses to each other influences PrP^{Sc} accumulation in dams with multiple fetuses of different genotypes. Our results suggest that there can be a partial or incomplete anastomosis between the fetal blood supplies to

the cotyledons among fetuses on the same side of the uterine horn, resulting in PrP^{Sc} accumulation in cotyledons with resistant genotypes.

METHODS

Animals and tissue collection. Ewes used in this study were naturally infected Suffolk or Suffolk × Hampshire sheep between 2 and 4 years of age. The sheep were sourced from multiple flocks from across the United States. The scrapie status of the ewes was determined ante-mortem by immunohistochemistry (IHC) of the third-eyelid lymphoid tissue (O'Rourke *et al.*, 2000). Post-mortem scrapie status was determined by IHC of the brainstem. Sheep with clinical signs of scrapie were euthanized humanely. All euthanized sheep were at 100 days or more gestation (full-term gestation is 144–150 days). For individual fetuses, between eight and ten placentomes (maternal caruncle plus fetal cotyledon) were collected from their respective placentae at necropsy. The blood supply from the fetal cotyledon was traced to the umbilicus of the fetus to ensure matching of the placentomes and fetuses. In addition, fetal cotyledons were genotyped and matched to the genotype of the fetus by genotyping a piece of fetal liver also collected at necropsy. Two placentomes were collected whole and stored in formalin until preparation for IHC. The remaining placentomes were gently separated manually into maternal caruncle and fetal cotyledon and stored at –20 °C until use. When necessary because of unexpected parturition, shed placentae were collected after parturition as soon as possible after they were passed. When spread out, the placentae duplicated the shape of the ewe's uterus with two uterine horns. For shed placentae, between 10 and 15 cotyledons were collected from each section of the placenta corresponding to a uterine horn in order to obtain cotyledons from each fetus contained within the placenta. Cotyledons and lambs were genotyped to determine fetal positioning *in utero*. Cotyledons were stored at –20 °C until use. Animal care, handling and use in this study were approved by the

Institutional Animal Care and Use Committee of Washington State University. The total number of dams and their respective fetuses used in the study are listed in Table 1.

Tissue processing and Western blot analysis. Samples were prepared and analysed by Western blot using a phosphotungstic acid precipitation method as described previously (Spraker *et al.*, 2004) with the following exception: mAbs F99/97.6.1 (VMRD) and P4 (R-Biopharm AG) were used for detection of PrP^{Sc}. Cotyledon samples from known scrapie-infected and -uninfected sheep as determined by Western blot and IHC of brain tissue were run as positive and negative controls, respectively.

IHC. IHC was performed as described previously (O'Rourke *et al.*, 2000; Tuo *et al.*, 2002). Briefly, tissues were fixed in 10% neutral-buffered formalin for 2–7 days, treated with 96% formic acid for 1 h, rinsed with water for at least 10 min and fixed in formalin for an additional 24 h before embedding in paraffin. Tissue sections placed on glass slides were deparaffinized and stained by an automated system (Benchmark; Ventana Medical Systems). Primary mAbs F99/97.6.1 (recognizing the epitope QYQRES) and F89/160.1.5 (recognizing the epitope IHFG) were applied as a cocktail of each antibody at 5 µg ml⁻¹. This was followed by the application of a biotinylated secondary antibody, streptavidin–horseradish peroxidase and detection with 3-amino-9-ethylcarbazole–H₂O₂. Tissues from scrapie-infected and -uninfected sheep were used as controls with each run. Negative controls included isotype-matched irrelevant IgG and blocking of the mAbs with synthetic peptides to which the mAbs were raised. PrP^{Sc} immunoreactivity was visualized by using a bright-field microscope. Photomicrographs were taken with a microscope equipped with a digital camera.

DNA isolation. DNA was isolated from 600 µl aliquots of blood by using a PureGene kit (Gentra), following the manufacturer's protocols. DNA was isolated from approximately 25 mg fetal cotyledon tissue by using a Qbiogene FastDNA kit (catalogue no. 6540-400) following the manufacturer's protocols.

Table 1. PrP^{Sc} accumulation and presence of *Sry* PCR product in fetal cotyledons according to dam genotype, fetal genotype and multiple births from dams with natural scrapie

Dam no.	<i>Prnp</i> genotype	Sibling fetal letter	<i>Prnp</i> genotype	Fetal sex	Uterine horn	PrP ^{Sc} in cotyledon	<i>Sry</i> PCR product
1	ARQ/ARQ	A	ARQ/ARR	F	1	–	–
		B	ARQ/ARQ	F	2	+	–
2	ARQ/ARQ	A	ARQ/ARR	F	1	–	–
		B	ARQ/ARQ	M	2	+	+
3	ARQ/ARQ	A	ARQ/ARR	M	1	+	+
		B	ARQ/ARQ	F	1	+	+
		C	ARQ/ARR	F	2	–	–
4	ARQ/VRQ	A	ARQ/VRQ	M	1	+	+
		B	ARQ/ARQ	F	2	–	–
5	ARQ/VRQ	A	ARQ/VRQ	M	1	+	+
		B	ARQ/VRQ	M	1	+	+
		C	ARQ/VRQ	F	2	+	–
		D	ARQ/ARQ	F	2	–	–
6	ARQ/VRQ	A	ARQ/VRQ	M	1	+	+
		B	ARQ/ARQ	F	1	+	+
		C	ARQ/VRQ	M	2	+	+
7	ARQ/ARQ	A	ARQ/ARQ	M	1	+	+
		B	ARQ/VRQ	F	2	–	–

Genotyping. Approximately 100 ng DNA was used as a template for amplification of the open reading frame of *Prnp* using the following primer pair: 5'-GGCATTGATGCTGACACC-3' and 5'-TACAGGGCTGCAGGTAGAC-3', corresponding to nucleotide positions 22234–22252 and 23106–23125 of GenBank accession no. U67922. Reactions were performed in a final volume of 90 µl under the following conditions: 1× Qiagen PCR buffer, 2.5 mM MgCl₂ and 2.5 U *Taq* polymerase (Qiagen *Taq*PCR Core kit; catalogue no. 201225). Amplification was performed under a temperature regime of 95 °C for 5 min, 62 °C for 30 s and 72 °C for 59 s for one cycle; 95 °C for 30 s, 62 °C for 30 s and 72 °C for 59 s for 30 cycles; and 95 °C for 30 s, 62 °C for 30 s and 72 °C for 7 min for one cycle. Amplified products were sequenced by standard dideoxynucleotide analysis (Amplicon Express) using primer pair 5'-CTGGGGTCAAGGTGGTAGCC-3' and 5'-GGTGGTACTGTGTGTTGCTTGA-3', corresponding to nucleotide positions 22553–22573 and 22838–22860 of GenBank accession no. U67922. Genotypes are shown as the deduced amino acid sequences at codons 136 (alanine, A, or valine, V), 154 (arginine, R) and 171 (glutamine, Q, or arginine, R) of the diploid genotype.

Sry PCR. Previous studies have used the *Sry* gene as a DNA marker for sex identification in a variety of mammals (Griffiths & Tiwari, 1993; Szatkowska *et al.*, 2004; Wilson & White, 1998). The *Sry* gene encodes a protein that has been identified as the testis-determining factor in mammals (Koopman *et al.*, 1991). Sex identification is based on the presence of an amplified PCR product in a male and absence of product in a female.

Approximately 100 ng DNA was used as a template for the amplification of the *Sry* region of the Y chromosome (Payen & Cotinot, 1993; Wilson & White, 1998). The primer sequences used were 5'-CTTCATTGTGTGGTCTCGTG-3' and 5'-CGGTATTGTCTCGGTGTA-3', corresponding to nucleotide positions 2–20 and 58–78 of a published bovine *Sry* sequence (Payen & Cotinot, 1993). Reactions were performed in a final volume of 90 µl under the following conditions: 1× Qiagen PCR buffer, 2.5 mM MgCl₂ and 2.5 U *Taq* polymerase (Qiagen *Taq*PCR Core kit). Amplification was performed under a temperature regime of 94 °C for 5 min, 60 °C for 30 s and 72 °C for 30 s for one cycle; 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s for 32 cycles; and 94 °C for 15 s, 60 °C for 30 s and 72 °C for 5 min for one cycle. *Sry* PCR products of approximately 200 bp were analysed by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide. A second PCR product present both in males and females, the *Prnp* gene, was amplified and used as an internal control by using the protocol listed above for *Prnp* gene amplification. DNA isolated from the blood of the lambs was also used in *Sry* amplification reactions to test for freemartinism in the female lambs; freemartinism is a condition that occurs in twins of different sexes in which the placental membranes that connect the fetus to the dam are shared and the placental fluids are exchanged between the two fetuses. The exchange of fluid and blood between the two fetuses mixes the antigens responsible for carrying the unique sex characteristics of each fetus and the twins develop with some sex characteristics of both the male and female. The female fetus is usually most affected and is sterile and masculinized. Blood was also sent to a commercial laboratory (UC Davis Veterinary Genetics Laboratory, Davis, CA, USA) for freemartin testing.

PrP^{Sc} detection by ELISA. A commercial test developed for lymphoid tissue [Chronic Wasting Disease (CWD) Antigen Test kit; IDEXX HerdChek] was used for detection of PrP^{Sc} in cotyledon tissue. This test is used for the post-mortem detection of PrP^{Sc} in white-tailed deer retropharyngeal lymph-node tissue, but is also suitable for the detection of PrP^{Sc} in sheep cotyledons: the kit utilizes a PrP^{Sc} ligand immobilized on the surface of the CWD antigen-capture plate and mAbs that recognize the ovine PrP protein. Briefly, 300 mg cotyledon tissue was homogenized twice in a

FastPrep (Qbiogene) instrument (also known as a ribolyser) for 30 s at the maximum speed (6.5 m s⁻¹) in a disruption tube containing ceramic beads and 0.9 ml distilled, deionized water. The ELISA was then conducted according to the manufacturer's protocols. Interpretation of samples analysed by ELISA was performed by comparing the *A*₄₅₀ of the cotyledon samples with that of negative- and positive-control samples supplied with the kit (a reference wavelength of 620 nm was used). Samples are considered positive if the mean *A*₄₅₀ values are greater than or equal to the cutoff value as calculated by the test manufacturer.

RESULTS

PrP^{Sc} detection in the placenta

PrP^{Sc} was detected by Western immunoblot, IHC and ELISA. When PrP^{Sc} was detected in a cotyledon belonging to a fetus, all other cotyledons tested from the fetus were also positive by all three tests (Table 1). Positive samples were determined by antibody binding to PrP^{Sc} and visualized as multiple bands on a Western immunoblot, as PrP^{Sc} plaques in fetal trophoblast cells by IHC or as absorbance readings above cutoff values in ELISA. Maternal caruncles were also collected at necropsy from dams 1, 2 and 5 and were found to be negative for PrP^{Sc} (data not shown). Dams 3, 4, 6 and 7 provided shed placentae, therefore only cotyledons were collected, as the caruncle is retained by the dam at parturition.

PrP^{Sc} accumulation in the placenta with multiple fetuses of genotype ARQ/ARR and ARQ/ARQ

The presence of PrP^{Sc} was detected in the cotyledons collected at necropsy corresponding to the fetus of genotype ARQ/ARQ (fetuses 1B and 2B) and not in the cotyledons from the fetus of genotype ARQ/ARR (fetuses 1A and 2A) in twins from scrapie-positive dams of genotype ARQ/ARQ (dams 1 and 2) (Table 1). This supports previous findings of these fetal genotypes from similar dams (Andréoletti *et al.*, 2002b; Tuo *et al.*, 2002). PCR products for the male-specific *Sry* gene were found in cotyledons corresponding to the male fetus, but not in those corresponding to female fetuses (data not shown). Placentae with all female fetuses were also found to be negative for *Sry* gene PCR products (data not shown).

Cotyledons from the shed placenta of a scrapie-positive dam (dam 3) of genotype ARQ/ARQ that gave birth to triplets of genotypes ARQ/ARQ (lamb 3B) and ARQ/ARR (lambs 3A and 3C) were tested for PrP^{Sc} (Table 1, Fig. 1). Sequence analysis of the *Prnp* and *Sry* genes in the cotyledons from the tissue corresponding to each uterine horn demonstrated that lambs 3A (male ARQ/ARR) and 3B (female ARQ/ARQ) shared one horn of the uterus and that lamb 3C (female ARQ/ARR) was alone in the other horn. ARQ/ARQ cotyledons from lamb 3B (Fig. 1, lane 5) and ARQ/ARR cotyledons from lamb 3A (Fig. 1, lanes 4, 6 and 7) had accumulation of protease-resistant anti-PrP immunoreactive bands with a molecular mass consistent with

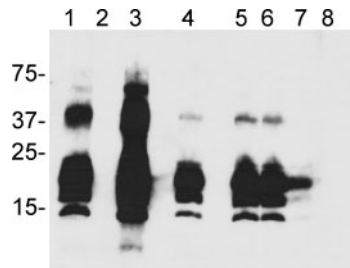


Fig. 1. Western immunoblot of fetal cotyledons from genotypically susceptible or resistant fetuses from an ARQ/ARQ dam (dam 3; Table 1). Lane 1, positive control; lane 2, negative control; lanes 4, 6 and 7, different cotyledons from lamb 3A (Table 1); lanes 3 and 5, lamb 3B (Table 1); lane 8, lamb 3C (Table 1). The positions of molecular mass standards (in kDa) are indicated on the left.

PrP^{Sc}. ARQ/ARR cotyledons from lamb 3C (Fig. 1, lane 8) showed no protease-resistant PrP immunoreactivity. Morphologically, PrP^{Sc} accumulated within trophoblast cells of the cotyledons of both lambs 3A and 3B (Fig. 2). No PrP^{Sc}-positive blood cells were observed in either lamb, indicating that the Western blot PrP^{Sc}-positive result in the genetically resistant lamb 3A was not due to blood cells originating in the genetically susceptible lamb 3B.

PrP^{Sc} accumulation in the placenta with multiple fetuses of genotype ARQ/ARQ and VRQ/ARQ

A scrapie-positive dam of genotype ARQ/VRQ (dam 4) delivered twin lambs. DNA sequencing of *Prnp* of the cotyledons from the shed placenta demonstrated that the female ARQ/ARQ fetus (lamb 4B) and the male ARQ/VRQ fetus (lamb 4A) developed in different uterine horns. PrP^{Sc} was detected in the cotyledons corresponding to the fetus of genotype ARQ/VRQ (lamb 4A) and not in the cotyledons from the fetus of genotype ARQ/ARQ (lamb 4B) (Table 1). PCR products for the male-specific *Sry* gene were found in cotyledons corresponding to the male fetus, but not in those corresponding to the female fetus.

Cotyledons collected at necropsy from a scrapie-positive dam (dam 5) of genotype ARQ/VRQ that was gestating quadruplet fetuses of genotypes ARQ/VRQ (lambs 5A, 5B and 5C) and ARQ/ARQ (lamb 5D) were sampled at necropsy and tested for PrP^{Sc}. PrP^{Sc} was detected in the cotyledons corresponding to the ARQ/VRQ fetuses (lambs 5A, 5B and 5C), but not in the cotyledons from the ARQ/ARQ fetus (lamb 5D) (Table 1). DNA sequencing of the cotyledons demonstrated that the male ARQ/VRQ fetuses (lambs 5A and 5B) developed in one uterine horn and that the female ARQ/VRQ and ARQ/ARQ fetuses (lambs 5C and 5D) developed in the other uterine horn. PCR products for the male-specific *Sry* gene were found in cotyledons corresponding to the male fetuses, but not in those corresponding to the female fetuses.

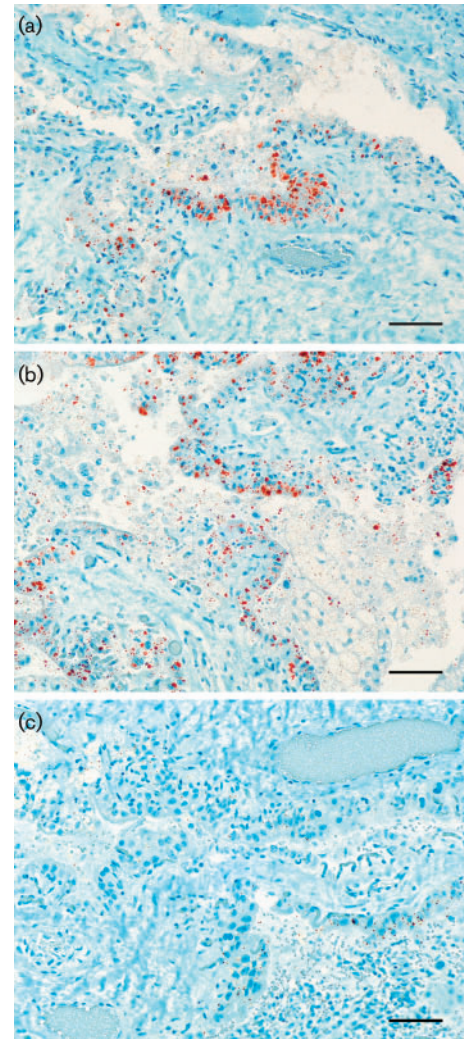


Fig. 2. IHC detection of PrP^{Sc} deposition in cotyledons from triplet fetuses from a scrapie-infected ARQ/ARQ ewe. PrP^{Sc} (red) in cytoplasm of trophoblasts in cotyledons from ARQ/ARQ fetus 3B (a) and ARQ/ARR fetus 3A (b) that shared the same uterine horn. PrP^{Sc} was not detected in cotyledons from ARQ/ARR fetus 3C (c) that occupied the other uterine horn by itself. Staining was by immunoperoxidase using mAb F89/F99 cocktail and Mayer's haematoxylin counterstain. Bars, 120 μm.

Cotyledons from the shed placenta of a scrapie-positive dam of genotype ARQ/VRQ (dam 6) that gave birth to triplets of genotypes ARQ/VRQ (lambs 6A and 6C) and ARQ/ARQ (lamb 6B) were tested for PrP^{Sc}. Sequence analysis of the *Prnp* and *Sry* genes in the cotyledons from the tissue corresponding to each uterine horn demonstrated that lambs 6A (male ARQ/VRQ) and 6B (female ARQ/ARQ) shared one horn of the uterus and that lamb 6C (male ARQ/VRQ) was alone in the other horn. PrP^{Sc} was detected in the cotyledons corresponding to the two ARQ/VRQ fetuses (lambs 6A and 6C), but PrP^{Sc} was also detected in the cotyledons from the ARQ/ARQ fetus (lamb 6B) (Table 1, Fig. 3). ARQ/VRQ cotyledons [lambs 6A (lanes 3, 4, 6 and 7)

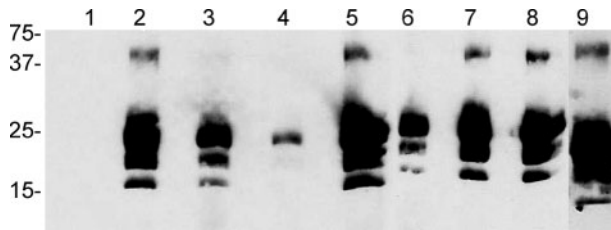


Fig. 3. Western immunoblot of fetal cotyledons from genotypically susceptible (ARQ/VRQ) or resistant (ARQ/ARQ) fetuses from an ARQ/VRQ dam (dam 6; Table 1). Lane 1, negative control; lane 2, positive control; lanes 5 and 9, different cotyledons from lamb 6A (Table 1); lanes 3, 4, 6 and 7, different cotyledons from lamb 6B (Table 1); lane 8, lamb 6C (Table 1). The positions of molecular mass standards (in kDa) are indicated on the left.

and 6C (lane 8)] and ARQ/ARQ cotyledons [lamb 6B (lanes 5 and 9)] had accumulation of protease-resistant anti-PrP immunoreactive bands with a molecular mass consistent with PrP^{Sc} (Fig. 3). A PCR product for the male-specific *Sry* gene was found in the female fetus (lamb 6B) that shared a uterine horn with the male fetus (lamb 6A). The male fetus (lamb 6C) that was alone in the other uterine horn also had a PCR product for the *Sry* gene (Table 1). Freemartin testing at a commercial laboratory was performed on blood from the three lambs and tested negative for freemartinism (data not shown).

A scrapie-positive dam of genotype ARQ/ARQ (dam 7) delivered twin lambs. DNA sequencing of the cotyledons from the shed placenta demonstrated that the male ARQ/ARQ fetus (lamb 7A) and the female ARQ/VRQ fetus (lamb 7B) developed in different uterine horns. PrP^{Sc} was detected in the cotyledons corresponding to the fetus of genotype ARQ/ARQ (lamb 7A) and not in the cotyledons from the fetus of genotype ARQ/VRQ (lamb 7B) (Table 1). PCR products for the male-specific *Sry* gene were found in cotyledons corresponding to the male fetus, but not in those corresponding to the female fetus.

DISCUSSION

This is the first study reporting results from births with multiple fetuses and the effect of fetal genotype on PrP^{Sc} accumulation in the case of births with multiple fetuses. We show that PrP^{Sc} can be detected in the cotyledons of some fetal genotypes considered resistant to scrapie when the fetus has shared the same uterine horn with a fetus of susceptible genotype where PrP^{Sc} has been detected. A PCR product specific for males was also found in the cotyledons from female fetuses that had shared the same uterine horn with a male fetus. Taken together, these results suggest a partial anastomosis between fetal cotyledon blood supplies *in utero*. This sharing of blood components between developing

fetuses is a possible source of prion infection in scrapie-resistant fetuses.

We detected PrP^{Sc} in cotyledons of genotype ARQ/ARQ (lambs 1B and 2B) and not in cotyledons of genotype ARQ/ARR (lambs 1A and 2A) in twin births with one fetus of each genotype. This followed the expected pattern of PrP^{Sc} distribution with ARQ/ARQ as a susceptible genotype for PrP^{Sc} accumulation and ARQ/ARR as a resistant genotype. In a triplet birth involving animals of the same genotypes, however, PrP^{Sc} was detected in cotyledons from an ARQ/ARR fetus (lamb 3A) that shared the uterine horn with an ARQ/ARQ fetus (lamb 3B) positive for the presence of PrP^{Sc}. The cotyledons from the ARQ/ARR female fetus had a male-specific PCR product for the *Sry* gene, suggesting exchange of blood cells between lambs 3A and 3B. However, the PrP^{Sc} positivity in the genetically resistant lamb 3A is probably not due to cross-contamination of blood cells from the genetically susceptible lamb 3B, as IHC localized PrP^{Sc} immunoreactivity in both fetuses to placental trophoblasts and not to blood cells. The third fetus in the opposite uterine horn of genotype ARQ/ARR was negative for the presence of PrP^{Sc} (lamb 3C). It is not known whether the genotypically resistant lambs from triplet births will go on to develop disease as adults when the cotyledons are positive for the presence of PrP^{Sc} in the shed placenta. Lambs 3A and 6B are being held at our facility under observation for signs of scrapie. To date, there have been only a few reports of scrapie-infected sheep carrying 171 arginine/arginine (RR) (Buschmann *et al.*, 2004; Ikeda *et al.*, 1995) and a few published reports of scrapie-infected sheep with 171 glutamine/arginine (QR) (Hunter *et al.*, 1997a; Ikeda *et al.*, 1995; Madec *et al.*, 2004). It would be interesting to know whether these reports were from sheep that had shared the same uterine horn with a susceptible fetus *in utero*.

There have been several published reports of ARQ/ARQ sheep remaining scrapie-free in flocks that are heavily stocked with sheep carrying 136 valine (Belt *et al.*, 1995; Goldmann *et al.*, 1991; Hunter *et al.*, 1991, 1997a; Laplanche *et al.*, 1993). Andréoletti and colleagues reported that placentae from ewes of genotype ARQ/VRQ are positive for the presence of PrP^{Sc} when the fetus is of genotype ARQ/VRQ (Andréoletti *et al.*, 2002b; Evoniuk *et al.*, 2005). Our results confirm those of Andréoletti *et al.* (2002b) and Evoniuk *et al.* (2005) and additionally showed that the cotyledons from a twin lamb of genotype ARQ/ARQ (lamb 4B), born to the same ARQ/VRQ dam (dam 4), were negative for the presence of PrP^{Sc}. However, twins of genotypes ARQ/VRQ (lamb 7B) and ARQ/ARQ (lamb 7A) showed the opposite PrP^{Sc} distribution pattern when born to a ewe of genotype ARQ/ARQ (dam 7); the cotyledons from the ARQ/VRQ fetus were negative and the cotyledons from the ARQ/ARQ fetus were positive for the presence of PrP^{Sc}. 136 Valine in the fetus was protective for PrP^{Sc} accumulation when the dam did not carry 136 valine, but was permissive for PrP^{Sc} accumulation when the dam also carried 136 valine. Conversely, 136 alanine in the fetus was protective for

PrP^{Sc} accumulation where the dam carried 136 valine, but was permissive for PrP^{Sc} accumulation when the dam did not carry 136 valine, except for dam 6, where there was potential exchange of blood cells between a susceptible ARQ/VRQ fetus and a resistant ARQ/ARQ fetus in the same uterine horn.

In a birth with triplets from an ARQ/VRQ dam (dam 6), the cotyledons from all of the fetuses were positive for the presence of PrP^{Sc}, including those from an ARQ/ARQ fetus (lamb 6B). Cotyledons with an ARQ/ARQ genotype were shown to be negative for the presence of PrP^{Sc} when conceived by an ARQ/VRQ dam (dam 4) in twin births from this study. The cotyledons from the female ARQ/ARQ triplet fetus (lamb 6B) showed a male-specific PCR product for the *Sry* gene, demonstrating a detectable amount of fetal blood exchange. A set of quadruplets from a necropsy at 120 days gestation had one fetus of genotype ARQ/ARQ (lamb 5D) and three fetuses of genotype ARQ/VRQ (lambs 5A, 5B and 5C). The cotyledons from all three of the ARQ/VRQ fetuses were positive for the presence of PrP^{Sc} and the cotyledons from the ARQ/ARQ fetus were negative for the presence of PrP^{Sc}. In this case, the fetuses that shared the same side of the uterine horn were of the same sex, so there was no PCR product for the *Sry* gene detected in the cotyledons from the female fetuses.

The ovine placenta is of the synepitheliochorial type and is composed of a flat apposition of chorionic epithelium (trophoblast) to uterine epithelium with a variable number of placentomes. The placentomes are composed of fetal chorionic villi (cotyledons) enmeshed with maternal endometrial crypts (caruncles). Binucleate trophoblast cells are common and unique to all ruminant placentae (Wooding *et al.*, 1997). It was previously shown that PrP^{Sc} is found in uterine endometrial epithelium, trophoblast cells and multinucleate cells in maternal endometrial crypts (Tuo *et al.*, 2002). The vasculature of each fetal cotyledon can be traced to individual fetuses' umbilical cords. Based on the results of this study, we speculate that there is some sharing of the blood supply between cotyledons on the same side of the uterus in some multiple births. This would explain the presence of a male-specific PCR product in female cotyledon tissue, but does not necessarily explain the presence of PrP^{Sc} in cotyledons of genotypes previously shown to be resistant to PrP^{Sc} deposition. Although post-partum contamination of the cotyledons with the resistant genotype by maternal tissue or fetal tissue from the twin cannot be ruled out in Western blot, ELISA or the genetic analysis, the demonstration of similar patterns of intracellular accumulation of PrP^{Sc} in the fetal trophoblast cells of genetically susceptible and genetically resistant twins is compelling evidence that PrP^{Sc} can accumulate in the cotyledon of a genetically resistant fetus under these conditions. This suggests a fetal-fetal blood sharing among cotyledons, as opposed to a maternal-fetal blood sharing where the maternal genotype at 136 has been shown to influence the accumulation of PrP^{Sc} in fetal cotyledons.

It has been suggested that transmission at lambing may be reduced or eliminated if infected ewes conceive offspring with scrapie-resistant genotypes. These results show that PrP^{Sc} can be present in cotyledons of fetuses with genotypes thought to be resistant to scrapie when it occurs in multiple births with fetuses sharing the same uterine horn.

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