Lymphoid follicles of the ileal Peyer's patch of lambs express low levels of PrP, as demonstrated by quantitative real-time RT-PCR on microdissected tissue compartments, in situ hybridization and immunohistochemistry

Lars Austbø, Arild Espenes, Ingrid Olsaker, Charles McL. Press and Grethe Skretting

The expression level of normal cellular prion protein (PrP\textsuperscript{C}) is thought to influence the transmission of transmissible spongiform encephalopathies (TSEs) from the peripheral entry site to the site of pathological changes in the central nervous system. In many TSEs, the clinical disease is preceded by a period in which the agent accumulates in lymphoid organs, particularly in association with follicular dendritic cells of lymphoid follicles. As the probable route of entry of the TSE agent is via the gut, the expression profile of PrP was examined in well-developed gut-associated lymphoid tissue of lambs, the ileal Peyer’s patch, by laser microdissection and real-time RT-PCR. Lymphoid follicles were found to have very low levels of expression, whilst highest levels were detected in the outer submucosa and the muscular layer. These findings were supported by in situ hybridization and immunohistochemistry, which showed specific labelling in nerve cells in ganglia of the submucosal (Meissner’s) and myenteric (Auerbach’s) plexi of the enteric nervous system. Based on the assumption that potential sites for conversion to the scrapie-related prion protein (PrP\textsuperscript{Sc}) should display high levels of expression of PrP\textsuperscript{C}, this study suggests that the accumulation of PrP\textsuperscript{Sc} in the lymphoid follicles of the Peyer’s patch is not preceded by PrP conversion in the same tissue compartment.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases including scrapie, bovine spongiform encephalopathy and Creutzfeldt–Jakob disease. These diseases are characterized by the conversion of a normal host-encoded protein, the prion protein (PrP\textsuperscript{C}), to an abnormal, insoluble and partially protease-resistant form, PrP\textsuperscript{Sc} (Prusiner et al., 1998). This change in protein structure results in heavy accumulation of PrP\textsuperscript{Sc} in the central nervous system (CNS), particularly at the terminal stage of the disease (Caughey et al., 2001). Normally, PrP mRNA is expressed widely in tissues, particularly in neurons (Caughey et al., 1988; Brown et al., 1990; Cagampang et al., 1999). The importance of PrP in prion diseases has been shown in studies using PrP-knockout mice. In the CNS, PrP expression in neurons is required for the development of neuropathology (Brandner et al., 1996), and in peripheral tissues, expression of PrP is important for efficient transmission of infectivity from the periphery to the CNS (Blättler et al., 1997). Studies in transgenic mice overexpressing PrP have shown that the pace and route of infection are influenced by the expression levels of the host-encoded PrP\textsuperscript{C} (Blättler et al., 1997; Glatzel & Aguzzi, 2000).

Uptake of the infectious agent from the alimentary tract is considered the natural route of infection in TSEs (Hadlow et al., 1982). Pathogenesis studies have shown that the gut-associated lymphoid tissue (GALT) is the first site of accumulation for PrP\textsuperscript{Sc} in scrapie in sheep and mice (Kimberlin & Walker, 1989; Andréoletti et al., 2000; Heggebo et al., 2000). The major component of the GALT in young sheep is a continuous aggregation of lymphoid follicles termed the ileal Peyer’s patch (PP) (Landsverk et al., 1991). During its life, the ileal PP is responsible for generating the vast majority of B lymphocytes in the circulation and peripheral lymphoid tissues (Reynolds & Morris, 1983; Gerber et al., 1986; Press et al., 1996) and for diversification of the pre-immune antibody repertoire (Reynaud et al., 1991; Lucier et al., 1998). This lymphoid organ involutes...
with age and is reduced dramatically by the age of 18 months. The follicles of the ileal PP consist predominantly of B lymphocytes supported by an extensive network of mesenchymal stromal cells including follicular dendritic cells (FDCs) and reticular cells, along with a population of tingible body macrophages and a few T lymphocytes (Halleraker et al., 1990; Nicander et al., 1991; Press et al., 1992). Most studies in scrapie-infected mice demonstrate that FDCs are the dominant cell type harbouring PrPSc in the lymphoreticular system (McBride et al., 1992; Brown et al., 1999; Mabbott et al., 2000). It has been shown that FDCs may enhance the spread of the infectious agent through surface accumulation of PrPSc (Shlomchik et al., 2001). However, there is still discussion as to whether the FDCs in germinal centres are the cells of the lymphoreticular system that sustain conversion of PrPC to PrPSc in TSEs (Shlomchik et al., 2001; Prinz et al., 2002).

At present, little is known about the molecular mechanisms involved in the uptake of the infectious agent from the gut lumen or its accumulation in lymphoid follicles. Indirect evidence for the involvement of GALT in sheep has been derived from oral challenge of lambs with the infectious agent. Heggebo et al. (2000) reported that PrP levels detected by immunohistochemistry were increased in follicles of the ileal PP as early as 1 week after challenge, whereas the presence of PrPSc was only detectable 5 weeks after challenge (Heggebo et al., 2003a). These investigators and others (Andréoletti et al., 2000; Heggebo et al., 2000, 2003a) have speculated that uptake occurs across the follicle-associated epithelium (FAE) overlying the dome of the ileal PP, a proposition supported by experimental studies using cultured M-cell monolayers (Heppner et al., 2001). However, both M-cell-dependent and -independent routes for the transport of PrPSc across the epithelium have been proposed (Ghosh, 2004; Huang & MacPherson, 2004). Both routes imply that, after uptake across the epithelium, PrPSc is transported to lymphoid tissues, possibly by migrating populations of dendritic cells (DCs) (Bruce et al., 2000; Huang et al., 2002). Others have suggested that neuroendocrine cell types, via their expression of PrPC, might play an important role in the internalization of PrPSc from gut lumen (Marcos et al., 2004).

More knowledge on PrP gene expression in GALT will provide an insight into how and where the infectious agent is taken up from the gut lumen and how the infection proceeds before it enters the CNS via the enteric nervous system (ENS) in the gut wall or by other pathways. It is, however, important to note that the site where PrPSc is produced might not be the same as the site where it is converted to PrPSc. Similarly, the site of conversion could be different from the eventual site of PrPSc accumulation.

The aim of the present work was to compare the expression of PrP mRNA with the presence of PrPC in the GALT of lambs at an age where they are most susceptible to scrapie and consequently most likely to be infected, using a combination of quantitative and distributional techniques.

We present the first quantification of PrP mRNA distribution in tissue compartments of the ileal PP by using laser-capture microdissection and real-time RT-PCR. These results are supported by in situ hybridization and immunohistochemistry.

**METHODS**

**Animals and tissue.** Sheep of the Norwegian white breed were collected randomly from healthy animals following slaughter at an abattoir (Felleslaktaetet, Oslo, Norway). Four sheep were studied, with ages ranging from a few weeks to 2 months; their genotypes were PrPVRQ/ARQ, PrPVRQ/ARR, PrPAHQ/ARR and PrPAHQ/ARR (the amino acids at positions 136, 154 and 171 of the ovine PrP gene are stated). Young lambs were selected, as the first few months of postnatal life are assumed to be the period in which the lambs are most susceptible to scrapie (Elsen et al., 1999; St Rose et al., 2006). Immediately after killing, tissue pieces from ileal PP were removed from the animals and placed with the mucosal surface down on liver slices to protect the mucosa during freezing. Slices of cerebellum, 5 mm in thickness, were also collected. These tissues were snap-frozen in 1,1,1-trifluoroethane/1,1,2 tetrafluoroethane/pentfluoroethane (R404A; Ausimont), chilled in liquid nitrogen and stored at $-80^\circ$C until use.

**Laser-capture microdissection.** Sections of 14 mm thickness were cut by using a cryostat (Leitz Cryostat 1720) and mounted on special membrane slides for laser microdissection (Molecular Machines and Industries). The sections were air-dried at room temperature and stored at $-80^\circ$C until use. Prior to microdissection, the membrane sections were stained with RNase-free haematoxylin and dried at room temperature. Laser-capture microdissection of tissue sections was performed by using the SLuCut laser microdissection system (Molecular Machines and Industries). The SLuCut equipment is provided with an automated UV dissection system coupled to video imaging. Tissue samples were removed securely (Fig. 1a, b) by using an adhesive membrane, which protects the tissue on the slide against cross-contamination. To preclude internal variation, several pieces of each desired compartment were microdissected to obtain an area corresponding to $1 \times 10^6$ mm$^2$ or approximately 500–1000 cells depending on the cell density, i.e. at least 10 different follicles from each animal. The microdissected compartments were follicle, dome, interfollicular area, outer submucosa (i.e. area of submucosa immediately adjacent to the inner muscular layer), muscular layer, FAE, lamina propria and villous epithelium (Fig. 1c).

**RNA extraction.** RNA from laser-captured tissue was isolated by using an Absolutely RNA Nanoprep kit (Stratagene). The kit allows rapid purification of high-quality total RNA from extremely small samples of cells ($10^4$ cells) harvested by laser-capture microdissection, with an expected yield of up to 100 ng. The manufacturer’s protocol was followed, including the optional DNase step. The RNA was eluted into 20 ml elution buffer and stored at $-80^\circ$C.

**Quantitative real-time RT-PCR.** Quantitative real-time RT-PCR was performed by using a One-step qPCR core kit (Eurogentec). Primers were designed to span across intron sections by using PrimerExpress 1.5 (Applied Biosystems). The expression level was measured by relative quantification using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. GAPDH has been shown to be expressed more stably across different tissues than other frequently used housekeeping genes (Foss et al., 1998; Garcia-Crespo et al., 2005). Each quantification target was amplified in triplicate and a control lacking the template for each mastermix was always included in the experiments.
Fig. 1. Laser-assisted microdissection of tissue compartments in the ileal PP. (a, b) Microdissection of the follicle-associated epithelium (FAE). Areas of about $1 \times 10^5 \mu m^2$ were isolated from 14 µm cryosections of ileal PP by laser microdissection. After laser-assisted cutting of each area (a), the samples were removed securely (b) by using an adhesive membrane into the cap of a 0.5 ml reaction tube and kept at $-20^\circ C$ until RNA extraction. Nuclei were stained with haematoxylin. Objective magnification, $\times 20$. (c) A section of the PP showing the micro-dissected areas: F, lymphoid follicles; D, dome; IF, interfollicular compartment; OS, outer submucosa; M, muscular layer; FAE, follicle-associated epithelium; LP, lamina propria; VE, villous epithelium. The tissue was counterstained with haematoxylin. Objective magnification, $\times 5$.

Primers and hybridization probes used for the quantitative real-time RT-PCR are as follows: *Ovis aries* PrP: forward, 5'-TCCCGAGACACAGATCTCAACCTT-3'; reverse, 5'-GATCCACTGGCTATGTTGCGCTATTT-3'; probe, 5'-FAM-ACCATGATGACTTCTATCTGCTGTGATTC-ACTG-TAMRA-3'. *Ovis aries* GAPDH: forward, 5'-TGATCCACCACATGGCAAAGT-3'; reverse, 5'-CCACGTACGACGACACCGCAT-3'; probe, 5'-FAM-TCCACGGCCACAGTCAGGCAGAA-TAMRA-3'. Real-time RT-PCR was carried out in an ABI PRISM 7700 (Applied Biosystems) using the following uniform temperature profile: 30 min at 94°C (reverse transcription), then 10 min at 95°C (denaturation), followed by 40 cycles of 30 s at 95°C, 15 s at 56°C and 60 s at 60°C. The same cycling profile was used for all real-time RT-PCRs. The data were analysed by using the Sequence Detection System v1.9.1 (Applied Biosystems). Statistical differences between the different tissue compartments were evaluated by using a paired t-test and the distribution of all datasets was tested by using the Kolmogorov & Smirnov test. Differences in expression between compartments were considered to be significant with values of probability ($P<$0.05 (InStat; GraphPad Software).

**In situ hybridization.** Frozen sections (12 µm) were cut with a cryostat (Leitz Cryostat 1720) and mounted on positively charged slides (Superfrost Plus; Menzel-Glaser). Frozen-tissue sections from the same lambs as were used for quantitative real-time RT-PCR were selected. The sections were air-dried and stored at $-80^\circ C$ until use. To increase the sensitivity, a cocktail of two digoxigenin (DIG)-labelled cRNA nucleotide fragments of the coding and the 3'-untranslated regions of *PRNP* mRNA was utilized, each covering approximately 700 bp of the ORF or the 3' UTR. PCR fragments containing T7 and SP6 promoters were prepared from plasmid clones of the two regions by using vector-derived M13 forward and reverse primers. The amplified PCR products were gel-purified and used as templates for synthesis of the DIG-labelled cRNA antisense and sense probes by using T7 or SP6 polymerases (Roche) following the manufacturer’s instructions. All RNA products were checked by gel electrophoresis and stored at $-80^\circ C$ until use. Probe concentrations were determined by using spot tests. *In situ* hybridization was carried out according to the method of Barthel & Raymond (1993) with some modifications. Briefly, the tissue sections were rehydrated, fixed in 4% paraformaldehyde in PBS, treated with 10 µg proteinase K ml $^{-1}$ (Sigma-Aldrich) for 5 min at 37°C followed by fixation in 4% paraformaldehyde in PBS, then treated with acetic anhydride and dehydrated. Approximately 100 µl hybridization mixture containing 100 ng of each DIG-labelled RNA probe was applied directly to each air-dried section and the sections were incubated in a humidity chamber with coverslips for 15 h at 60°C. Non-specific binding of probes was removed by digestion for 30 min at 37°C with 20 µg RNase A ml $^{-1}$ (Sigma-Aldrich). The probes were detected by using an anti-DIG antibody coupled to alkaline phosphatase. Labelling was visualized by using NBT/BCIP as substrate. For each of the analysed tissue sections, sense probes were applied to serial sections as a negative control to confirm the specificity of the hybridization.

**Immunolabelling for PrP$^{\text{C}}$ on frozen sections.** Frozen sections (10 µm) were placed on positively charged slides (Superfrost Plus; Menzel-Glaser), allowed to dry for 1 h and fixed in 10% formalin/calcium for 15 min. The following monoclonal antibodies (mAbs) of the IgG, isotype were used to detect PrP: L42, P4 (both kindly provided by Dr Martin Groschup, Greifswald-Insel Reims, Germany) and 6H4 (Prionics). In the following steps, a TSA-indirect kit (NEN Life Science Products) was used according to the manufacturer’s instructions with some modifications. All incubations were performed in a humid chamber at room temperature and sections were washed in PBS with 0.05% Tween 20 between incubations unless otherwise stated. To block non-specific binding of antibodies and binding of streptavidin in the TSA kit to endogenous biotin, sections were incubated in avidin (Vector Laboratories) diluted 1:6 in TNB blocking buffer [0.1 M Tris/HCl (pH 7.5), 0.15 M NaCl, 0.5% blocking reagent supplied in the TSA kit] for 30 min. After gentle removal of the blocking solution, sections were incubated overnight at 4°C with primary antibody diluted in biotin (Vector Laboratories): TSB 1:6. To detect binding of primary antibodies, the sections were incubated in biotinylated sheep anti-mouse Ig (RPN 1001; Amersham Biosciences) followed by a streptavidin-horseradish peroxidase (HRP) complex (P0397; DakoCytomation). Signal enhancement was performed by using biotinyl–thyramide followed by streptavidin–HRP; both reagents were supplied in the TSA kit. Finally, signals were developed with aminoethylcarbazole (AEC) as substrate. As negative-control sections, primary antibodies were replaced by an irrelevant antibody of the same isotype (IgG 1) (BD Pharmingen) or dilution buffer. Microphotographs were captured by using a Spot RT Slider digital camera (Diagnostic Instruments Inc.) mounted on a Leica DMRXA microscope (Leica Microsystems Wetzlar GmbH).

**Detection of FDCs by immunohistochemistry.** Frozen sections (8 µm) were placed on positively charged slides (Superfrost Plus; Menzel-Glaser), allowed to dry for 1 h and fixed in 4% formaldehyde for 2 min followed by 70% ethanol for 10 min. Anti-FDC mAb CNA.42 (M7157; DakoCytomation) was used to detect an antigen expressed predominantly by FDCs in various species including...
sheep (Raymond et al., 1997; Lezmi et al., 2001). Sections were labelled by using a polymeric labelling kit (UltraVision LPValue detection system; Lab Vision Corporation) according to the manufacturer’s instructions. Sections were incubated overnight at 4°C with the CNA.42 antibody diluted 1:100 in TBS [0·1 M Tris/HCl (pH 7·5), 0·15 M NaCl] containing 1% BSA. Labelling was detected by using AEC from the kit. Control sections were incubated overnight at 37°C in neuraminidase solution [neuraminidase (N5631-IUN; Sigma-Aldrich) at 0·01 U ml⁻¹ in 50 mM sodium acetate, 154 mM NaCl, 9 mM CaCl₂, pH 5·5] before immunolabelling, as the CNA.42 reactive epitope is known to be destroyed by neuraminidase (Raymond et al., 1997).

Detection of FDCs by 5′-nucleotidase histochemistry. Enzyme activity for 5′-nucleotidase was examined by using the method described by Müller-Hermelink et al. (1974) with minor modifications. In brief, frozen sections (7 μm) were air-dried at room temperature for 1–2 h and fixed for 5 min at 4°C in 4% formaldehyde containing 68 mM CaCl₂ adjusted to pH 7·2 with NaOH. Reactivity for 5′-nucleotidase was detected by incubation with a solution composed of 1 mM adenosine 5′-monophosphate, 200 mM Tris/maleate buffer (pH 7·4), 1·3 mM Pb(NO₃)₂, 100 mM MgSO₄·7H₂O and 96 mM sucrose. The sections were incubated for 40 min at 37°C, followed by treatment with a 0·2% (NH₄)₂S solution for 1 min. The addition of 100 mM NaF to the incubation solution inhibited the reaction.

RESULTS

PrP mRNA levels in different compartments of the ileal PP

The distribution of PrP mRNA expression in the ileal PP was investigated by quantitative real-time RT-PCR on mRNA isolated from distinct tissue compartments. Tissues from young lambs between 2 and 8 weeks of age (n=4) were selected to ensure that the lymphoid follicles of the ileal PP were fully developed. Samples from follicle, dome, interfollicular area, outer submucosa (i.e. area of submucosa immediately adjacent to the inner muscular layer), muscular layer, FAE, lamina propria and villous epithelium (Fig. 1c) were laser-microdissected. PrP mRNA expression was detected in all examined compartments of the ileal PP, with distinct differences in the expression levels between the compartments (Fig. 2). Lowest expression was seen in the follicles and the villous epithelium. The dome, interfollicular area, FAE and the lamina propria showed a two- to threefold higher expression level compared with the level in the follicles. The highest expression level of PrP mRNA was found in the outer submucosa, and muscular layer. In these compartments, the levels were approximately five- to tenfold higher than in the follicles. All animals in this experiment displayed the same pattern regarding the distribution and levels of expression, and no major differences in the expression level in these samples could be seen between the sheep with different PrP genotypes. Pair-specific comparisons of expression levels in the different dissected tissue compartments are presented in Table 1.

PrP expression pattern in the ileal PP

In situ hybridization was performed to determine the localization of cells expressing PrP mRNA in the ileal PP.

The results revealed specific labelling of nerve cells of the myenteric and submucosal nerve plexi of the ileal PP (Fig. 3). Although PrP mRNA expression was detected in the follicles, villous and T-cell areas by quantitative real-time RT-PCR, labelling was not observed in these tissue compartments by using in situ hybridization (data not shown). As brain tissue is known to express high amounts of PrP mRNA (Tichopad et al., 2003; Ning et al., 2005), the cerebellum was used as a positive-control tissue to ensure that the probes were specific. In cerebellum, strong labelling was present in Purkinje cells and some scattered cells of the molecular and granule-cell layers (data not shown), which is in agreement with previous reports in sheep and human (Kubosaki et al., 2000; McLennan et al., 2001). In negative-control sections, in which the antisense PrP probe was replaced with a sense probe, no labelling was observed in any sections from the ileal PP or the cerebellum. When each of the two antisense RNA probes was applied separately, they both produced positive PrP mRNA-specific labelling (data not shown).

Immunohistochemical investigation of the ileal PP by using anti-PrP antibodies revealed strong immunolabelling in the myenteric and submucosal plexuses, as well as some weaker labelling in the capsule of the lymphoid follicles (Fig. 4a). The distribution of PrP in the lamina propria and interfollicular area suggested the labelling of nerves or smooth-muscle cells (Fig. 4b). Labelling was demonstrated also in some large, mononuclear cells in these areas. Most follicles showed little or no labelling. In negative-control sections, no labelling caused by endogenous peroxidase or non-specific

![Fig. 2. Analysis of PrP mRNA levels in the ileal PP. Quantitative real-time RT-PCR of PrP mRNA levels in the sections. Highest expression levels were detected in the outer submucosa and muscular layer, whilst the follicles displayed the lowest expression levels. PrP expression levels are presented relative to GAPDH expression (mean ± SD, n=4).](image-url)
interactions caused by the IgG₁ isotype-control antibody were observed (data not shown).

**FDCs in the ileal PP**

Both immunohistochemistry and 5'-nucleotidase enzyme histochemistry were used to confirm the presence of FDCs in the lymphoid follicles of the sheep ileal PP. Activity for the enzyme 5'-nucleotidase is a marker for FDCs in sheep (Halleraker et al., 1990) and there was strong staining for 5'-nucleotidase in the light central zone of the lymphoid follicles (Fig. 5a). A similar distribution of immunohistochemical labelling was observed with the CNA.42 antibody (Fig. 5b). This distribution of staining, obtained by using both of these methods, indicated the presence of FDCs in the light central zones of all follicles in all animals examined, and that at least 80% of the follicles in a section seemed to be cut through the light central zone. No 5'-nucleotidase staining was detected in NaF-inhibited sections. In neuraminidase-treated sections, labelling with the CNA.42 antibody was abolished.

**DISCUSSION**

In GALT of sheep, accumulation of PrP Sc has mainly been observed within the lymphoid follicles (Andreéleoti et al., 2000; Heggebø et al., 2000). Given the assumption that potential sites of PrP Sc conversion should display high levels of expression of PrP C (McBride et al., 1992), it was unexpected to find that the follicles showed very low levels of

### Table 1. Pair-specific comparisons between PrP mRNA expression levels of the different laser-dissected tissue compartments in the ileal PP of lambs

Statistical differences were evaluated by using a paired t-test (n=4) (InStat; GraphPad Software). Differences in expression with P<0.05 between compartments were considered to be significant and designated + in the table. PrP expression levels relative to GAPDH expression (mean ± SD) are presented.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Expression level relative to GAPDH</th>
<th>Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Follicles</td>
<td>0.0357 ± 0.016</td>
<td>3. Interfollicular area</td>
</tr>
<tr>
<td>2. Dome</td>
<td>0.1129 ± 0.020</td>
<td>4. Outer submucosa</td>
</tr>
<tr>
<td>3. Interfollicular area</td>
<td>0.2106 ± 0.015</td>
<td>5. Muscular layer</td>
</tr>
<tr>
<td>4. Outer submucosa</td>
<td>0.2986 ± 0.043</td>
<td>6. FAE</td>
</tr>
<tr>
<td>5. Muscular layer</td>
<td>0.1129 ± 0.016</td>
<td>7. Lamina propria</td>
</tr>
<tr>
<td>6. FAE</td>
<td>0.0816 ± 0.029</td>
<td>8. Villous epithelium</td>
</tr>
</tbody>
</table>

**Fig. 3.** PrP mRNA expression in ileal PP. *In situ* hybridization was performed by using an antisense cRNA PrP probe. PrP transcripts were detected in nerve cells (arrowheads) (a) in submucosa, (b) the muscular layer and (c) in the interfollicular area in close contact with the follicle capsule. (d–f) A sense probe was used as a negative control. Nuclei were stained with haematoxylin. Bars, 50 μm.
PrP mRNA. Indeed, the lymphoid follicles had the lowest level of expression of all compartments examined (Fig. 2). In mice, it has previously been reported that PrP mRNA levels may not correlate with PrPC levels in cells of the CNS (Ford et al., 2002a). Our results indicate, however, that the low levels of PrP mRNA in the follicles of sheep ileal PP were supported by the absence of labelling for PrPC by immunohistochemistry.

The expression of PrP C by FDCs has been reported to be critical for the conversion and accumulation of PrPSc in lymphoid follicles (Brown et al., 1999) and subsequent neuroinvasin in mouse scrapie (Mabbott et al., 2000). Similarly, the accumulation of PrPSc in sheep scrapie was found to be associated with FDCs in lymphoid follicles early in infection (Jeffrey et al., 2000; Heggebø et al., 2002). However, the expression level of PrP in FDCs has previously not been investigated in sheep. In cattle, FDCs isolated from lymphoid tissues were found to react only with one mAb against PrP, 6H4, indicating that a special isoform of PrPC was present in this type of cell (Thielen et al., 2001). In sheep, even though both 5'-nucleotidase enzyme activity and immunohistochemistry confirmed the presence of FDCs, they were not labelled by using 6H4 or two other mAbs in immunohistochemistry on frozen sections, suggesting a possible difference in the amount of PrPC present in FDCs between cattle and sheep.

The observed low levels of PrP expression in lymphoid follicles and FDCs of the ileal PP of sheep suggest that the PrPSc accumulation found in these sites during scrapie infection (Jeffrey et al., 2000; Heggebø et al., 2002) was not derived from conversion of PrPC produced by FDCs. It is more likely that preformed PrPSc is transported into the follicles and subsequently trapped on FDCs via receptors such as the complement receptor (Mabbott et al., 2001). Another possibility is that the levels of PrPC in FDCs are below the detection limits of the techniques used in the present study, and that both the conversion and accumulation of PrPSc still might take place in FDCs during the prolonged incubation period of scrapie. A third possibility, not investigated in this study, is that PrP expression may be increased in infected animals.

It is possible that the presence of DCs in the dome and the interfollicular regions can account for the significantly higher levels of PrP expression that were present in these compartments compared with the levels detected in lymphoid follicles (Kelsall & Strober, 1996; Huang & MacPherson, 2004; Defaweux et al., 2005). DCs are known to express PrPC (Burthem et al., 2001; Sugaya et al., 2002) and they form a dense layer of cells in the dome in the PP just beneath the FAE, where they are in close contact with M cells (Halleraker et al., 1990; Press et al., 1992; Kelsall & Strober, 1996). It has previously been shown that DCs can acquire PrPSc in vitro and that a small subpopulation of migrating DCs is able to take up and transport PrPSc from the gut lumen through the lymphatics to the lymphoid tissue (Huang et al., 2002). PrPSc accumulation in the dome and subsequently in central FDCs of follicles in the early phases of scrapie has been reported (Andréoletti et al., 2000). These accumulation patterns indicate that conversion of PrPSc might take place in the dome, a tissue compartment with higher levels of PrP mRNA expression levels than the follicles, before the misfolded protein is transported to and accumulates in association with FDCs of the follicles.

The highest levels of PrP mRNA expression were observed in the outer submucosa and the muscular layer and were

---

**Fig. 4.** PrPC protein expression in ileal PP. (a, b) PrPC was detected by immunohistochemistry in the ganglia of the enteric nervous system (arrowheads). In the (a) interfollicular area and (b) lamina propria, long, thin fibres, possibly representing nerves, were present (arrowheads). Nuclei were stained with haematoxylin. Bars, 100 μm.

**Fig. 5.** Detection of FDCs in the ileal PP. (a) 5'-Nucleotidase enzyme histochemistry in the ileal PP revealed a reticular pattern of staining in the light central zone of the ileal lymphoid follicles. Bar, 100 μm. (b) Immunohistochemistry using anti-FDC mAb CNA.42 showed a similar pattern of labelling, indicative of FDCs in the light central zone of the ileal lymphoid follicles. Nuclei were stained with haematoxylin. Bar, 100 μm.
probably the result of the high PrPC expression in the submucosal (Meissner’s) and myenteric (Auerbach’s) plexi of the ENS, as shown in mice (Ford et al., 2002b) and sheep (Heggebø et al., 2003b). The high expression levels that were detected by real-time RT-PCR in samples microdissected from the outer submucosa and the muscular layer were supported by in situ hybridization and immunohistochemistry. Both techniques revealed extensive labelling in neuronal cell bodies and satellite cells of the myenteric and submucosal nervous plexi, structures with substantial accumulation of PrPSc during scrapie (van Keulen et al., 1999; Heggebø et al., 2002). In the small intestine of mice, specific labelling of enteroendocrine cells and small enterocytes in the epithelium or myofibroblasts in the lamina propria has been reported (Ford et al., 2002b). Additionally, González et al. (2005) showed a faint and inconsistent FDC-like pattern of PrP immunolabelling in the lymphoid follicles. Labelling of these cell populations was not detected in the present work. This discrepancy may be due to the sensitivity of the in situ hybridization and immunohistochemistry protocols used in our study. It should be noted that species differences may account for varying levels of PrP expression, as may the differing functions of the ileal PP as opposed to conventional mucosal lymphoid tissue represented by jejunal PP in sheep and PP in mice (Reynolds & Morris, 1983; Landsverk et al., 1991).

To our knowledge, the present study is the first report on the expression levels and distribution pattern of PrP mRNA in the ileal PP in sheep. The finding that the follicles displayed the lowest levels of expression of PrP mRNA in the ileal PP focused attention on the relationship between PrPC expression, PrPSc conversion and accumulation of PrPSc. The molecular mechanisms by which PrPSc is taken up and transferred to nerves, and to what extent FDCs, M cells and DCs are involved in these processes, await further clarification, in particular the demonstration that the expression of PrPC is involved in the process of transfer of PrPSc from the gut lumen to subepithelial tissues. The refinement of laser-dissection techniques and an improvement in sensitivity of in situ hybridization protocols, along with accompanying studies on experimentally challenged scrapie animals, may enhance our understanding of these fundamental events in the peripheral pathogenesis of scrapie in sheep.

ACKNOWLEDGEMENTS

The authors thank Ragnhild Fugleseit for providing the tissue sections and performing the enzyme histochemistry and immunohistochemistry work. This study was supported by grants from the Norwegian Research Council (146916/140).

REFERENCES


