

Accumulation of Pathogenic Prion Protein (PrP^{Sc}) in Nervous and Lymphoid Tissues of Sheep with Subclinical Scrapie

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Abstract. All sheep older than 1 year of age from a flock of the Rygja breed in which clinical scrapie was detected for the first time in two animals (4%) were examined for accumulation of pathogenic prion protein (PrP^{Sc}) by immunohistochemistry in the obex, the cerebellum, and the medial retropharyngeal lymph node. In addition, six lambs, 2–3 months old, all offspring of PrP^{Sc}-positive dams, were examined for PrP^{Sc} in the ileal Peyer's patch (IPP), the distal jejunal lymph node, the spleen, and the medial retropharyngeal lymph node (RPLN). In this flock, 35% (17/48) of the adult sheep showed accumulation of PrP^{Sc}, an eightfold increase compared with clinical disease. All positives carried susceptible PrP genotypes. Three sheep had deposits of PrP^{Sc} in the RPLN and not in the brain, suggesting that this organ, easily accessible at slaughter, is suitable for screening purposes. Two 7-year-old clinically healthy homozygous V₁₃₆Q₁₇₁ ewes showed sparse immunostaining in the central nervous system and may have been infected as adults. Further, two littermates, 86-days-old, showed PrP^{Sc} in the IPP. Interestingly, one of these lambs had the intermediate susceptible PrP genotype, VA₁₃₆QR₁₇₁. In addition to early immunolabeling in the dorsal motor nucleus of the vagal nerve, a few of the sheep had early involvement of the cerebellum. In fact, a 2-year-old sheep had sparse deposits of PrP^{Sc} in the cerebellum only. Because experimental bovine spongiform encephalopathy (BSE) in sheep seems to behave in a similar manner as natural scrapie, these results, particularly regarding spread of infectivity, may have implications for the handling of BSE should it be diagnosed in sheep.

Key words: Brain; immunohistochemistry; lymphoid tissue; PrP^{Sc}; sheep; subclinical scrapie.

Scrapie is a transmissible spongiform encephalopathy (TSE) in sheep and goats. At the molecular level the TSEs, or prion diseases, are characterized by accumulation of the pathogenic prion protein (PrP^{Sc}), a modified form of the host-encoded prion protein (PrP^C).⁸ Ultimately, scrapie is a neurodegenerative disease with vacuolation of neurons and neuropil, neuronal loss, and gliosis.¹² According to the prion hypothesis, the transmissible agent of all prion diseases, the prion, is composed largely, if not solely, of PrP^{Sc}.³⁹ However, verified disease has also been described without detection of PrP^{Sc},³¹ or with detection of very small amounts of it.³⁴ The normal form of the prion protein, PrP^C, is found in a wide range of tissues,⁶ and expression of PrP^C in peripheral nervous and lymphoid tissue seems to be a prerequisite for transfer of infectivity and spread of the disease to the central nervous system (CNS).^{7,40}

In natural scrapie the gastrointestinal tract is consid-

ered to be the major route of infection.^{17,38} The earliest accumulations of PrP^{Sc} are found in the ileal Peyer's patch (IPP), as well as in the draining mesenteric lymph node,¹ and this gut-associated lymphoid tissue is a probable port of entry for the scrapie agent in sheep.²⁰ Accumulation of PrP^{Sc} in the enteric nervous tissue has been reported to appear later but still in an early phase of the disease.^{1,50} PrP^{Sc} also aggregates in other lymphoid tissues such as spleen, tonsils, and retropharyngeal lymph nodes in sheep with both clinical and nonclinical disease, and the primary sites of replication of the scrapie agent are likely to be the tissues mentioned.^{1,25,26,44,48}

Transport of prion infectivity along the splanchnic nerves and the vagal nerve has been indicated in several studies.^{3,28,29,35,51} The earliest traces of PrP^{Sc} in the brain have been found in the dorsal motor nucleus of the vagal nerve (DMNV) when naturally infected lambs are 9 months old, and at this stage it was associated with few morphologic changes.¹ The scrapie agent seems to reach the spinal cord at approximately the same time, because deposits were found in the DMNV and the intermediolateral column of the tho-

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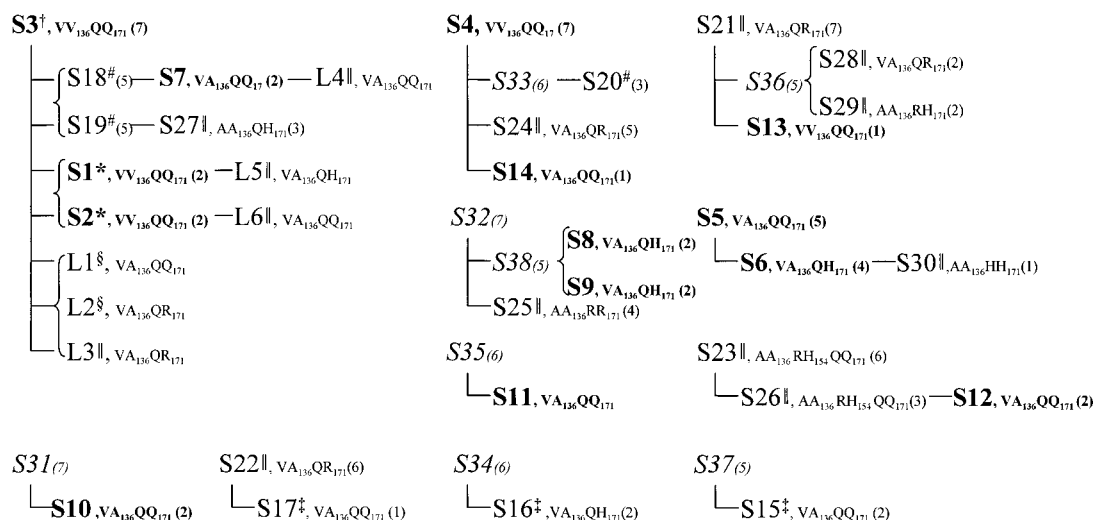


Fig. 1. Descent of diseased sheep. Only animals examined by histopathology and IHC, sheep with suspect clinical signs, and dams of PrP^{Sc}-positive sheep are included. S = sheep; L = lamb. The number in parentheses indicates age. PrP genotypes are given if known. Bold text: PrP^{Sc} positive in the brain and the lymphoid tissue. Italic text: No obtained material, no history of disease. * Clinical cases. † Dubious clinic. ‡ PrP^{Sc} positive in the RPLN only. § PrP^{Sc} positive in the IPP only. || Sheep investigated with negative result. # Sheep with suspect clinical signs but no obtained material.

racic segments T8–T10 in naturally infected lambs at the age of 10 months in another study.⁵¹

Polymorphisms in the PrP gene at codons 136, 154, and 171 are strongly correlated to susceptibility and disease development in scrapie.¹⁴ In several breeds, such as Cheviot, Romanov, or Rygja, the presence of valine (V) at codon 136 enhances susceptibility,^{24,30,46} and a change from glutamine (Q) to arginine (R) at codon 171 renders the animal more resistant to the disease irrespective of breed.¹⁵

Morphologic changes and accumulation of PrP^{Sc} in the brain of sheep with clinical scrapie have been described.^{16,36,42,49,53} Recently, however, focus has turned to studies of PrP^{Sc} accumulations and early pathologic events occurring in the clinically healthy animal. Such studies are of great importance, not only for understanding the pathogenesis of sheep scrapie, but also for targeting tissues for screening purposes to obtain the earliest possible diagnosis. Because scrapie behaves differently between breeds and according to disease-linked polymorphisms in the PrP gene, we have undertaken a comprehensive immunohistochemical and morphologic study of a flock of the Rygja breed in which scrapie was diagnosed. We have previously characterized the PrP genetics of this breed in relation to natural scrapie,⁴⁶ and we are pursuing studies of this breed by using a model of experimental scrapie.²⁰ Lately, there have been a few reports on preclinical PrP^{Sc} accumulation and early lesions in the CNS in sheep with natural infection,^{18,27,42} and a low level of preclinical infection and scrapie-like pathology in sheep flocks has been described.^{5,33} This is the first

report describing whole-flock distribution of PrP^{Sc} accumulations in nervous and lymphoid tissues, and scrapielike pathology in sheep with various PrP genotypes during an outbreak of natural scrapie in a Rygja flock. In Norway the whole flock is culled when a scrapie diagnosis is confirmed, offering favorable conditions for such investigations. As experimental bovine spongiform encephalopathy (BSE) in sheep seems to behave very much like natural scrapie,^{11,26} the results, particularly regarding spread of infectivity, also may have implications for the handling of BSE should it be diagnosed in sheep.

Materials and Methods

Sheep

In 1999, scrapie was diagnosed in a pair of twin sheep (Nos. 1 and 2) in a flock of 49 Rygja sheep, both showing typical clinical signs of scrapie.⁴⁷ Shortly after, the whole flock was culled, except the dam (sheep No. 3) of the twins and her granddaughter (sheep No. 7), and six 2- to 3-month-old lambs, all offspring of the aforementioned ewes. These animals were brought to an isolation facility for observation and subsequent autopsy. One ewe died of unknown cause between the time of scrapie diagnosis and the culling of the flock and was not examined. The table of descent of the diseased sheep is shown in Fig. 1.

The flock was established in 1992, when 20 ewes were recruited from two different farms. In 1993, 10 additional sheep were purchased from one of these farmers. The flock formerly held at the premises and all recruited animals were classified as scrapie contacts before 1999, according to a national scrapie surveillance program.

Table 1. Anti-PrP antibodies and tissues included in the study.

Antibody	Reference	Peptide Sequence	Dilution	Adult Sheep*				Lambs†			
				Obex	Cerebellum	RPLN	Ileum	RPLN	DJLN	Ileum	Spleen
F89/160.1.5	O'Rourke et al. ³⁷	142–145	1 : 200 + 1 : 2,500	48	18	48	2	5	6	6	6
L42	Hardt et al. ¹⁹	145–163	1 : 500	18	18	—	2	5	6	6	6
R521	Garssen et al. ¹³	94–105	1 : 1,500	18	18	48	2	5	6	6	6
R505	Garssen et al. ¹³	100–111	1 : 1,500	48	48	48	—	—	—	—	—

* Brain sections from adult sheep carrying susceptible PrP genotypes (*n* = 18) were selected for examination with all four antibodies. Only ileum sections from sheep Nos. 3 and 7 were examined. — = not examined.

† The RPLN from lamb 2 was not examined. — = not examined.

Sampling of material

From 48 adult sheep, the medulla oblongata, the cerebellum, and the medial retropharyngeal lymph node (RPLN) were collected. In addition, ileum was taken from the two ewes received in the isolation facility. From the six lambs, ileum with the IPP, the distal jejunal lymph node (DJLN), the spleen, and the RPLN were taken for the study. Tissue samples for histology and immunohistochemistry (IHC) were immersion fixed in 4% formaldehyde immediately after death and were subsequently dehydrated and paraffin embedded. For PrP genotyping, blood samples were taken.

PrP genotyping

PrP genotyping was performed by direct sequencing as described by Tranulis et al.⁴⁶

Immunohistochemistry

Tissue sections were mounted on 3-aminoalkyltriethoxysilane-coated glass slides and dried for 48 hours at 37 C. IHC was performed using the polyclonal anti-PrP antibodies R505 and R521¹³ (kindly provided by J. Langeveld), the monoclonal anti-PrP antibodies L42¹⁹ (kindly provided by M. Groschup), and F89/

160.1.5³⁷ (Table 1). On the sections stained with R505 and R521, a streptavidin–biotin–peroxidase IHC was performed as described by van Keulen et al.⁴⁹ After rehydration, tissue sections immunolabeled with F89/160.1.5 and L42 were immersed in formic acid and autoclaved at 121 C in 0.01 M citric acid (pH 6.1) or distilled water. Endogenous peroxidase was blocked by use of 0.3% H₂O₂ in methanol for 30 minutes at room temperature. Tissue sections were then incubated with 20% normal goat serum in phosphate-buffered saline (PBS), pH 7.2, for 20 minutes, to block nonspecific binding sites. Proteinase K (Dako, S3020, California, USA) digestion for 2 minutes was included after the endogenous peroxidase–blocking step on tissue sections from the lambs, in addition to parallel runs without digestion. Sections were incubated with primary antibodies at room temperature for 15 minutes (F89/160.1.5, dilution 1:200) or overnight at 4 C (F89/160.1.5, dilution 1:2,500, and L42). Further steps were performed with the EnvisionTM+ kit (Dako, K4005). Digital images of sections from the brain and the ileum were captured using a digital camera (Spot RT230-2 Slider, Diagnostic Instruments, Inc., Michigan, USA) mounted on a microscope (Leica DM RA/RXA, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

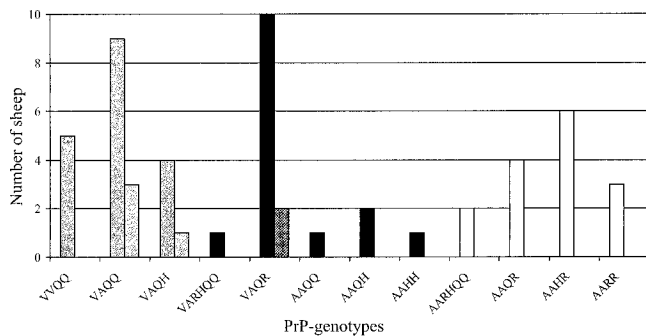


Fig. 2. PrP genotypes in the flock. PrP-genotypes are given with alleles 136 and 171, and in addition allele 154 only when it differs from RR. Dotted bars show the PrP-genotypes of the lambs. Grey bars: Susceptible PrP-genotypes. Black bars: Intermediate susceptible PrP-genotypes. White bars: Low susceptibility PrP-genotypes.

Table 2. Lambs: Individual data and immunohistochemical results.

Lamb	PrP Genotype	Age (days)	Days with Dam	PrP ^{Sc} in IPP*
1	VA ₁₃₆ QQ ₁₇₁	86	84	++
2	VA ₁₃₆ QR ₁₇₁	86	84	+
3	VA ₁₃₆ QR ₁₇₁	86	84	—
4	VA ₁₃₆ QQ ₁₇₁	97	95	—
5	VA ₁₃₆ QH ₁₇₁	84	30	—
6	VA ₁₃₆ QQ ₁₇₁	65	50	—

* The DJLN, the spleen, and the RPLN were negative in all six lambs. — = Negative; + = immunolabeling in 2–4 follicles; ++ = immunolabeling in > 4 follicles.

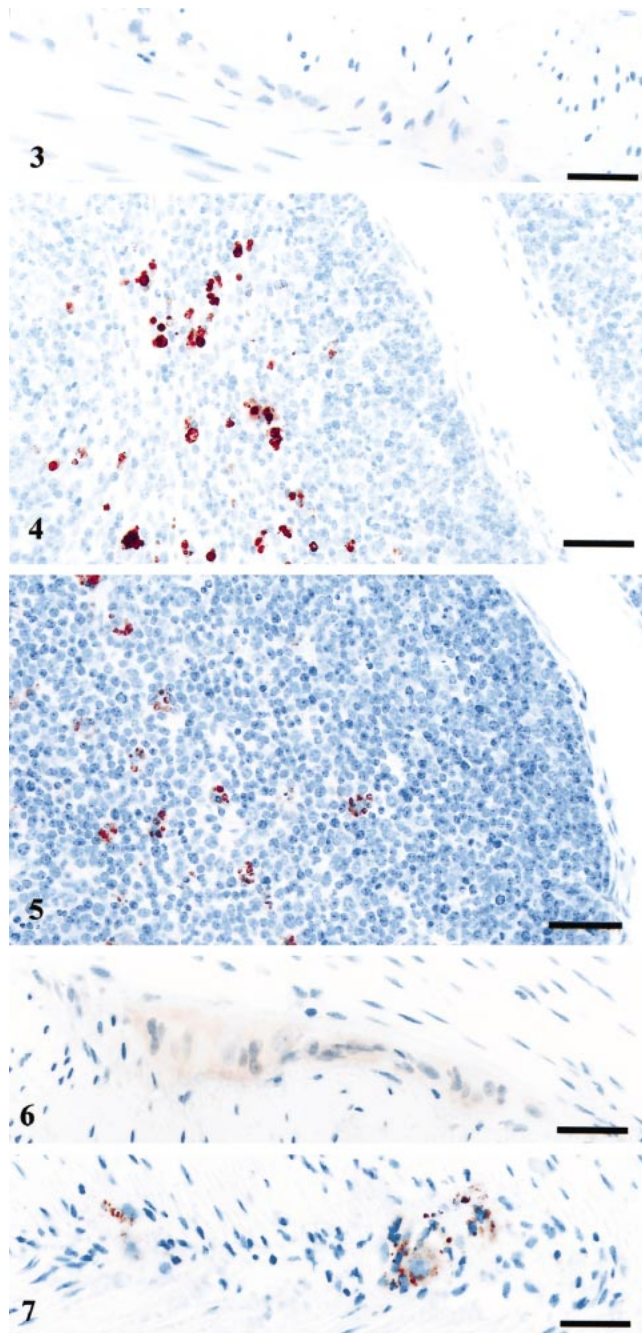


Fig. 3. Enteric nervous system; negative control lamb. Weak and diffuse labeling of ganglion cells in Auerbach's plexus. Anti-PrP antibody L42, EnvisionTM+ kit. Mayer's hematoxylin counterstain. Bar = 30 μ m.

Fig. 4. IPP; lamb No. 1, PrP genotype VA₁₃₆QQ₁₇₁. Strong PrP^{Sc} immunolabeling of tingible body macrophages in a lymphoid follicle. Anti-PrP antibody L42, EnvisionTM+ kit. Mayer's hematoxylin counterstain. Bar = 40 μ m.

Fig. 5. IPP; lamb No. 2, PrP genotype VA₁₃₆QR₁₇₁. Evident PrP^{Sc} immunolabeling of tingible body macrophages in a lymphoid follicle in the IPP. Anti-PrP antibody L42, EnvisionTM+ kit. Mayer's hematoxylin counterstain. Bar = 40 μ m.

Controls

All runs included at least one positive and one negative control section. The positive controls used were confirmed clinical scrapie cases. Negative control tissue was selected from lambs (lymphoid tissue) and sheep (brain) raised in scrapie-free flocks. To characterize nonspecific immunolabeling, PBS replaced the primary antibody on one section in each run.

Histopathology and lesion profiling in CNS

One section from the obex and the cerebellum was stained with hematoxylin and eosin. In the obex, nine areas (DMNV, Nucleus tractus solitarius [NTS], Nucleus n. hypoglossi, Nucleus cuneatus accessorius, Nucleus tractus spinalis n. trigemini [NTS-5], reticular formation, midline raphe, Nucleus ambiguus, and Nucleus olivaris) were examined. Each area was given a score for intraneuronal vacuolation, by counting the number of vacuolated neurons (score = number of vacuolated neurons), vacuolation in neuropil (0–4: score 0 representing six vacuoles in neuropil; score 1, 6–10 vacuoles; score 2, 11–20 vacuoles; score 3, 21–40 vacuoles; and score 4, > 40 vacuoles), and astrocytosis (graded 0–3: 0, nothing; 1, little; 2, moderate; 3, prominent). Caudal parts of the cerebellum were sampled. The whole cerebellum section and each of the nine areas in the obex were given a semiquantitative visual intensity score (0, negative; 1, little; 2, moderate; 3, widespread) of PrP^{Sc} deposition on serial sections.

Results

PrP genotypes

Scrapie susceptibility was graded basically as described by Dawson et al.⁹ Altogether 18 (38%, mean age 2.7 years) of the adult sheep had highly susceptible, 15 animals (31%, mean age 3.7 years) had intermediate, and 15 (31%, mean age 3.7 years) had low-susceptibility PrP genotypes. Three lambs had PrP genotype VA₁₃₆QQ₁₇₁, one lamb had the genotype VA₁₃₆QH₁₇₁, and two lambs had the genotype VA₁₃₆QR₁₇₁. The distribution of PrP genotypes is shown in Fig. 2.

←

Fig. 6. Enteric nervous system; lamb No. 3, PrP genotype VA₁₃₆QR₁₇₁. Diffuse labeling of ganglion cells in Auerbach's plexus. Anti-PrP antibody L42, EnvisionTM+ kit. Mayer's hematoxylin counterstain. Bar = 30 μ m.

Fig. 7. Enteric nervous system; sheep No. 3, PrP genotype VV₁₃₆QQ₁₇₁. Granular labeling of ganglion cells and satellite cells in Auerbach's plexus. Anti-PrP antibody F89/160.1.5, EnvisionTM+ kit. Mayer's hematoxylin counterstain. Bar = 30 μ m.

Table 3. Semiquantitative estimation of HP and IHC in the brain of all sheep with positive CNS findings.*

Sheep No.	Age	PrP genotype	DMNV		NTS		Nucleus n. hypoglossi		Nucleus cunetaus accessorius		NTS-5	
			HP	IHC	HP	IHC	HP	IHC	HP	IHC	HP	IHC
3†	7	VV ₁₃₆ QQ ₁₇₁	+/-	++	-	+/-	-	+	-	-	-	+
4	7	VV ₁₃₆ QQ ₁₇₁	++	++	-	-	-	+/-	-	-	-	+
1‡	2	VV ₁₃₆ QQ ₁₇₁	+++	+++	-	+	+	+	++	++	+	++
2‡	2	VV ₁₃₆ QQ ₁₇₁	+++	+++	+	+	-	++	-	++	++	++
13	1	VV ₁₃₆ QQ ₁₇₁	++	++	-	+	+/-	+/-	-	+/-	-	+/-
5	6	VA ₁₃₆ QQ ₁₇₁	++	+++	++	++	+	++	++	++	++	++
7	2	VA ₁₃₆ QQ ₁₇₁	+++	+++	++	++	++	++	++	++	+++	+++
12	2	VA ₁₃₆ QQ ₁₇₁	+++	+++	+	+	-	++	+	+	+	++
11	2	VA ₁₃₆ QQ ₁₇₁	++	+++	+	++	-	+	++	++	-	++
10	2	VA ₁₃₆ QQ ₁₇₁	-	-	-	-	-	-	-	-	-	-
14	1	VA ₁₃₆ QQ ₁₇₁	+/-	+	-	-	-	-	-	-	-	+/-
6	4	VA ₁₃₆ QH ₁₇₁	-	++	-	-	-	+/-	-	-	-	+/-
8	2	VA ₁₃₆ QH ₁₇₁	+	+++	-	+	-	+	-	-	-	+
9	2	VA ₁₃₆ QH ₁₇₁	+/-	+++	-	+	-	+	+	-	-	+

* HP = histopathology—lesions: +/- = < 6 vacuoles in neuropil; + = score 1–2 on vacuoles in neuropil, no perikaryonal vacuoles; ++ = score 2–3 on vacuoles in neuropil, 1–2 perikaryonal vacuoles; +++ = score 3–4 on vacuoles in neuropil, ≥ 1 perikaryonal vacuoles. IHC—PrP^{Sc} labeling intensity: +/- = minimal; + = little; ++ = moderate; +++ = strong.

† Dubious clinic.

‡ Sheep with clinical signs of scrapie.

§ Perivascular amyloid.

Control slides

Lymphoid tissue. Without proteinase K in the IHC protocol, some of the anti-PrP antibodies produced weak immunolabeling of a few Goblet cells, lymphoid cells, and ganglion cells (Fig. 3) in ileum sections of the negative controls. When proteinase K was included in the protocol, the immunolabeling was completely removed from the negative controls, indicating successful destruction of residual PrP^C or possible cross-reacting epitopes. Because proteinase K treatment was used to confirm disease specificity of labeling, the deposits in the positive controls and flock animals were designated as PrP^{Sc}. All four antibodies detected PrP^{Sc} in lymphoid tissues in the positive controls.

Brain. None of the anti-PrP antibodies used in this study produced immunolabeling in the negative controls. The positive control tissue showed reproducible patterns of PrP^{Sc} accumulations with all the anti-PrP sera used.

PrP^{Sc} in lymphoid tissue and enteric nervous system

Lambs. Two littermates carrying the PrP genotypes VA₁₃₆QQ₁₇₁ and VA₁₃₆QR₁₇₁ had deposits of PrP^{Sc} in the IPP (Table 2). Immunolabeling of several lymphoid follicles in each section was evident with intense and granular staining of macrophage-like cells (Figs. 4, 5). The L42 antibody without proteinase K pretreatment gave diffuse immunolabeling of some of the ganglion cells in ileum in all the six lambs, presumably representing PrP^C and not disease specific PrP^C (Fig.

6). The DJLN, the spleen, and the RPLN were negative in all six lambs.

Adult sheep. Altogether 17 (35%) of the adult sheep had PrP^{Sc} deposits in the RPLN; all positives had susceptible PrP genotypes. One sheep with a susceptible PrP genotype was found negative. Interestingly, two of the subclinical PrP^{Sc}-positive sheep with the highly susceptible VV₁₃₆QQ₁₇₁ PrP genotype were as old as 7 years. In three animals, 15 months (sheep No. 17) and 2 years old (sheep Nos. 15 and 16), PrP^{Sc} was found in the RPLN only. Ten of the PrP^{Sc}-positive sheep were from the same 2-year-old cohort.

In the RPLN, PrP^{Sc} was found mainly in the germinal centers; labeling involved presumed tingible macrophages and follicular dendritic cells. Single positive cells were occasionally found in the interfollicular and paracortical areas.

Sheep Nos. 3 and 7 showed granular immunolabeling of satellite and ganglion cells in Meissner's and Auerbach's plexa in ileum (Fig. 7), and in sheep No. 7 a residual ileal lymphoid follicle showed strong immunolabeling.

Detection of PrP^{Sc} in the brain

Of the 17 adult sheep that were positive for PrP^{Sc} in the RPLN, 14 also showed PrP^{Sc} deposits in the brain. All sheep with PrP^{Sc} deposits in the obex had immunolabeling in DMNV and NTS-5. One sheep (No. 10) was negative in the obex but had consistently punctuate deposits of PrP^{Sc} in the cerebellum when

Table 3. Extended.

Reticular Formation		Midline Raphe		Nucleus ambiguus		Nucleus olivaris		Cerebellum	
HP	IHC	HP	IHC	HP	IHC	HP	IHC	HP	IHC
-	+/-	-	+	-	-	-	-	-	-
-	-	-	+/-	-	+	-	-	-	-
+	+	+++	++	++	++	++	++	+	++
-	++	+	++	+	++	++	++	++	+++
-	+/-	-	+/-	-	-	-	-	+§	++
-	++	+	++	+	++	-	++	++§	+++
++	++	+	++	++	++	-	++	+/-	++
-	+	-	++	+	+	-	+	+/-	+++
-	+	-	++	-	+	-	+	-	+
-	-	-	-	-	-	-	-	-	+/-
-	-	-	-	-	-	-	-	-	-
-	-	-	+	-	-	-	-	-	+/-
-	-	-	+	-	+	-	-	-	-
-	+	-	++	-	+	-	-	-	-

immunolabeled with the F89/160.1.5 and L42 antibodies. Six sheep, including the two clinical cases, were PrP^{Sc} positive in all the nine areas examined in the obex (Table 3).

The PrP^{Sc} deposits were most intense in the DMNV. Sheep presumed to be in an early CNS phase showed deposits of PrP^{Sc} at the ventral border in this nucleus (Fig. 8). These animals had sparse immunolabeling in other areas in the obex. Other sheep, which were in a more advanced stage of disease, had a crescent distribution of staining, reaching dorsolaterally (Fig. 9). A third type of pattern was a diffuse distribution of staining comprising the whole nucleus (Figs. 10, 11). Sheep with the diffuse and the crescent type of staining had widespread PrP^{Sc} deposits in the obex. In sheep with ventral staining in DMNV, all but one had dorsal, sparse staining in N. n. hypoglossi and a low intensity of immunolabeling in midline raphe. The immunostaining in NTS-5 was confined mostly to the dorso-lateral perimeter of the nucleus.

PrP^{Sc} was associated with glial cells interspersed in neuropil, perivascularly and along the ependyme at the ventral margin of the fourth ventricle. Two glia-associated patterns were recognized: a stellate labeling (Fig. 10, arrow) and granular deposits near the glial nuclei (Fig. 9, arrow), the latter assumed to be intracytoplasmic. Both patterns were seen in all nine areas in the obex.

A number of different neuropil-labeling patterns were recognized. Granular deposits of PrP^{Sc} in the neuropil were regularly found in DMNV (Figs. 9–11), dorsally in NTS-5, in N. cuneatus accessorius, and sparsely in N. olivaris. Perineuronal staining (Fig. 11) was common, and in several of the sheep with widespread PrP^{Sc} deposits, there was a linear pattern (Fig.

11) of immunolabeling, likely the accumulation of PrP^{Sc} around neurites. These neuropil-staining patterns were frequently seen ventrally in DMNV, N. ambiguus, reticular formation, and midline raphe. All the animals had granular PrP^{Sc} deposits to some extent in the neuronal perikarya.

Nine sheep had PrP^{Sc} deposits in the cerebellum, and one sheep had sparse immunolabeling in the cerebellum only, indicating an early involvement of this part of the brain. PrP^{Sc} deposits were detected in all three gray matter layers and in the deep cerebellar nuclei. Two sheep had vascular amyloid-like deposits of PrP^{Sc} in the molecular layer. Subpial staining was quite prominent in several cerebellum sections. A stellate pattern was seen in the molecular layer and the deep cerebellar nuclei. Granular PrP^{Sc} deposits were found in all gray matter layers. Perineuronal and intraneuronal labeling was found in the Purkinje cell layer and the deep cerebellar nuclei. Sheep with sparse immunolabeling had granular deposits restricted to the granular and Purkinje cell layers.

There were no obvious differences in labeling patterns between the three groups of PrP genotypes that were positive for PrP^{Sc}, but the VA₁₃₆QH₁₇₁ animals had less PrP^{Sc} deposits than did the other two PrP genotypes (Table 3).

Histopathology in the brain

Vacuolar pathology in the obex and the cerebellum was usually associated with accumulation of PrP^{Sc}, but areas with PrP^{Sc} deposits were frequently without light microscopic pathology (Table 3). Five sheep with immunolabeling in the obex or the cerebellum had very few or no lesions at all in the obex (Fig. 12); three of these had the PrP genotype VA₁₃₆QH₁₇₁.

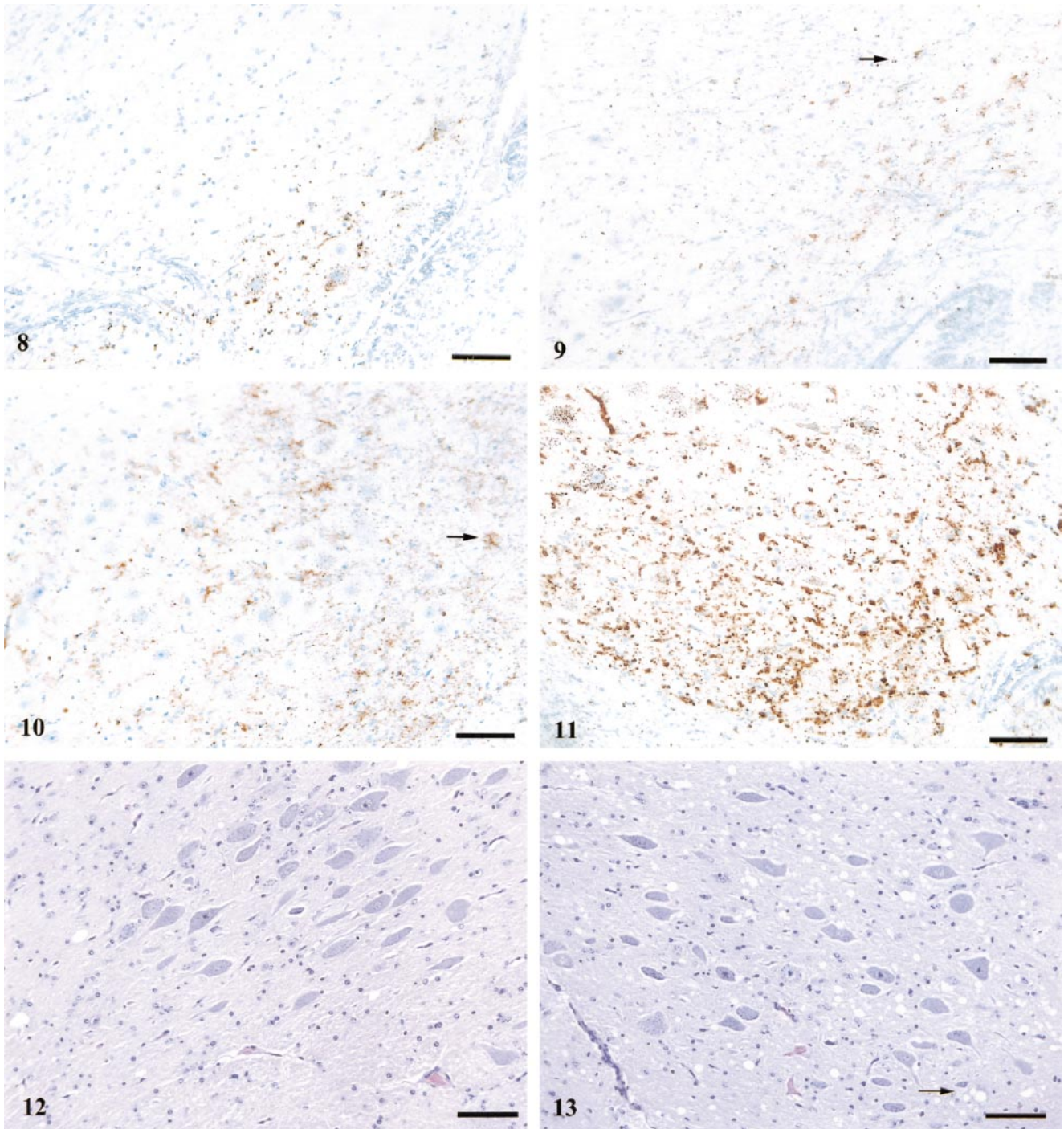


Fig. 8. DMNV; sheep No. 3, PrP genotype VV₁₃₆QQ₁₇₁, 7 years old. Section shows intraneuronal, intragial, and granular neuropil PrP^{Sc} labeling at the ventral margin of the nucleus. Anti-PrP antibody F89/160.1.5, Envision[™]+ kit. Mayer's hematoxylin counterstain. Bar = 80 μ m.

Fig. 9. DMNV; sheep No. 8, PrP genotype VA₁₃₆QH₁₇₁, 2 years old. Sections show a predominantly stellate and granular PrP^{Sc} labeling and some intraneuronal and intragial (arrow) immunostaining in a crescent distribution. Anti-PrP antibody F89/160.1.5, Envision[™]+ kit. Mayer's hematoxylin counterstain. Bar = 80 μ m.

Fig. 10. DMNV; sheep No. 12, PrP genotype VA₁₃₆QQ₁₇₁, 2 years old. Section shows stellate (arrow) and neuropil PrP^{Sc} deposits and some intraneuronal immunostaining diffusely in the nucleus. Anti-PrP antibody F89/160.1.5, Envision[™]+ kit. Mayer's hematoxylin counterstain. Bar = 80 μ m.

Fig. 11. DMNV; sheep No. 2, PrP genotype VV₁₃₆QQ₁₇₁, 2 years old. Section shows a diffuse distribution of intraneuronal and intragial granular PrP^{Sc} deposits and also granular deposits in the neuropil, linear and perineuronal immunostaining mainly ventrally in the nucleus. Anti-PrP antibody F89/160.1.5, Envision[™]+ kit. Mayer's hematoxylin counterstain. Bar = 80 μ m.

The most extensive lesions were found in the DMNV, and the second most affected area was N. cuneatus accessorius. Vacuolation in the DMNV showed the same distribution as that described for PrP^{Sc}: ventral vacuolation, vacuoles spreading dorsally, and scattered vacuoles in the whole nucleus (Fig. 13, arrow shows intraneuronal vacuoles). Astrocytosis correlated well with the vacuolar changes in all areas.

Six animals had vacuolar pathology in the cerebellum. Two sheep (Nos. 5 and 13) also had vascular amyloid-like changes in the molecular layer.

The three sheep with immunolabeling in lymphoid tissue only had no histopathologic changes in the brain. Those animals negative for PrP^{Sc} in any of the organs examined generally had minor or no lesions in the obex.

Discussion

To our knowledge, this is the first report describing the distribution of PrP^{Sc} in nervous and lymphoid tissues in a naturally infected scrapie flock at a given time. Elsen et al.¹⁰ studied a flock in which the animals were kept alive until the appearance of clinical signs. Our data show that the prevalence of PrP^{Sc}-positive sheep in a sheep flock with few clinical scrapie cases (4%) may still be considerably high. In this case, 35% of the sheep greater than 1 year of age showed accumulation of PrP^{Sc}, all contained within the group of animals carrying susceptible PrP genotypes. Only one sheep with a susceptible PrP genotype came out as PrP^{Sc} negative. It is of particular interest that two of the PrP^{Sc}-positive sheep with a highly susceptible PrP genotype were 7 years old and clinically healthy when culled. A possible explanation for the apparently slow development of disease in these animals, as opposed to younger flock-mates of similar PrP genotype, is that the older animals were infected as adults. Alternatively, the older sheep may have been exposed to a lower infective dose initially and, therefore, experienced a slower propagation of PrP^{Sc}. Newly born lambs in an infected environment are believed to receive a high dose of infectivity and may have a more rapid disease development. A previous study reported that infection with the scrapie agent occurred with higher incidence in lambs exposed from birth than in lambs moved to an infected environment when 3 and 9 months of age.²² Scrapie susceptibility also may be influenced by flank-

ing regions of the PrP gene²³ or by other still unknown genes.³²

Three sheep had deposits of PrP^{Sc} in the RPLN, and not in the brain, suggesting that this organ, easily accessible at slaughter, is suitable for screening purposes. Ten of the positive sheep (59%) were 2 years old, and these account for 67% of the 2-year-old cohorts still alive at the time the flock was culled. Seven PrP^{Sc}-positive animals, lambs included, were the first or second generation after the diseased dams. This could not be verified in the other 10 sheep, including a 15-month-old sheep (No. 13). This indicates the importance of an intense infection pressure in the environment combined with a susceptible PrP genotype in the transmission of the disease.

In accordance with recent studies^{1,20} our data highlights the importance of the IPP as an organ involved in early uptake of the scrapie agent. In lambs the IPP reaches its peak development at the age of 3 months, after which it gradually involutes until 18 months of age.⁴¹ In the present work two 86-day-old littermates had PrP^{Sc} deposits in the IPP. Surprisingly, one of these lambs had the intermediate susceptible PrP genotype VA₁₃₆QR₁₇₁, whereas the second positive lamb and the dam had highly susceptible PrP genotypes. To the best of our knowledge, this is the first observation of PrP^{Sc} deposits in lymphoid tissue in sheep with the VA₁₃₆QR₁₇₁ PrP genotype. Andréoletti et al.¹ studied lymphoid tissue from 2-, 4-, 6-, and 9-month-old, naturally infected VA₁₃₆QR₁₇₁ lambs, and in contrast to VV₁₃₆QQ₁₇₁ lambs, none of these showed any immunolabeling. Orally challenged VA₁₃₆QR₁₇₁ lambs seem to take up PrP rapidly at a young age, as Heggebø et al.²⁰ showed that some follicles in the IPP had strong immunolabeling 1 and 5 weeks after exposure. Based on the putative role of the IPP in absorption and dissemination of the scrapie agent, infection after involution of this tissue also may be less effective and may lead to a slower development of clinical disease.

In the brain, all sheep with PrP^{Sc} accumulation in DMNV had concomitant, but generally weaker, labeling in NTS-5. Ten sheep had PrP^{Sc} deposits in NTS. Early immunolabeling in DMNV suggests a retrograde spreading of PrP^{Sc} along efferent fibers of N. vagus and that this nucleus may be the site of entry of the scrapie agent into the brain.^{5,42,51} However, DMNV is also a target for vagal afferents in sheep, and a spread

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Fig. 12. DMNV; sheep No. 3, PrP genotype VV₁₃₆QQ₁₇₁, 7 years old. Section shows a few vacuoles in the neuropil at the perimeter of the nucleus. HE. Bar = 80 μm.

Fig. 13. DMNV; sheep No. 7, PrP genotype VA₁₃₆QQ₁₇₁, 2 years old. Section shows widespread vacuoles predominantly in the neuropil; arrow points to a neuron with perikaryonal vacuoles. An increased number of glial cells are also present. HE. Bar = 80 μm.

along these fibers cannot be excluded. Further dissemination of the agent through neuroanatomic connections between DMNV, NTS, N. n. hypoglossi, and NTS-5 is possible because such connections are established in the lamb.⁵² An additional port of entry of the agent may be any of the cranial nerves associated with the oro-pharyngeal cavity. However, studies on natural scrapie in sheep revealed PrP^{Sc} in Ganglion trigeminale subsequent to accumulation in the brain, suggesting an anterograde spread along afferent nerve fibers.⁵¹

In the present study nine sheep showed accumulation of PrP^{Sc} in the cerebellum, and one sheep had deposits in the cerebellum only. Two of these animals were assumed to be in a very early phase of CNS involvement. Secondary trigemino-cerebellar connections have been established in sheep, and NTS-5 projects to all lobules of the cerebellar hemisphere.⁴³ In addition, cerebellar afferent projections from DMNV have been described in the cat.⁵⁴ The early involvement of the cerebellum of sheep in this study could be explained by these neuroanatomic connections.

This study also suggests that clinically normal VA₁₃₆QQ₁₇₁ sheep may accumulate more PrP^{Sc} than do homozygous V₁₃₆Q₁₇₁ animals before developing clinical disease. Our data indicate that the dissemination rate of PrP^{Sc} to the brain varies with the PrP genotype, being fastest in the V₁₃₆Q₁₇₁ homozygous sheep. However, a rapid spread to the brain also was observed in heterozygous VA₁₃₆QQ₁₇₁ sheep. Among adult sheep, VA₁₃₆QH₁₇₁ animals appear to have a slower dissemination of the scrapie agent to the brain than do the other two PrP^{Sc}-positive V₁₃₆Q₁₇₁ groups. The three sheep with the A₁₃₆H₁₇₁ allele had markedly reduced accumulation of PrP^{Sc} in the brain compared with the other two groups.

The most likely mode of introduction of the disease in this flock may be by recruitment of already infected sheep, as suggested in an epidemiologic study.²¹ The age distribution of susceptible sheep in the present study indicates introduction of the disease in the near past.⁴ As already mentioned, two of the sheep purchased as lambs in 1992 had sparse accumulation of PrP^{Sc} in the brain 7 years later and probably had not been infected as lambs. A third sheep (No. 5) was purchased as a lamb in 1993. This ewe had widespread immunolabeling in the obex and the cerebellum 6 years later and may have been infected as a lamb, thus introducing the infectivity. Second, there is a possibility that the environment was already contaminated when the sheep were stocked up, although there was no previous history of scrapie-like signs at the farm. A third possible entry of the disease is through feeds. Feeding of meat and bone meal to ruminants has been banned since 1990.⁴⁵ Such feeds, however, have been used for pigs and poultry until 2000, and cross-con-

tamination at the mill, during transport, or at the farm cannot be entirely ruled out.²

This study has shown that most of the susceptible animals in a scrapie-affected flock were infected, but only a small number showed clinical signs at a given time. PrP^{Sc} may be detected in lymphoid and nervous tissues of old, clinically normal, but highly scrapie-susceptible, sheep. VA₁₃₆QR₁₇₁ lambs are capable of accumulating PrP^{Sc} in gut-associated lymphoid tissue, but no adult sheep of this PrP genotype had any detectable accumulation of PrP^{Sc} in the RPLN or the brain.

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