

PrP^{Sc} accumulation in placentas of ewes exposed to natural scrapie: influence of foetal *PrP* genotype and effect on ewe-to-lamb transmission

Olivier Andréoletti,¹ Caroline Lacroux,¹ Armelle Chabert,¹ Laurent Monnereau,¹ Guillaume Tabouret,¹ Frédéric Lantier,² Patricia Berthon,² Francis Eychenne,³ Sylvie Lafond-Benestad,⁴ Jean-Michel Elsen⁵ and François Schelcher¹

¹UMR 959 INRA-ENVT, Physiopathologie Infectieuse et Parasitaire des Ruminants, Ecole Nationale Vétérinaire, 23 Chemin des Capelles, 31076 Toulouse Cedex 3, France

²INRA, Laboratoire de Pathologie Infectieuse et Immunologie, Nouzilly, France

³INRA, Domaine de Langlade, Pompertuzat, France

⁴NVI, Department of Pathology, Oslo, Norway

⁵INRA, Station d'Amélioration Génétique des Animaux, Auzeville, France

Placentas from scrapie-affected ewes are known to be infectious. Nevertheless, placenta infectivity in such ewes is not systematic. Maternal transmission to lambs is highly suspected but contamination of the foetus in utero has not been demonstrated. Using ewes from a naturally scrapie-infected flock, it was demonstrated that abnormal prion protein (PrP^{Sc}) accumulation in the placenta (i) is controlled by polymorphisms at codons 136, 154 and 171 of the foetal *PrP* gene and (ii) is restricted mainly to placental foetal trophoblastic cells. In order to go deeper into the role of the placenta in scrapie transmission, the pattern of PrP^{Sc} dissemination was established in susceptible lambs (genotype VRQ/VRQ) sampled from 140 days post-insemination to the age of 4 months from either VRQ/VRQ ewes with PrP^{Sc}-positive placentas or ARR/VRQ ewes with PrP^{Sc}-negative placentas. In both VRQ/VRQ lamb groups, PrP^{Sc} spatial and temporal accumulation patterns were similar, suggesting post-natal rather than in utero contamination.

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders that occur in sheep (scrapie), cattle (bovine spongiform encephalopathy, BSE) and humans (Creutzfeldt–Jakob disease, CJD) and share similar characteristics: long incubation periods and a progressive and chronic clinical course resulting in death (Fraser, 1976). The accumulation of an abnormal isoform (PrP^{Sc}) of a normal cellular protein (PrP^C) in organs from infected individuals correlates with the presence of infectivity and is currently considered the biochemical marker of the disease (McKinley *et al.*, 1983; Race *et al.*, 2001). According to the prion hypothesis, PrP^{Sc} is an infectious protein, thought to be the causative agent of TSE (Prusiner, 1982).

Ovine susceptibility to TSE is controlled mainly by polymorphism at the *PrP* gene encoding the PrP protein. The major mutations associated with susceptibility or resistance are located at codons 136 (A or V), 154 (R or H) and 171 (R, Q or H) (Clouscard *et al.*, 1995; Hunter *et al.*, 1996). Animals with *PrP* genotypes V¹³⁶R¹⁵⁴Q¹⁷¹/VRQ, ARQ/VRQ and ARQ/ARQ are the most susceptible to scrapie, whereas homozygous or heterozygous AHQ and heterozygous ARR animals show only marginal susceptibility. ARR/ARR sheep are considered to be fully clinically resistant (Hunter *et al.*, 1996, 1997).

Under natural conditions, the transmission of the TSE agent between individuals is still poorly understood. A case control study in BSE-affected cattle has suggested that maternal transmission may occur at low levels. The existence of post-natal transmission was not evaluated (Wilesmith *et al.*, 1994, 1997) but no infectivity was detected in the placentas of clinically BSE-affected cows (Wrathall, 1997). Concerning human CJD (sporadic and variant), transmission from mother

Author for correspondence: Olivier Andréoletti.
Fax +33 5 61 19 38 34. e-mail o.andreoletti@envt.fr

to child has never been observed until now (Berrebi *et al.*, 1997), despite the detection of placenta infectivity in one case (Tamai *et al.*, 1992).

Scrapie in small ruminants is certainly the best-documented model for TSE transmission. Numerous epidemiological data support the possibility of maternal transmission. Offspring from scrapie-affected ewes show a higher risk of developing scrapie than offspring from asymptomatic ewes (reviewed by Detwiler, 1992; Hoinville, 1996). Unfortunately, most of the original data were collected from dams and sires with a possibly misclassified infectious status and unknown *PrP* genotype (Dickinson *et al.*, 1965). However, recent and reliable observations argue strongly for maternal transmission, as lambs born from scrapie-incubating ewes present a higher risk of developing scrapie (Hoinville, 1996; Elsen *et al.*, 1999).

The hypothesis of scrapie transmission via germinal cells has been assessed in experimental infection models using the technique of embryo transfer. Results obtained from scrapie in sheep (Foster *et al.*, 1992, 1996; Foote *et al.*, 1993) or BSE in goats (Foster *et al.*, 1999) did not lead to any striking evidence on this possible pathway of transmission of the TSE agent in small ruminants.

The presence of the scrapie agent in placentas from clinically scrapie-affected goats (Pattison *et al.*, 1972, 1974) and ewes (Onodera *et al.*, 1993) was demonstrated by mouse bioassays. PrP^{Sc} accumulation was detected in foetal chorion from pregnant scrapie-incubating ewes. However, the placenta from a scrapie-incubating ewe can be found to be PrP^{Sc}-positive in its first gestation and negative in the following one (Race *et al.*, 1998; Tuo *et al.*, 2001). Until now, no clear explanation for this phenomenon has been proposed and the transmission pathway of scrapie from dams to offspring remains uncertain as there is no direct evidence for scrapie infectivity in lambs either in utero or post-partum.

Using sheep naturally exposed to scrapie, we identified the PrP^{Sc}-accumulating cell subsets in the placenta and demonstrated the role of the foetal *PrP* genotype in the control of PrP^{Sc} accumulation in this organ. We also investigated the presence of the abnormal PrP isoform in susceptible VRQ/VRQ lambs born from either scrapie-incubating dams accumulating PrP^{Sc} in their placentas or scrapie-negative dams with PrP^{Sc}-negative placentas. Our results suggest that the most likely pathway of scrapie transmission from ewes to lambs is an early lateral post-natal event rather than in utero transmission.

Methods

■ **Animals.** The INRA Langlade flock is a closed flock of 700 Romanov sheep in which a high annual incidence (30%) of natural scrapie has been developing since 1993 (Elsen *et al.*, 1999). All animals were born and bred in this flock and so exposed to natural scrapie. Animals were cared for following European Union recommendations for animal welfare and under the supervision of the local INRA ethics committee.

The *PrP* gene polymorphism at codons 136 (A/V), 154 (R/H) and

171 (R/Q) was determined for each animal used in this study (Labogena) by the RFLP-PCR method (Cloucard *et al.*, 1995).

Ewes with the chosen *PrP* genotypes (ARR/ARR, ARR/VRQ, ARQ/VRQ and VRQ/VRQ) were inseminated with either ARR/ARR or VRQ/VRQ ram semen, according to the experimental group. No natural mating or contact occurred with the rams.

As described previously, the presence of the scrapie agent in all ewes was investigated by immunohistochemical (IHC) detection of PrP^{Sc} in palatine tonsil biopsies (Dickinson *et al.*, 1965; Schreuder *et al.*, 1996, 1998; Roels *et al.*, 1999). Finally, 80 lambing ewes were included in this study and were sampled twice, 1 month prior to artificial insemination (AI) and 1 month after lambing. Each animal showed an identical PrP^{Sc} accumulation result in the two consecutive tonsil biopsies. No clinical signs of scrapie were observed during the experimental period in any of the animals. At lambing, all ARQ/VRQ and VRQ/VRQ ewes were between 15 and 20 months old (first lambing), while ARR/VRQ and ARR/ARR ewes were between 18 and 48 months old (first to fourth lambing). In this flock, VRQ/VRQ and ARQ/VRQ scrapie-affected sheep usually expressed clinical signs around 24 and 32 months, respectively (unpublished data).

■ **Experimental group and tissue collection.** The influence of foetal *PrP* genotype on PrP^{Sc} accumulation in the placenta was investigated by producing groups of ARQ/VRQ, VRQ/VRQ, ARR/VRQ and ARR/ARR lambs. Dams ($n = 36$) were assessed for PrP^{Sc} deposition in their peripheral lymphoid tissues (tonsil biopsy) and rams with the appropriate *PrP* genotype were used for AI purposes (Table 1). At lambing, at least two cotyledons and intercotyledonary foetal chorion for each placenta were sampled and fixed in a 10% neutral-buffered formalin solution for 10 days. Thereafter, paraffin-embedded samples were processed for IHC. Similar samples from the same location were frozen at -80°C before PrP^{Sc} detection by ELISA.

The hypothesis for in utero PrP^{Sc} dissemination from scrapie-affected ewes to the conceptus (uterofetal unit) was investigated using pregnant ewes (140 days post-AI – normal duration of gestation in sheep is 145–150 days) and their foetuses. Tissues were collected from two groups of three PrP^{Sc}-positive tonsils from VRQ/VRQ ewes inseminated with semen from either VRQ/VRQ or ARR/ARR rams. A negative control group comprising three ARR/ARR (PrP^{Sc}-negative tonsils) ewes inseminated with ARR/ARR semen was also included. Animals were euthanized by exsanguination after a 10 mg/kg pentobarbital sodium salt intravenous injection. From the ewes, placentomes, intercaruncular uterine wall, palatine tonsils, spleen and brain were sampled; from the foetus, umbilical cord, liver, hepatic lymph node and different areas of the digestive tract were subjected to IHC analysis for PrP^{Sc} detection.

In order to investigate the influence of placental PrP^{Sc} accumulation on the timing of lamb infection, VRQ/VRQ lambs were produced from either ARR/VRQ ewes (PrP^{Sc}-negative tonsils, group 1, $n = 13$ ewes) or VRQ/VRQ ewes (PrP^{Sc}-positive tonsils, group 2, $n = 8$ ewes). A third group, used as a control, comprised ARR/VRQ lambs born from VRQ/VRQ ewes (PrP^{Sc}-positive tonsils, $n = 8$) that had been inseminated with ARR/ARR ram semen.

Three lambs from each group were euthanized (exsanguination following 10 mg/kg pentobarbital sodium salt intravenous injection) at birth (before any suckling) and at 10, 21, 64, 104 and 144 days of age. For each lamb, 23 different tissues were sampled, including the umbilical system and the digestive tract (Table 2). IHC analysis for PrP^{Sc} detection was performed on all tissues.

■ **Immunohistochemistry.** After paraffin-embedding, tissue sections (3 μm thick) were dried overnight at 56°C before being deparaffinized and rehydrated. Sections were first incubated in 98% formic acid (Merck)

Table 1. PrP^{Sc} accumulation in the placenta according to the presence of PrP^{Sc} in ewe tonsils and to PrP genotype of ewes and lambs

Lambs were produced and bred in a naturally scrapie-affected flock by appropriate mating between genetically susceptible or resistant dams and sires.

| Ewe PrP genotype | PrP ^{Sc} in tonsils | Ram PrP genotype | Lamb PrP genotype | PrP ^{Sc} in placenta |
|------------------|------------------------------|------------------|------------------------------------|-------------------------------|
| ARQ/VRQ (n = 9) | + | VRQ/VRQ | ARQ/VRQ (n = 7) VRQ/VRQ (n = 6) | + |
| ARQ/VRQ (n = 3) | - | VRQ/VRQ | ARQ/VRQ (n = 1) VRQ/VRQ (n = 4) | - |
| ARQ/VRQ (n = 5) | + | ARR/ARR | ARR/ARQ (n = 3) ARR/VRQ (n = 5) | - |
| ARQ/VRQ (n = 3) | - | ARR/ARR | ARR/ARQ (n = 4) ARR/VRQ (n = 4) | - |
| ARR/ARR (n = 5) | - | VRQ/VRQ | ARR/VRQ (n = 5) | - |
| ARR/ARR (n = 6) | - | ARR/ARR | ARR/ARR (n = 9) | - |
| ARR/VRQ (n = 5) | - | VRQ/VRQ | VRQ/VRQ (n = 4) ARR/VRQ (n = 7) | - |

Table 2. PrP^{Sc} IHC detection in tissues from VRQ/VRQ lambs naturally exposed to scrapie

Group 1 comprised lambs born from ARR/VRQ ewes with PrP^{Sc}-negative placentas. Group 2 comprised lambs born from VRQ/VRQ ewes with PrP^{Sc}-positive placentas. Each group constituted three animals. Animals were sampled at birth (data not shown, all organs negative) and 10, 21, 64, 104 and 144 days later. A third group (data not shown) comprised ARR/VRQ lambs born from VRQ/VRQ ewes inseminated with ARR/ARR ram semen. No PrP^{Sc} was detected in any tissue from these ARR/VRQ lambs throughout the experiment. LN, lymph node; MLN, mesenteric lymph node; PP, Peyer's patch.

| Organ | 10 days | | 21 days | | 64 days | | 104 days | | 144 days | |
|-----------------|---------|---------|---------|---------|---------|---------|----------|---------|----------|---------|
| | Group 1 | Group 2 | Group 1 | Group 2 | Group 1 | Group 2 | Group 1 | Group 2 | Group 1 | Group 2 |
| Thymus | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Liver | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Abomasum | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Duodenum | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Jejunum | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Ileum | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Caecum | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Colon | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| PP-duodenum | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| PP-jejunum 25% | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| MLN-jejunum 25% | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| PP-jejunum 50% | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| MLN-jejunum 50% | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| PP-jejunum 75% | 0/3 | 0/3 | 0/3 | 0/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| MLN-jejunum 75% | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| PP-ileum | 0/3 | 0/3 | 1/3 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| MLN-ileum | 0/3 | 0/3 | 0/3 | 0/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| PP-caecum | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| Ileo-caecal LN | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| Palatin tonsils | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| Hepatic LN | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 2/3 | 2/3 | 3/3 | 3/3 |
| Spleen | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 2/3 | 1/3 | 3/3 | 3/3 |
| Mediastinal LN | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 3/3 | 2/3 | 3/3 | 3/3 |
| Prescapular LN | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 3/3 | 1/3 | 3/3 | 3/3 |

for 30 min at room temperature and then autoclaved for 20 min at 121 °C in 10 mM citrate buffer (pH 6.1).

PrP^{Sc} single-labelling protocol. Single PrP^{Sc} immunolabelling was carried out using mouse monoclonal antibody (mAb) 8G8 (IgG2a, raised against the human recombinant PrP protein and specifically recognizing the 95–108 aa sequence, kindly provided by J. Grassi, CEA Saclay, France), used at a 1:2000 dilution of the ascites fluid (Krasemann *et al.*, 1996).

Non-specific binding sites were blocked by incubating sections with 20% normal goat serum in 0.1 M TBS (pH 7.6) for 20 min. Primary antibody was then applied for 60 min at room temperature. A 30 min incubation with a biotinylated secondary goat antibody (1:100 dilution) specific for mouse immunoglobulins was performed before applying a streptavidin–alkaline phosphatase complex (1:100 dilution) for 30 min. Immunostaining was achieved using BCIP/NBT (Dako).

PrP^{Sc} double-labelling protocol. PrP^{Sc}-positive cell phenotyping was achieved using anti-CD and anti-cytokeratin antibodies. Antibodies were mAbs specific for: human CD68 [clone Ki-M6 (monocytes/macrophages), used at a dilution of 1:150; Serotec]; human CD20-like [clone BLA36 (B lymphocytes), used at a dilution of 1:50; Novocastra] or a rabbit anti-human CD3 antiserum [A0452 (T lymphocytes), used at a dilution of 1:100; Dako]. An anti-cytokeratin rabbit serum [A0575 (large spectrum cytokeratin), used at a dilution of 1:1600; Dako] was used for uninucleated trophoblastic epithelial cell characterization (Lee *et al.*, 1990).

In the PrP^{Sc} double-labelling protocol, anti-PrP antibodies were either mAb 8G8 or a rabbit polyclonal antibody (pAb), R521-7 (van Keulen *et al.*, 1995), diluted 1:1000 (raised against ovine PrP sequence peptide 94–105 and kindly provided by L. van Keulen, CIDC, Lelystad, The Netherlands). A 'two-step' method based on the simultaneous application of two primary antibodies followed by a simultaneous incubation with both secondary antibodies was applied as described previously (Andréoletti *et al.*, 2000). Revelation was performed sequentially using first the alkaline phosphatase substrate BCIP/NBT followed by the peroxidase chromogen using the AEC⁺ red end product (Dako).

Controls. In order to characterize non-specific immunolabelling, each IHC run included negative serum controls in which the primary antibody was either omitted or replaced by normal rabbit or mouse serum. In addition, mouse mAbs were replaced by isotype-matched mAbs irrelevant to the tissue under investigation. Reproducibility of immunostaining was assessed using serial tissue sections from the same sample included in two successive IHC runs. For double-labelling, cross-reactivity controls were performed for each couple of primary antibodies and each sample in order to verify the absence of interspecies reactivity of secondary antibodies toward primary antibodies. The absence of a possible affinity between the two secondary antibodies was also checked.

■ **PrP^{Sc} detection by ELISA.** A commercially available BSE detection test (Platelia BSE; Bio-Rad) was used according to the manufacturer's recommendations. This test is used currently for the post-mortem diagnosis of BSE in bovine brain tissue but is also suitable for the diagnosis of scrapie in sheep: mAbs used in this immunometric assay also recognize the ovine PrP protein. The procedure established for bovine brain tissue was applied directly to sheep tissue. Briefly, 350 ± 40 mg tissue was subjected first to three cycles of homogenization of 45 s each (maximum speed in an Ribolyser; Coger) in 1.5 ml of 5% glucose solution. Of the homogenate, 500 µl was filtered through a 25 gauge needle and incubated for 10 min at 37 °C with 500 µl of buffer A containing proteinase K. PrP^{Sc} was recovered as a pellet after the addition of 500 µl of buffer B and centrifugation for 5 min at 20000 g at room

temperature. Supernatants were discarded and the pellets were dried. Finally, the pellet was dissolved in buffer C1 (5 min at 100 °C) and diluted 1:6 in R6 reagent. A synthetic PrP peptide specifically recognized by the two mAbs included with the immunometric assay was used as an internal standard to compare interassay results and to quantify ovine PrP^{Sc} in samples.

A total of 18 placentas was assessed for PrP^{Sc} by this method. Eight were collected from ARQ/VRQ ewes (PrP^{Sc}-positive tonsils) inseminated with VRQ/VRQ ram semen. Three others were sampled from ARQ/VRQ ewes (PrP^{Sc}-positive tonsils) inseminated with semen from an ARR/ARR ram and the last seven were from ARR/ARR ewes (PrP^{Sc}-negative tonsils) inseminated with semen from ARR/ARR rams. Moreover, tissues from five clinically scrapie-affected ARQ/VRQ ewes (obex, spleen, mesenteric lymph node, ileal Peyer's patch and tonsil) were tested as positive controls and results were compared to accumulation levels of PrP^{Sc} in the placenta.

Results

PrP^{Sc} accumulation in the placenta according to the PrP genotype of the foetus

Results on PrP^{Sc} IHC detection in all placentas tested were confirmed by ELISA (data not shown). When PrP^{Sc} accumulation was detected in a placental cotyledon from a ewe, all other cotyledons tested from this placenta were also positive.

The absence of any detectable PrP^{Sc} accumulation in the tonsils of dams ($n = 22/36$, Table 1) was associated with PrP^{Sc}-negative results in placental structures and, thus, irrespective of the PrP genotype of the foetus ($n = 38$) or dam. PrP^{Sc} accumulation in the placenta was only identified in ewes with PrP^{Sc}-positive tonsils ($n = 14/36$, Table 1) and carrying a foetus with a susceptible PrP genotype. PrP^{Sc} deposition was detected by IHC in the placentas of all genetically susceptible offspring ($n = 13$) from these ewes ($n = 9$). Scrapie-incubating susceptible ewes ($n = 5$) inseminated with scrapie-resistant ram semen resulted in heterozygous ARR foetuses ($n = 8$) and no PrP^{Sc} accumulation was observed in the corresponding placenta (Table 1).

PrP^{Sc}-accumulating cell subsets in the placenta

In the placenta, using both IHC and ELISA techniques, PrP^{Sc} accumulation was found to be restricted to cotyledonary tissues. In the intercotyledonary foetal chorion or the maternal uterine wall, no PrP^{Sc} was detected. PrP^{Sc} deposition in placentomes was multifocal (Fig. 1A) and localized within epithelial uni- (Fig. 1B) and multinucleated cells (Fig. 1D). Deposits were granular, intracytoplasmic and often massive. These epithelial cells were identified as trophoblasts using PrP^{Sc}/cytokeratin double-labelling (Fig. 1C). No apparent cellular modification was associated with PrP^{Sc} accumulation in trophoblastic cells.

Using the PrP^{Sc} double-labelling protocol, only a few CD68-positive cells were found to accumulate PrP^{Sc} in caruncular endometrial chorion from animals with PrP^{Sc}-positive placentas. Some of them were in close contact with

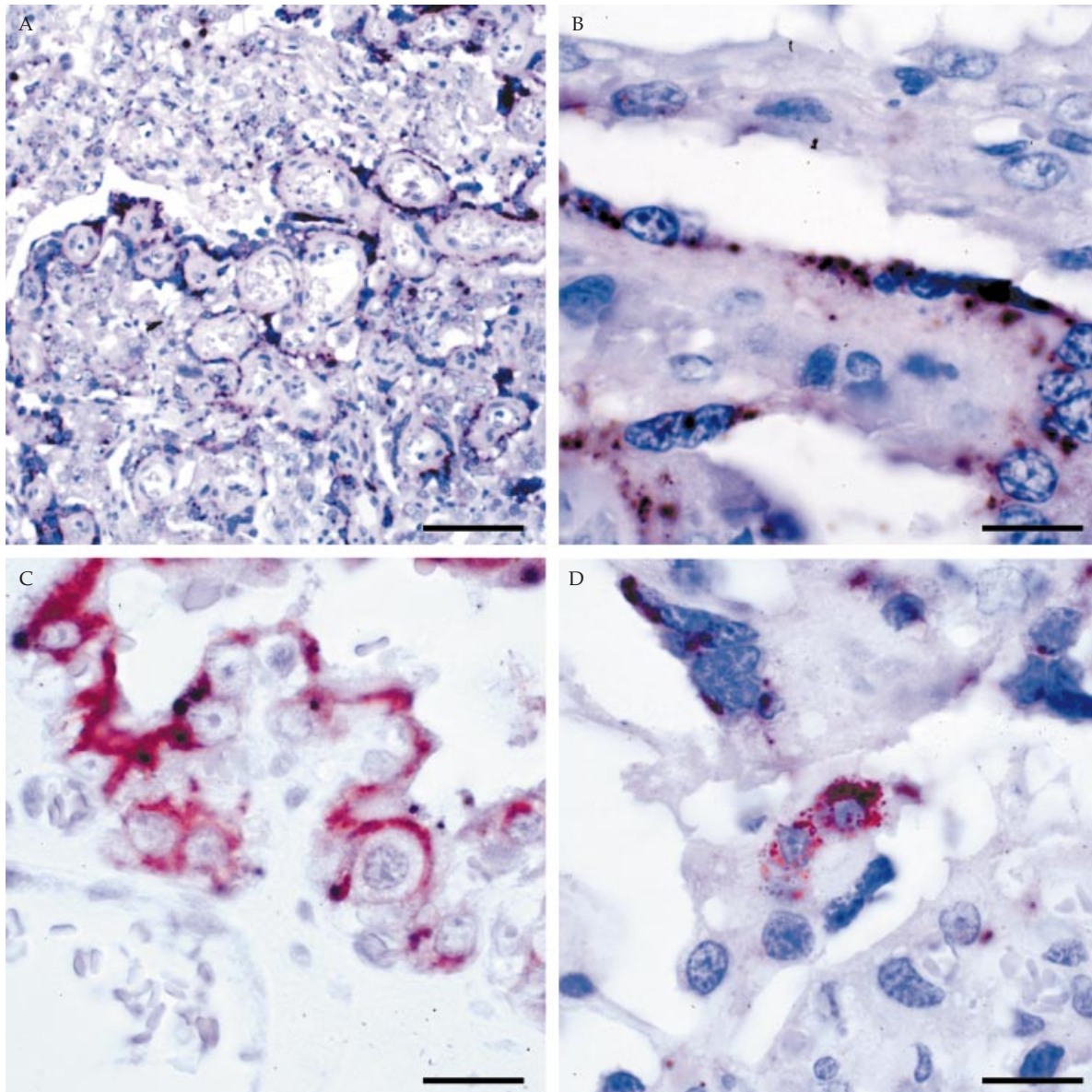


Fig. 1. IHC detection of PrP^{Sc} deposition (alkaline phosphatase–BCIP/NBT, black deposits) in cotyledons from a scrapie-incubating ARQ/VRQ ewe using mAb 8G8 (A–C) or pAb R521-7 (D). PrP^{Sc} accumulation is massive, multifocal (A, bar 100 μ m) and located mainly in epithelial uninucleated (B, bar 10 μ m) or multinucleated (D, bar 10 μ m) cells. Double labelling using anti-cytokeratin rabbit serum (peroxidase–AEC, red deposits) indicates that epithelial PrP^{Sc}-positive cells are trophoblasts (C, bar 10 μ m). Some CD68-positive cells (peroxidase–AEC, red deposits) from maternal placental cells also accumulate PrP^{Sc} in their cytoplasm (D, bar 10 μ m).

PrP^{Sc}-positive trophoblastic cells (Fig. 1D). In scrapie-incubating ewes with PrP^{Sc}-negative placentas, no PrP^{Sc}-accumulating and CD68-positive cells were observed. No CD3- or CD20-positive lymphocytes were found to contain PrP^{Sc} granules in any animals investigated.

Levels of PrP^{Sc} within cotyledons

According to ELISA data obtained from eight PrP^{Sc}-positive placentas of ARQ/VRQ ewes and different organs from five ARQ/VRQ ewes at the clinical stage of scrapie

(Fig. 2), the relative concentration of PrP^{Sc} from 47 mg fresh cotyledon was found to be equivalent to 1 mg obex. Similarly, 8 and 4 mg positive cotyledon appeared, respectively, to be equivalent to 1 mg tonsil and 1 mg spleen from clinically scrapie-affected sheep.

PrP^{Sc} dissemination to the conceptus

In late gestation (140 days post-AI), no PrP^{Sc} accumulation was detected using IHC in caruncular and intercaruncular endometrial epithelial cells or in myometrium from scrapie-

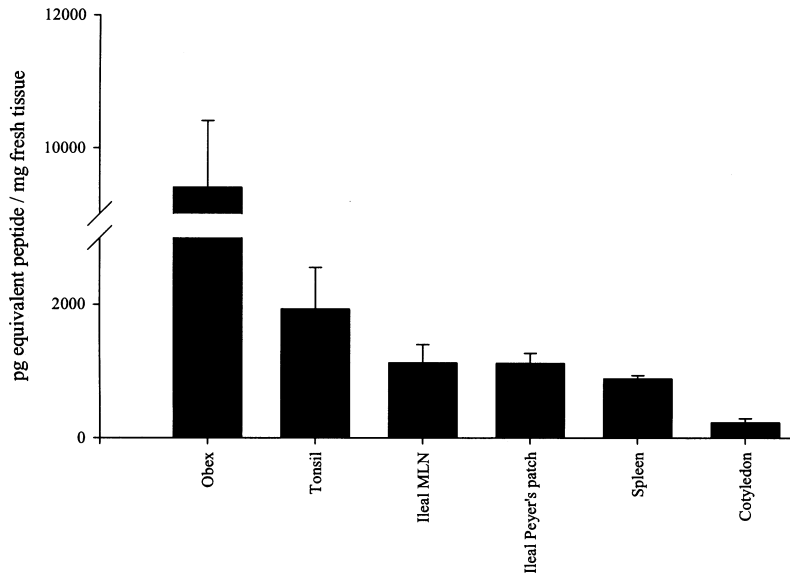


Fig. 2. ELISA quantification of PrP^{Sc} in equivalent concentrations (pg) (same absorbance reading) of synthetic peptide (human PrP, aa 79–92) per mg of fresh tissue (mean \pm SD) in: (i) the obex (9404 ± 1012 pg), palatine tonsils (1930 ± 628 pg), ileal mesenteric lymph node (1125 ± 273 pg), ileal Peyer's patch (1118 ± 152 pg) and spleen (889 ± 47 pg) from five clinically scrapie-affected ARQ/VRQ ewes and (ii) the cotyledons (232 ± 62 pg) from the placentas of eight scrapie-incubating ARQ/VRQ ewes during their first gestation.

affected VRQ/VRQ ewes with PrP^{Sc}-positive placentas. Despite the massive levels of PrP^{Sc} accumulation in placental trophoblastic cells, evaluation of structures related to foetal/placental circulation systems (umbilical cord, liver and hepatic lymph node) or to amniotic liquid (foetal digestive tract) before lambing showed no apparent PrP^{Sc} deposition. Samples from scrapie-incubating VRQ/VRQ ewes, inseminated with ARR/ARR ram semen, confirmed results obtained from similar ARQ/VRQ ewes, since no PrP^{Sc} accumulation was observed in either the placenta or foetal tissue.

Scrapie infection of lambs in relation to PrP^{Sc} accumulation in the placenta

VRQ/VRQ lambs could be divided into two groups depending on the genotype of the ewe. Group 1 lambs were born from ARR/VRQ ewes (PrP^{Sc}-negative tonsil biopsies) inseminated with a VRQ/VRQ ram, whereas those of group 2 were born from VRQ/VRQ ewes (PrP^{Sc}-positive tonsil biopsies) inseminated with a VRQ/VRQ ram. No PrP^{Sc} accumulation was detected in tissues from offspring at 8 days before lambing, 0 days or 10 days post-partum, whatever their group. At 21 days of age, PrP^{Sc} was detected in ileal Peyer's patches from one of three lambs in both groups, suggesting a primary site of entry through ileal gut-associated lymphoid tissues (GALT).

At 64 days of age, all animals in each group were PrP^{Sc}-positive for at least one tissue sample. PrP^{Sc}-positive tissues were more numerous in animals from group 2 than from group 1 but no major discrepancy was observed in the tissue distribution of the abnormal protein between these groups. From 104 days of age, PrP^{Sc} deposits extended progressively to all GALT formations and then to other secondary lymphoid organs, such as hepatic, prescapular, mediastinal lymph nodes

and spleen, with no observable differences between the two groups (Table 2). Over the same period, there was no PrP^{Sc} accumulation detected in any of the tissues sampled from ARR/VRQ control lambs (group 3).

Discussion

Our study confirms the presence of PrP^{Sc} in placental cotyledons from naturally scrapie-affected ewes during the incubation period of the disease. These results strengthen the role of the placenta in scrapie contamination of the environment (Pattison *et al.*, 1972, 1974; Hoinville, 1996). During the incubation period of scrapie, genetically susceptible ewes can accumulate massive amounts of PrP^{Sc} and disseminate the agent in the environment through their placenta as early as their first gestation and long before the clinical onset of disease, which currently occurs between 24 and 30 months in this flock (Elsen *et al.*, 1999). These observations are consistent with results published previously (Race *et al.*, 1998).

The apparent absence of PrP^{Sc} accumulation in placentas from scrapie-incubating ewes carrying ARR lambs, as assessed by IHC and ELISA, is consistent with and extends the similar observations reported recently from Suffolk sheep in the USA (Tuo *et al.*, 2002) and is of primary importance for the control of scrapie. The introduction of the ARR *PrP* allele in infected flocks should not only reduce the incidence of the disease by making the animals less genetically susceptible (Dawson *et al.*, 1998) but should also prevent the environmental spread of the scrapie agent by the placental route from infected ewes. Moreover, a *PrP* genotype genetic control from the conceptus on placental PrP^{Sc} accumulation is a coherent explanation for previous observations (Race *et al.*, 1998) from scrapie-incubating ewes, in which the placentas were found to be either PrP^{Sc}-positive or -negative according to the lambing year.

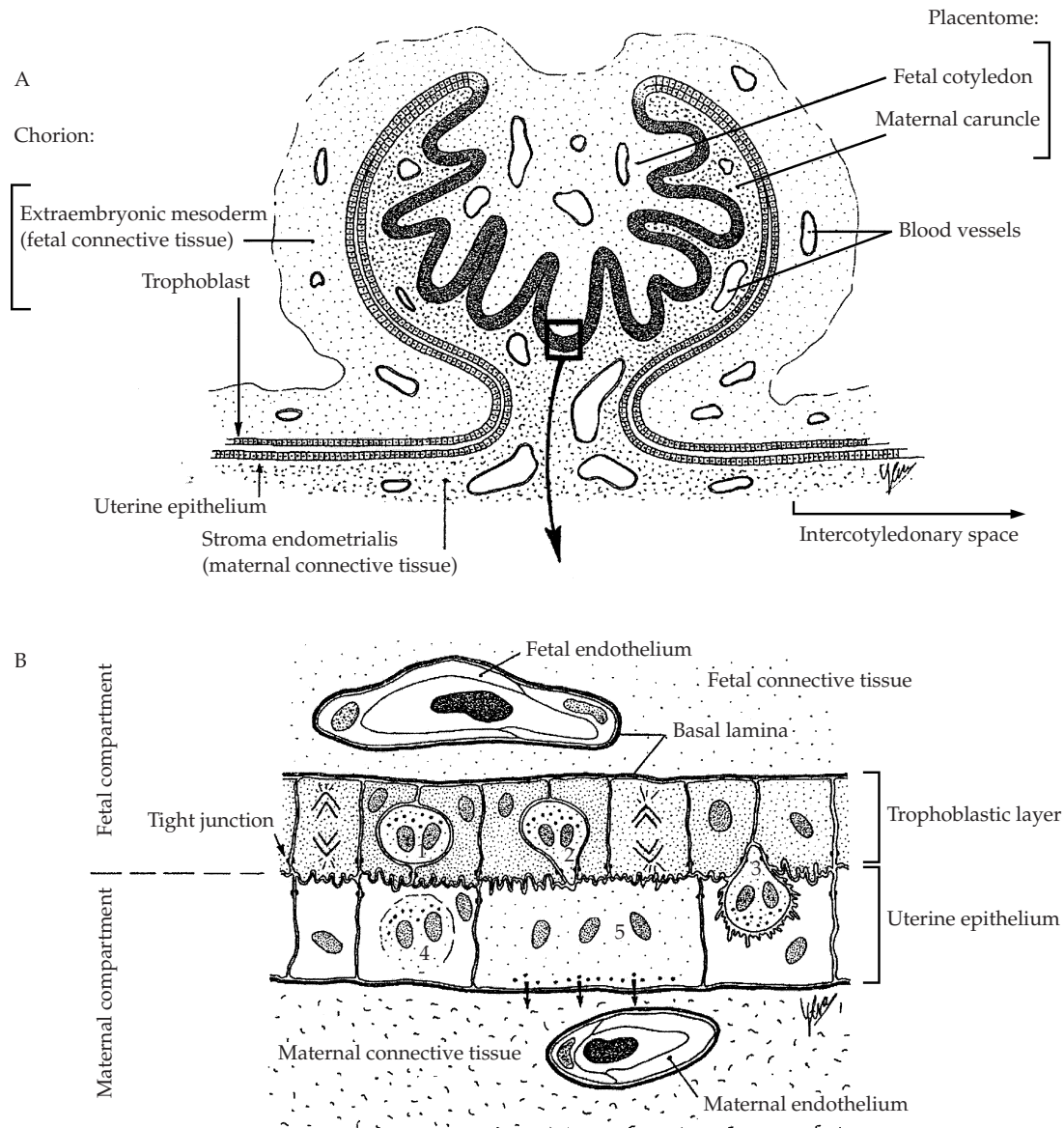


Fig. 3. Schematic diagram of the synepitheliochorial sheep placenta. (A) Growth structure of a placentome: tufts of foetal chorionic villi (cotyledon) are intimately enmeshed with preformed maternal endometrial crypts (caruncle) and so form a placentome. In the intercotyledonary space, the chorion remains smooth, its superficial trophoblastic layer being flat apposed to the uterine epithelium. (B) Diagram of the layers of tissue that constitute the foeto-maternal barrier at the level of a placentome, showing, in particular, BNCs and their migration (1). BNCs possess a large population of characteristic granules that mainly contain placental lactogen. BNCs migrate through a tight junction of the trophoblast epithelium, while maintaining their structure by the insertion of membrane vesicles, thus allowing the cell to form a pseudopodium past the tight junction (2). This pseudopodium increases its size and develops a close apposition to the plasmalemma of an uterine epithelial cell (3). As it migrates across the tight junction, the BNCs form a part of it, thus maintaining the ionic barrier seal between maternal and foetal compartments. BNCs then fuse with the cell membrane to which it was closely apposed (4) and then form a foeto-maternal hybrid TNC (5). This process permits the exocytosis of foetal granules to the maternal side of the placenta (arrows).

However, these results need confirmation on a larger scale before any final conclusion can be drawn. Furthermore, in rodent models, some authors have reported infectivity in spite of any detectable PrP^{Sc} accumulation (Somerville & Dunn, 1996; Lasmezaz *et al.*, 1997; Manousis *et al.*, 2000; Race *et al.*, 2001).

Ovine placenta is of the synepitheliochorial type (Fig. 3). It results from the imbrication of foetal chorionic villi (cotyledons) with maternal preformed endometrial crypts (caruncles). Cotyledons and caruncles combine to form placentomes, while in the intercotyledonary space, the chorion remains smooth, its superficial trophoblastic layer being immediately adjacent to

the uterine epithelium. Sheep chorion is also characterized by the presence of binucleated trophoblastic cells (BNCs), which constitute 15–20% of the trophoblastic epithelial cells (Wooding, 1983, 1984). At the level of the placentome, BNCs have been shown to fuse with the maternal caruncular epithelial cell membrane to form a foeto–maternal hybrid trinucleate cell (TNC). The continuous migration of BNCs and fusion into TNCs produces hybrid foeto–maternal syncytial plaques of limited extent, which completely replace the uterine epithelium in the placentomes throughout the gestation period. The trophoblast-derived nuclei, whether in uninucleated cells, BNCs, TNCs or syncytial plaques, are genotypically identical (Wooding *et al.*, 1997). In our study, PrP^{Sc}-accumulating epithelial cells were only placentomal multi- and uninucleated trophoblasts. The influence of the foetal *PrP* genotype on PrP^{Sc} accumulation in the placenta is consistent with the *PrP* genotype of the foetal trophoblastic cells.

The progressive regression of the maternal epithelial cells at placentomes level during gestation, resulting in a direct physical contact between trophoblastic cells and maternal uterine chorion (Wooding *et al.*, 1997). This direct contact could explain the trophoblast contamination at the placentome level, while intercaruncular trophoblastic cells remained negative; this is in agreement with previous observations (Tuo *et al.*, 2001). Following this hypothesis, early deposition of PrP^{Sc} in trophoblastic cells after foetal implantation and its relationship with maternal epithelial cell disappearance is an interesting aspect and remains to be explored.

In PrP^{Sc}-accumulating placentas, CD68/PrP^{Sc}-positive cells were identified in caruncular endometrial chorion. Some of these CD68-positive cells were in very close contact with positive trophoblastic cells. Previous observations in lymphoid organs from scrapie-incubating sheep showed some CD68-positive cells accumulating PrP^{Sc} and circulating via the lymphoid system (Androletti *et al.*, 2000). Studies in mice have pointed to a role for dendritic (CD68-positive) cells in the spread of the scrapie agent also through the lymphoid system (Beringue *et al.*, 2000; Huang *et al.*, 2002). However, the lack of PrP^{Sc}-positive cells within the placental chorion from scrapie-incubating ewes with PrP^{Sc}-negative placentas, and the absence of CD68/PrP^{Sc}-positive cells in uterine chorion intercotyledonary spaces from ewes with PrP^{Sc}-positive placentas, does not sustain the hypothesis of trophoblastic cell contamination by CD68-positive cells. BNCs produce and deliver proteins through granule secretions (Wooding, 1992; Wooding *et al.*, 1993), which can be phagocyted by maternal uterine mucosal chorion CD68-positive cells, making PrP^{Sc} accumulation within this cell subset possible. In this case, the contamination pathway of trophoblastic cells, via peripheral nerve fibres or circulating cells, remains to be determined.

Endometrial epithelial and trophoblastic cells share the same environment in the uterus of a pregnant ewe. However, one set of cells (endometrial epithelium) remains PrP^{Sc}-negative, while the other (trophoblast) accumulates large

amounts of PrP^{Sc}. This question of cellular permissiveness to PrP^{Sc} accumulation and the comparison of trophoblastic cells with other epithelial cells (permissive/non-permissive) will be interesting to investigate.

In late gestation, no apparent PrP^{Sc} was found within VRQ/VRQ foetuses, while PrP^{Sc} deposition was observed within the placentas of their VRQ/VRQ dams. Further experiments (bioassays) are required to assess definitively the absence of scrapie infectivity in the foetus. This result suggests the absence of in utero transmission via foetal blood circulation or amniotic liquid, which are the two most common routes of foetus infection. Placental connections in sheep ensure total isolation of the foetus from the chorionic structure (trophoblast) and is a possible explanation for non-contamination of lambs in utero (Foote *et al.*, 1993; Tuo *et al.*, 2001). However, no definitive statement can be made for transplacental PrP^{Sc} dissemination in case of placentitis or accidental loss of the continuity of the foeto–maternal barrier.

Placentation in cattle and humans differs from sheep. In the bovine species, there is no fusion of embryonic trophoblastic cells with maternal uterine cells. The absence of contact between trophoblastic cells and maternal chorion (Guillomot, 1995; Wooding *et al.*, 1997), combined to the apparent absence of the prion agent in peripheral tissues of BSE-affected cattle, could explain the absence of PrP^{Sc} accumulation in the placentas in this species. In humans, the placenta is characterized by a total fusion between the embryonic and maternal structures (haemochorial) (Enders & Blankenship, 1999). Such a structure would be more likely to (i) allow contamination of the embryonic placenta and (ii) facilitate possible embryonic contamination in utero. Infectivity studies on a placenta from a pregnant CJD patient has been reported once (Tamai *et al.*, 1992). However, no effective transmission of the disease has been noticed yet in children (who are now 29, 17, 14 and 10 years of age) born from CJD-affected mothers (Berrebi *et al.*, 1997), and previous studies in an experimental primate model argued against a vertical transmission in case of haemochorial placentation (Amyx *et al.*, 1981).

No difference was observed in the spatial PrP^{Sc} dissemination pattern in genetically susceptible VRQ/VRQ lambs, born from ewes with (group 2) or without (group 1) PrP^{Sc} deposition in their placentas and tonsils. Ages at first detection of PrP^{Sc} in ileal Peyer's patches did not differ between the two groups of lambs, suggesting that these lambs could have been infected after birth by contact with infectious material in the surroundings rather than by in utero contamination. However, TSE infections have been reported to occur in the absence of detectable PrP^{Sc} (Somerville & Dunn, 1996; Lasmezaz *et al.*, 1997; Manousis *et al.*, 2000). According to this hypothesis, our data do not allow the dismissal of the theoretical possibility that lambs of group 1 could have been infected by their dams. Nevertheless, until now, no infectivity from such PrP^{Sc}-negative ARR/VRQ ewes was detected by mouse bioassay (unpublished data).

Previous epidemiological studies involving separation of lambs from their dams at different ages after lambing demonstrated clearly the influence of dam-to-offspring contact length on lamb scrapie contamination efficiency (Hourrigan *et al.*, 1979; Hoinville, 1996; Elsen *et al.*, 1999).

Our data highlight that lambing scrapie-infected ewes mated with genetically susceptible rams are a source of early and highly efficient lateral contamination, even for susceptible lambs born from scrapie-free ewes. The use of genetically resistant (ARR/ARR) rams in infected flocks will not only improve the ovine genetic resistance to scrapie on a long-term basis but also directly and dramatically reduce the factors of exposure.

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