Early accumulation of PrPSc in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie

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The immune system is known to be involved in the early phase of scrapie pathogenesis. However, the infection route of naturally occurring scrapie and its spread within the host are not entirely known. In this study, the pathogenesis of scrapie was investigated in sheep of three PrP genotypes, from 2 to 9 months of age, which were born and raised together in a naturally scrapie-affected Romanov flock. The kinetics of PrPSc accumulation in sheep organs were determined by immunohistochemistry. PrPSc was detected only in susceptible VRQ/VRQ sheep, from 2 months of age, with an apparent entry site at the ileal Peyer’s patch as well as its draining mesenteric lymph node. At the cellular level, PrPSc deposits were associated with CD68-positive cells of the dome area and B follicles before being detected in follicular dendritic cells. In 3- to 6-month-old sheep, PrPSc was detected in most of the gut-associated lymphoid tissues (GALT) and to a lesser extent in more systemic lymphoid formations such as the spleen or the mediastinal lymph node. All secondary lymphoid organs showed a similar intensity of PrPSc-immunolabelling at 9 months of age. At this time-point, PrPSc was also detected in the autonomic myenteric nervous plexus and in the nucleus parasympathicus nervi X of the brain stem. These data suggest that natural scrapie infection occurs by the oral route via infection of the Peyer’s patches followed by replication in the GALT. It may then spread to the central nervous system through the autonomic nervous fibres innervating the digestive tract.

Introduction

Scrapie, the ovine prion disease, is the most common natural form of the transmissible spongiform encephalopathies (TSEs), which also include Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy in cattle. TSEs are infectious neurodegenerative diseases (Asher et al., 1976; Fraser, 1976) in which the accumulation of an abnormal isoform (PrPSc) of the prion protein (PrP) is the key event identified at the molecular level (Büeler et al., 1993; Brandner et al., 1996; Blättler et al., 1997; Harris, 1999). The prion protein, the product of the PrP gene (Oesch et al., 1985), is constitutively expressed in its non-pathogenic form, PrPC, by numerous cell types (Bendheim et al., 1992; Horiuchi et al., 1995). According to the prion hypothesis (Prusiner, 1982), PrPSc itself is the causative agent of TSE, although this is still in debate. Notably, TSE infections have been reported in the absence of detectable PrPSc (Somerville & Dunn, 1996; Lasmézas et al., 1997; Manousis et al., 2000) and the protease-resistant prion protein generated in vitro is unable to produce the disease in bioassays (Hill et al., 1999). Although the related pathological mechan-
isms are not well-defined. PrPSc deposition in tissues correlates with infectivity (McKinley et al., 1983; Race et al., 1998) and PrPSc deposits are currently the only molecular marker specific for TSE infections.

In natural prion diseases, as well as in experimental models, TSE pathogenesis in its earlier phase involves the immune system (Hadlow et al., 1982; Kitamoto et al., 1989, 1991; McBride et al., 1992; van Keulen et al., 1996; Klein et al., 1998; Mabbott et al., 1998). The primary site of scrapie infection and its spread within the host still remain unclear. The most widely accepted hypothesis is that natural transmission of scrapie occurs by peripheral contaminations, either through the oral route (Pattison et al., 1974) or through skin lesions (Taylor et al., 1996). In the very early phase of the disease, detection of scrapie infectivity in lymphoid tissues of the oropharynx (tonsils) and intestine (Peyer’s patches), as well as in the spleen and the draining lymph nodes, suggests that primary replication of the infectious agent occurs in these tissues. The lymphatic pathway would then allow scrapie dissemination to the whole immune system. Replication in the intestine and lymphoid organs usually occurs for many months, even years, before reaching the central nervous system (CNS). At the cellular level, replication of the scrapie agent in lymphoid tissues correlates with the presence of PrPSc deposits in macrophages and follicular dendritic cells in the germinal centres of B follicles (McBride et al., 1992; van Keulen et al., 1996; Brown et al., 1999). Infection of the CNS is indicated by PrPSc accumulation in astrocytes and neurons (van Keulen et al., 1995).

Ovine susceptibility to scrapie is mainly controlled by the PrP gene, encoding the protein PrP (Clouscard et al., 1995; Hunter et al., 1996; Bossers et al., 1996). However, the effects of PrP genotypes on scrapie susceptibility can vary between flocks and breeds of sheep (Dawson et al., 1998) and can also depend on the scrapie agent isolates (Foster & Dickinson, 1988; Foster et al., 1993; Goldmann et al., 1994). The main polymorphic PrP gene sites associated with susceptibility or resistance are codons 136 (A or V), 154 (R or H) and 171 (R, Q or H). Thus, in a recent study of a Romanov flock affected by natural scrapie (Elsen et al., 1999), the most susceptible genotypes were found to be VRQ/VRQ (V136R, R154Q, Q171 homozygous), with a disease incidence of 80%, and ARQ/ARQ, with a disease incidence of 45%. Those animals with the VRQ/ARR genotype showed a moderate disease susceptibility with less than 5% of the group affected, while ARR/ARR sheep were highly resistant with no scrapie occurring within this group.

In this study, the kinetics of PrPSc accumulation were determined by immunohistochemistry (IHC) analysis of the intestinal, lymphoid and nervous tissues from groups of genotyped sheep, bred in a naturally scrapie-infected flock (Elsen et al., 1999). The sheep were sequentially autopsied from 2 to 9 months after birth in order to identify those tissues and cells that support the early replication of the scrapie agent and which might allow its dissemination to the lymphoid tissues and the CNS.

**Methods**

**Animals.** The Institut National de la Recherche Agronomique (INRA) flock in Langlade is a closed flock of 700 French Romanov sheep in which an outbreak of natural scrapie occurred in 1993. Since that date, more than 450 cases of scrapie have been observed. The biological and epidemiological features of the outbreak have been subjected to detailed investigations (Elsen et al., 1999). For this study, an appropriate mating of PrP-genotyped parents was achieved and the sheep produced were genotyped at birth (Labogena). Selected sheep were equally distributed between three PrP genotypes of (i) high susceptibility (VRQ/VRQ), (ii) intermediate susceptibility (VRQ/ARR), and (iii) resistant (ARR/ARR). These sheep were raised under the normal conditions for a sheep-producing flock with no physical separation between purposely bred animals and other animals and were removed from the flock just before culling.

**Necropsy and tissue collection.** Animals were raised according to the requirements of the INRA Animal Care and Ethics Committee. All procedures on the animals were performed by workers accredited to the French Ministry of Agriculture and were aimed at limiting animal pain and distress. Sheep were euthanized by an intravenous injection of T61 (Hoechst) followed by exsanguination.

Our study was performed using 56 Romanov sheep. At 2 months of age four animals of each PrP genotype were culled. From 2 to 6 months of age, four animals of the VRQ/VRQ genotype were culled every month and four animals each of the VRQ/ARR and ARR/ARR genotypes were culled every 2 months. The remaining twelve animals were culled at 9 months of age. Collected samples included several lymphoid organs, brain, sections of spinal cord, peripheral nerves and sections of the digestive tract. Samples (encephalon, spleen, palatine tonsils, prescapular and mesenteric lymph nodes) from clinically scrapie-affected sheep from the main flock were used as positive controls (three 5-year-old VRQ/ARR and three 18-month-old VRQ/VRQ animals). Three clinically normal adult ARR/ARR animals, from 3 to 7 years of age, were used as negative controls.

Tissues were fixed in neutral-buffered 4% formalin (final concentration) for 6 days before paraffin-embedding.

**Immunohistochemical staining.** Tissue sections (2 μm thick) were collected onto adhesive-treated ChemMate Capillary Gap microscope slides (DAKO) and dried overnight at 56°C before being deparaffinized and rehydrated. A section stained with haematoxylin and eosin was used as a reference control.

For IHC, tissue sections were first incubated in 98% formic acid (MERCK) for 30 min at room temperature and then subjected to a 5 min proteolysis at 37°C in a 0.1 M Tris buffer solution (TBS) (pH 7.6) containing 0.1% trypsin (200 FIP-U/g) (MERCK). Sections were then autoclaved for 5 min at 121°C in 10 mM citrate buffer (pH 6.0) and allowed to cool for 20 min. Endogenous peroxidase was inhibited using a 1:100 dilution of 30% (w/w) hydrogen peroxide in methyl alcohol for 30 min at room temperature. Washing steps were performed with tap water. Tissue sections were then incubated with 20% normal goat serum in TBS for 20 min to block non-specific binding sites. PrPSc-immunolabelling was carried out using either a rabbit polyclonal antibody...
Early oral contamination in natural scrapie

or a mouse monoclonal antibody (MAb). Rabbit antiserum R521, raised against the ovine PrP peptide sequence 94–105 (van Keulen et al., 1995), was used at a 1:1000 dilution. MAb 2G11 (IgG2a), used at a 1:2 supernatant dilution, was raised against a synthetic peptide (146-R151R152-182) of the ovine PrP peptide and specifically recognizes the R152-R153 sequence. Incubation with primary antibody (1 h) was followed by a 30 min incubation with a biotinylated secondary goat antibody (1:100 dilution) specific for either rabbit or mouse immunoglobulin heavy chains. A streptavidin–peroxidase complex (1:100 dilution) was then applied for 30 min. Colour development was performed using diaminobenzidine (DAB) (ChemMate Detection Kit Peroxidase/DAB, DAKO). Each step was followed by washes with 1 % skimmed milk and 0–05 % Tween 20 in TBS. Sections were counterstained with Mayer’s haematoxylin. All steps were carried out at room temperature.

Double-immunolabelling. Anti-CD antibodies were either MAb specific for human CD68 (clone Ki-M6, monocytes–macrophages, Serotec, 1:150 dilution), human CD20-like (clone BLA36, B lymphocytes, Novocastra, 1:50 dilution), human follicular dendritic cells (clone CNA.42, kindly provided by G. Delsol; Raymond et al., 1997) or a rabbit anti-human CD3 antiserum (A 0452, T lymphocytes, DAKO, 1:100 dilution). Anti-PrP antibodies were the same as above (polyclonal antiserum R521 or MAb 2G11). Secondary antibodies were also used as described above. PrPSc deposits were demonstrated using an alkaline phosphatase activity complex followed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (BCIP/NBT, dark blue/purple labelling). Anti-CD antibody labelling was revealed using a peroxidase activity complex and 3-amino-9-ethylcarbazole substrate chromogen (AEC, red labelling).

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

Results

Negative and positive controls

In clinically scrapie-affected VRQ/VRQ sheep, both anti-PrP antibodies (polyclonal antiserum R521 and MAb 2G11) detected PrPSc accumulations in the CNS and lymphoid tissues. No PrPSc-labelling was observed in the negative serum control sections. In clinically scrapie-affected VRQ/ARR animals, PrPSc deposits were detected only in the CNS and were not observed in lymphoid tissues. All of the ARR/ARR animals were clinically normal and no PrPSc deposition was found in either the CNS or lymphoid tissues.

Excellent PrPSc-immunolabelling was obtained on samples from clinically scrapie-affected VRQ/VRQ sheep, using MAb 2G11, with no non-specific background staining (Fig. 1). Using our IHC method this MAb appeared not to immunostain the cellular PrP expressed in healthy sheep tissue sections resulting in specific labelling of the ovine PrPSc.

In the CNS, the heaviest accumulation of PrPSc was observed in the posterior brain stem and was generally more pronounced in grey rather than in white matter. PrPSc deposits were prominently located in neuronal perikarya and axons. The neuropil was also labelled and numerous glial cells stained...
Among the lymphoid tissues examined, the highest level of 
PrP<sup>Sc</sup> was detected in the palatine tonsils. Low intensity 
labelling was obtained in mesenteric and prescapular lymph 
nodes and in the spleen. PrP<sup>Sc</sup> deposits were located mainly 
(> 95%) in primary and secondary B follicles. Some cells in 
the follicular mantle and paracortical area were also positive. 
Rarely interfollicular and sinusal cells were labelled. In 
centrofollicular areas labelling appeared as a fine reticular 
pattern, suggesting a cytoplasmic deposit in follicular dendritic 
cells. A granular deposition was observed close to round cells 
with a morphology typical of immature B lymphocytes. Some 
macrophage-like cells were strongly positive.

Labelling observed with R521 was roughly similar to the 
labelling intensity seen with MAb 2G11. However, reactions 
in the CNS appeared to be weaker and less extensive. 
Conversely, labelling in the lymphoid tissues was more 
extensive and more intense than that seen with MAb 2G11. In 
both the nervous and lymphoid tissues, the cellular distribution 
of PrP<sup>Sc</sup> deposits remained identical to that observed with 
MAb 2G11.

**Kinetics of PrP<sup>Sc</sup> accumulation in sheep tissues during 
the early phase of natural scrapie**

From 2 to 9 months of age, no PrP<sup>Sc</sup> deposition was 
observed in any of the tissue samples from VRQ/ARR and 
ARR/ARR sheep, whereas PrP<sup>Sc</sup>-immunolabelling was 
observed in the lymphoid and nervous tissues from VRQ/VRQ 
animals (Tables 1 and 2). No clinical signs of scrapie were seen 
in any of the experimental sheep. The kinetics of PrP<sup>Sc</sup> 
accumulation in tissues from the VRQ/VRQ sheep is presented 
below.

**Table 1. Number of PrP<sup>Sc</sup>-affected sheep in relation to 
the host PrP genotype and age**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (months)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>ARR/ARR* (resistant)</td>
<td>0/4</td>
</tr>
<tr>
<td>VRQ/ARR* (intermediate)</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* No case of scrapie was observed at time of writing from animals at 20 months of age.
† Animals from the same cohort have expressed clinical signs of scrapie at 18 months of age.

3-month-old (four animals). One sheep in this group was 
entirely negative for PrP<sup>Sc</sup>. The other three animals showed 
identical patterns of PrP<sup>Sc</sup> deposition in their lymphoid tissues. 
In Peyer’s patches, PrP<sup>Sc</sup> deposits were located mostly in the 
dome areas, of which more than 70% were immunolabelled 
and depending on the animal and on the sample, 50 to 100% of 
B follicles were positive (Fig. 2). In the dome areas, CD68 
cells contained PrP<sup>Sc</sup> granulation, whereas both CD20 and 
CD3 cells were negative (Fig. 3). The corresponding draining 
mesenteric lymph nodes were strongly positive and showed 
more than 95% of B follicles that were positive. The hepatic 
and duodeno-mesenteric lymph nodes were very slightly 
positive. The spleen and the mediastinal lymph node showed 
only a weak labelling intensity and this was only in some B 
follicles. In the upper digestive tract, palatine tonsils (100% of 
fOLLicles were positive), mandibular and retropharyngeal lymph 
nodes (70% to 100% of follicles were positive) were strongly 
labelled, whereas in the parotideal lymph node less than 10% 
of follicles were positive. The prescapular lymph node was 
strongly positive. The precrural lymph node showed minimal 
labelling with less than 5% of follicles that were positive. 
The thymus and the third eyelid were both negative. At the cellular 
level, PrP<sup>Sc</sup>-labelling always showed the same pattern: de-

4-month-old (four animals). At four months of age, PrP<sup>Sc</sup> 
accumulation was observed in all four animals. In animals 
arbitrarily named ‘a’ and ‘b’, the labelling pattern was similar 
to that described at 3 months of age in terms of both 
PrP<sup>Sc</sup>- 
positive tissues and staining intensity. However, in the domes 
of the Peyer’s patches, while PrP<sup>Sc</sup> 
was still located in CD68 
cells, a clear decrease of labelling, both in intensity and extent,
Table 2. PrPSc IHC detection in lymphoid tissues from susceptible VRQ/VRQ sheep with natural scrapie

Sheep with a positive reaction in the examined tissues are indicated as the number of positive sheep out of the four sheep examined at each time period. However, when less than four sheep were positive, the number of animals is followed by the identification of the positive sheep in brackets, arbitrarily named a, b, c and d. Positive sheep were scored on the basis of PrPSc-labelling intensity and are indicated as negative (−), minimal to slight (+), moderate (+++) or strong (++++).

<table>
<thead>
<tr>
<th>Organ*</th>
<th>2-month-old</th>
<th>3-month-old†</th>
<th>4-month-old</th>
<th>5-month-old</th>
<th>6-month-old</th>
<th>9-month-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive sheep</td>
<td>PrPSc-labelling</td>
<td>Positive sheep</td>
<td>PrPSc-labelling</td>
<td>Positive sheep</td>
<td>PrPSc-labelling</td>
</tr>
<tr>
<td>Thymus</td>
<td>0</td>
<td>−</td>
<td>0</td>
<td>−</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>Third eyelid</td>
<td>0</td>
<td>−</td>
<td>0</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Parotid LN</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>2 (a,b)</td>
<td>+</td>
</tr>
<tr>
<td>Mediastinal LN</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>2 (a,b)</td>
<td>+</td>
</tr>
<tr>
<td>Hepatic LN</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>2 (a,b)</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>2 (a,b)</td>
<td>+</td>
</tr>
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<td>Precrural LN</td>
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<td>3</td>
<td>+</td>
<td>2 (a,b)</td>
<td>+</td>
</tr>
<tr>
<td>Palatine tonsils</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Retropharyngeal LN</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Mandibular LN</td>
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<td>+++</td>
<td>3 (a,b,d)</td>
<td>++</td>
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<tr>
<td>Prescapular LN</td>
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<td>−</td>
<td>3</td>
<td>+++</td>
<td>2 (a,b)</td>
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<tr>
<td>PP-duodenum</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>3 (a,b,d)</td>
<td>++</td>
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<tr>
<td>MLN-duodenum</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+</td>
<td>2 (a,b)</td>
<td>+</td>
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<tr>
<td>PP-jejunum 25%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>3 (a,b,d)</td>
<td>++</td>
</tr>
<tr>
<td>MLN-jejunum 25%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>3 (a,b,d)</td>
<td>++</td>
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<tr>
<td>MLN-jejunum 50%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
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<td>+</td>
</tr>
<tr>
<td>PP-jejunum 50%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
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<td>+</td>
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<tr>
<td>MLN-jejunum 50%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
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<tr>
<td>PP-jejunum 75%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
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<td>+</td>
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<tr>
<td>MLN-jejunum 75%</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
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<td>+</td>
</tr>
<tr>
<td>PP-ileum</td>
<td>1 (a)</td>
<td>++</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>MLN-ileum</td>
<td>1 (a)</td>
<td>+</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>PP-caecum</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Ileo-coecal LN</td>
<td>0</td>
<td>−</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

* LN, lymph node; MLN, mesenteric lymph node; PP, Peyer’s patch.
† One of the four 3-month-old sheep was negative in all examined tissues.
ND, Not determined.
was observed compared to 3-month-old animals. Labelling was comparatively stronger in the Peyer’s patch B follicles.

In animals ‘c’ and ‘d’, the situation was intermediate between those observed in 2- and 3-month-old animals. In animal ‘c’, which appeared to be the least scrapie-infected sheep from that group, a marked labelling was observed only in distal jejunal, ileal and caecal Peyer’s patches, whereas proximo-jejunal and duodenal Peyer’s patches remained negative. Among the mesenteric lymph nodes, only those draining

Fig. 2. IHC detection of PrPSc deposition (peroxidase/DAB, brown deposits) in the dome of an ileal Peyer’s patch (a, bar 15 µm) and in the draining mesenteric lymph node (b, c) from a 3-month-old VRQ/VRQ sheep, using the rabbit antiserum R521. In the mesenteric lymph node, PrPSc-positive cells are located in both the interfollicular and centrofollicular areas (b, bar 25 µm) and in the subcapsular sinus (c, bar 15 µm).

Fig. 3. Identification of PrPSc-positive cells in the dome of the ileal Peyer’s patch from a 3-month-old VRQ/VRQ sheep. The double-labelling of PrPSc (alkaline phosphatase/BCIP/NBT, dark blue deposits) with CD20 B lymphocytes (a, bar 15 µm), CD3 T lymphocytes (b, bar 15 µm) or CD68 macrophages (c, bar 15 µm) (peroxidase/AEC, red deposits) reveals an exclusive localization of PrPSc granulation within CD68-positive cells.
Early oral contamination in natural scrapie

PrPSc-positive Peyer’s patches were labelled (Fig. 4). The palatine tonsils and retropharyngeal lymph nodes were also positive. All the other organs examined were negative. In the fourth animal, ‘d’, all of the examined gut-associated lymphoid tissues (GALT), as well as their draining lymph nodes were strongly positive. An intense labelling in the palatine tonsils, mandibular and retropharyngeal lymph nodes was observed, whereas the prescapular lymph node was negative. In the spleen, a few macrophage-like cells migrating through arteriolar walls in the white pulp were positive but without any clear labelling of B follicles. The other examined samples remained negative. The labelled cell populations were identical to those described for the previous animals. However, a large number of cells with intracytoplasmic PrPSc-positive granulation was observed apparently migrating through cortical sinuses and paracortex in the positive lymphoid organs of animals ‘c’ and ‘d’.

5-month-old (four animals). With the exception of the thymus, all the lymphoid tissues, including the third eyelid, from all four animals were PrPSc-positive. Peyer’s patches were still strongly labelled at the B follicle level, whereas labelling in the dome dramatically decreased. In the spleen the number of positive B follicles increased but still remained below 20% and PrPSc-labelling was more intense. In the precrural and parotideal lymph nodes, which were weakly positive in 3- and 4-month-old animals, the labelling was also stronger, with no more than 40 to 60% of the follicles involved. Tonsils were still very strongly positive in 100% of B follicles. The cellular distribution of PrPSc was identical to that described at previous ages except that a dramatic reduction of circulating sinus labelled cells was observed.

6-month-old (four animals). In tissue samples from one of the four animals (sheep ‘d’), the pattern of PrPSc deposition was equivalent to that described for the two least scrapie-infected animals at 4 months of age: the GALT and their draining lymph nodes were positive whereas lymphoid organs unrelated to GALT, such as the spleen or mediastinal and hepatic lymph nodes, remained negative. With the exception of the
Table 3. IHC detection of PrPSc in the autonomic myenteric nervous plexus of the digestive tract

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sheep</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oesophagus</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Rumen</td>
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<tr>
<td>Abomasum</td>
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<tr>
<td>Duodenum</td>
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<td>+</td>
<td>+</td>
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9-month-old (four animals). All four animals were positive in all the investigated lymphoid tissues except the thymus. About 100% of B follicles were PrPSc-immunolabelled, including those in the spleen and those in other lymphoid organs which at previous time-points were only weakly stained. The cellular pattern of PrPSc deposits in lymphoid tissues was similar to that observed at 5 months of age. Only some CD68-positive cells in a few domes of the Peyer’s patches contained PrPSc deposits.

Discussion

PrPSc deposits are associated with TSE pathogenesis and IHC detection of this pathological PrP isoform is routinely used as a hallmark of these diseases (Kitamoto et al., 1987; Lantos et al., 1992). In our study, similar patterns of PrPSc accumulation were observed using either the rabbit antiserum R521 or the mouse MAb 2G11. R521 has been used previously for IHC studies on ovine scrapie (van Keulen et al., 1995, 1996; Schreuder et al., 1996, 1998). Moreover, MAb 2G11 appears to be highly specific for PrPSc, with no labelling of the cellular PrP under our experimental conditions (Andreoletti et al., 1999), and therefore constitutes a useful tool for the IHC detection of ovine PrPSc. Our method, using the rabbit antiserum R521, has been evaluated in a procedure for the standardization of IHC protocols for PrPSc detection in ovine palatine tonsils (European proposal FAIR-PL 97–6013) and showed a marked improvement in the quality of ovine PrPSc-labelling compared to other methods.

At the clinical stage of the disease, PrPSc was detected in both the lymphoid tissues and in the CNS of VRQ/VRQ sheep, whereas in VRQ/ARR sheep, PrPSc deposits were seen only in the CNS. Similar findings have been reported previously in natural scrapie of Texel sheep (van Keulen et al., 1996; Schreuder et al., 1996, 1998). Taken together, these data suggest that sheep carrying the PrP allele ARR do not accumulate PrPSc in the lymphoid tissues.

The kinetics of scrapie dissemination in ovine lymphoid tissues have only been slightly documented to date (Hadlow et al., 1982). Our study revealed a very early PrPSc deposition in VRQ/VRQ sheep. In one animal of this genotype, PrPSc was detected as early as 2 months of age in the ileal Peyer’s patches and in the ileal mesenteric lymph node. Between 2 and 6 months of age, PrPSc-labelling extended to all other GALT, the palatine tonsils and their draining lymph nodes. During this time, GALT-unrelated lymphoid tissues became positive in some individuals, although PrPSc-labelling was still weaker both in its intensity and in its extent. Finally, at 9 months of age, PrPSc was deposited in all the investigated lymphoid tissues, except for the thymus.

PrPSc accumulation in the palatine tonsil biopsies has been previously observed in naturally exposed Texel VRQ/VRQ sheep, all observed lymphoid tissues of the three other animals were positive. In most secondary lymphoid organs, 80 to 100% of B follicles were strongly positive. In the spleen and in the precrural and mediastinal lymph nodes, the amount of PrPSc-positive B follicles reached 60 to 90%. The cellular pattern of PrPSc deposits in lymphoid tissues was similar to that observed at 5 months of age. Only some CD68-positive cells in a few domes of the Peyer’s patches contained PrPSc deposits.
sheep at the age of 4 months, more than 20 months before the occurrence of clinical signs (Schreuder et al., 1998). In our study, tonsils were found to be already PrPSc-positive in most of the VRQ/VRQ sheep at the age of 3 months. All these data strongly argue for a very early scrapie infection by the oral route under natural conditions and point to the ileal Peyer’s patch as a likely primary entry site of the scrapie agent. After experimental oral ingestion of infectious brain material in mice (Maignien et al., 1999) and in deer (Sigurdson et al., 1999), Peyer’s patches and the draining mesenteric lymph nodes became PrPSc-positive 45 days post-inoculation. Comparison of our results to these data suggests that, at least for the oral route, natural contamination may be as efficient as experimental inoculation.

The distribution of PrPSc deposits among Peyer’s patch cells showed a sequential pattern: early steps of scrapie infection first involved cells located in dome areas between the specialized lymphoepithelium and B follicles. Subsequently, PrPSc deposits were visualized in both mantle and centrofollicular cells of B follicles and, at later times, were detected mainly in centrofollicular cells. During this later period, PrPSc deposits in the domes of the Peyer’s patches decreased progressively. It seems therefore, that after penetration through Peyer’s patches, PrPSc spreads to the centrofollicular area where it can persist in its target follicular dendritic cells.

In the domes of the Peyer’s patches, double-labeling techniques demonstrated an exclusive localization of PrPSc granulation in CD68 cells and no PrPSc accumulation was seen in B or T lymphocytes. In humans, the CD68 epitope defined with the MAb Ki-M6 is specifically expressed by macrophages (Parwaresch et al., 1986). In sheep lymphoid tissues, some CD68 cells showed a morphology indicative of interdigitating dendritic cells rather than macrophages, although these two cell types are derived from a common progenitor (Randolph et al., 1999). Nevertheless, it has been proposed that phagocytic cells present in the domes of the Peyer’s patches, such as CD68 cells, could act as antigen-transporting cells from the lymphoepithelium to follicular dendritic cells (Szakal et al., 1983; Berney et al., 1999). Therefore, the presence of PrPSc deposits in these cells may provide a clue to the early stages of infection. Uptake of the scrapie infectious agent from the digestive tract could be achieved by lymphoepithelial M cells, just as with the uptake of bacteria or inert particles (Owen, 1999; Beekes & McBride, 2000). Then CD68 cells may transport the infectious agent to follicular dendritic cells where a primary replication, or at least a concentration, may occur. Whether PrPSc granulation in CD68 cells results from a passive transport of an exogenous PrPSc or from an endogenous replication is still unknown, although the amount of PrPSc accumulated in those cells may favour the second hypothesis. According to recent studies in mice, prion replication depends on PrP-expressing follicular dendritic cells without any direct involvement of bone marrow-derived cells such as lymphocytes or myeloid cells (Brown et al., 1999). However, cellular mechanisms of scrapie pathogenesis could differ from one species to another and, in our opinion, myeloid cells (such as macrophages or interdigitating dendritic cells) could also be involved in scrapie replication in sheep. Further investigations are required to address this question.

Entry of the scrapie agent into ileal Peyer’s patches was followed by its spread within the whole GALT. Numerous cells with cytoplasmic PrPSc granulation were observed apparently migrating through lymph node sinusal formations, indicating a lymphatic/vascular dissemination pathway. A less extensive PrPSc accumulation was observed in more systemic lymphoid organs such as the spleen. This suggests that prion dissemination depends on cells migrating preferentially through gut-associated lymphoid formations.

PrPSc deposits were observed in the autonomic myenteric nervous system only after their dissemination in lymphoid tissues. It must be noted that the dissemination through the enteric nervous system showed a pattern similar to that observed in the GALT, with a primary involvement of ileal structures, followed by a spread to more proximal and distal segments. On this point, our observations are in agreement with those previously obtained in Texel sheep (van Keulen et al., 1999a, b). Furthermore, the preferential accumulation of PrPSc in neurons close to PrPSc-positive Peyer’s patches could suggest that a scrapie passage from lymphoid structures to the nervous system occurs there at the level of the nervous fibres innervating these lymphoid tissues (Beekes & McBride, 2000). Lymphoid organs could thus act as a primary replication site and as a reservoir for the scrapie agent, facilitating infection of the nervous system. Comparison of VRQ/ARR and VRQ/VRQ sheep in the Langlade flock may offer some facts supporting this view. VRQ/ARR animals, of which fewer than 5% eventually develop the disease, do not accumulate PrPSc in their lymphoid tissues, whereas VRQ/VRQ animals, of which 80% die of scrapie after a relatively short incubation period, do accumulate PrPSc in their lymphoid system. Thus, it seems that replication and accumulation of the scrapie agent in lymphoid tissues is required for an efficient and rapid spread of infection to the nervous system. After reaching the autonomous nervous system, the scrapie agent could reach the CNS through an axonal pathway (Race et al., 2000). Our observation of a primary deposition in the nucleus parasympathicus nervi X, which is the origin of the autonomic nervous system innervating the digestive tract, is a clue which supports this hypothesis. In contrast to a previous report (van Keulen et al., 1999b), in which spinal cord was described as the first site of CNS accumulation in sheep with natural scrapie, we did not observe any PrPSc deposition in spinal cord segments. This is probably due to a non-systematic sampling of the spinal cord in our study and does not interfere with our hypothesis. Furthermore, in hamsters orally infected with scrapie an independent pathway to the brain has been demonstrated, bypassing the spinal cord after the uptake of the scrapie agent via the gastro-intestinal tract (Baldauf et al., 1997).
The incubation period and pathogenesis of scrapie results from interactions of the agent isolate with the host PrP genotype. Several scrapie strains have been identified in sheep (Bruce & Fraser, 1991; Bruce et al., 1997) and the allelic distribution of the PrP gene differs between flocks and breeds of sheep (Dawson et al., 1998). In the context of our naturally scrapie-infected flock of Romanov sheep (Elsen et al., 1999), the data obtained from the VRQ/VRQ sheep strongly suggest that an early natural scrapie infection occurs via the oral route and point to the ileal Peyer’s patch as a likely primary entry site of the scrapie agent. Subsequently, it appears that replication and dissemination of the infectious agent takes place in the secondary lymphoid organ system via the lymphatic/vascular pathway. Infection of the autonomic myenteric nervous system, possibly facilitated through infection of the GALT, could finally lead to a spread of the scrapie agent to the CNS. The level of infectivity in the GALT could influence the efficiency of infection of the autonomic nervous system and its dissemination of the infectious agent takes place in the secondary lymphoid organ system via the lymphatic/vascular pathway. Infection of the autonomic myenteric nervous system, possibly facilitated through infection of the GALT, could finally lead to a spread of the scrapie agent to the CNS.

This work has been supported by the EU (grant FAIR-PL 97–6013) and the French TSE Interministerial Committee (grant ACC2).

The authors wish to deeply thank Dr E. Monks (Veterinary Research Laboratories, Dublin, Ireland) for critical reading of the manuscript. We gratefully acknowledge the Experimental Unit of Langlade (Département de Génétique Animale, INRA), especially Francis Eychenne and Eric Lecloux, for their expert care of the provided animals. We should like also to acknowledge Thierry Delaunay for production of MAb 2G11. We are grateful to the assistants of the National Veterinary School of Toulouse for their help in sheep necropsies. Many thanks to Colette Chetcutti and Abdelkader Bouzar for their technical assistance in performing all the IHC tissue sections and to Labogena (Jouy-en-Josas, France) for ovine PrP genotyping.

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Early oral contamination in natural scrapie


Received 16 May 2000; Accepted 12 September 2000