

Correspondence

Charles Press

Charles.Press@veths.no

Disease-associated PrP in the enteric nervous system of scrapie-affected Suffolk sheep

Ragna Heggebø,¹ Lorenzo González,² Charles McL. Press,¹ Gjermund Gunnes,¹ Arild Espenes¹ and Martin Jeffrey²

¹Department of Morphology, Genetics and Aquatic Biology, Norwegian School of Veterinary Science, PO Box 8146 Dep., N-0033 Oslo, Norway

²Lasswade Veterinary Laboratory, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ, UK

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Disease-associated prion protein (PrP^d) in the enteric nervous system (ENS) of 20- to 24-month-old Suffolk sheep in the late subclinical and early clinical phase of scrapie was studied. Sites in the alimentary tract extending from the forestomachs and abomasum to the colon from scrapie-affected sheep (PrP^{ARQ/ARQ}) and scrapie-resistant sheep (PrP^{ARR/ARQ} and PrP^{ARR/ARR}) were examined. PrP^d was found only in scrapie-affected sheep and was most prominent in the ENS when abundant deposits of PrP^d were also present in adjacent lymphoid nodules. Immunolabelling with the nerve fibre markers PgP 9.5 and neuron-specific enolase and the satellite cell marker glial fibrillary acidic protein revealed the extensive ganglionated networks of the myenteric and submucosal plexi. Fewer nerve fibres were present in the lamina propria, T-cell dominated interfollicular areas and dome regions of Peyer's patches. A substantial network of nerve fibres was detected in many lymphoid nodules of both the scrapie-affected and scrapie-resistant sheep. Nerve fibres were also detected within the capsule of lymphoid nodules. Electron microscopy revealed the presence of nerves in the lymphoid nodules, showing a close association with follicular dendritic cells, lymphocytes and tingible body macrophages. In demonstrating that lymphoid nodules in the Peyer's patches of scrapie-affected sheep possess a substantial network of nerve fibres, the present study shows that nodules provide close contact between nerve fibres and cell populations known to contain abundant PrP^d, including follicular dendritic cells and tingible body macrophages, and that gut-associated lymphoid nodules in sheep may represent an important site for neuroinvasion.

INTRODUCTION

Scrapie is a fatal neurodegenerative disease of sheep and goats that is characterized by the accumulation of a protease-resistant conformer of the cellular prion protein PrP^C, which is considered identical to the infectious agent (Prusiner, 1982). The uptake of the scrapie agent from the alimentary tract is the most likely natural route of infection but the processes involved in the movement of the invading agent from the gut lumen to the central nervous system (CNS) are only partially understood. While the early accumulation of disease-associated PrP (PrP^d) in lymphoid tissues has been shown to facilitate neuroinvasion (Lasmézas *et al.*, 1996), it is probably the involvement of peripheral nerves that represents the final common pathway for neuroinvasion *in vivo* (Race *et al.*, 2000). Studies in PrP-knockout mice have shown that a non-haematopoietic PrP-positive cell-type is required for neuroinvasion, arguing against a central role for haematogenous routes of infection (Race *et al.*, 2000). It has also been shown that neuroinvasion can occur without the involvement of lymphoid tissues and bypass the spinal cord to enter the CNS via the vagus nerve and dorsal motor nucleus of the vagus (Beekes *et al.*, 1998). Indeed, the vagus nerve would appear to be the primary route for the transfer of infectivity to the CNS when animals are infected via the oral route (Baldauf *et al.*, 1997; Beekes *et al.*, 1996).

The scrapie agent accumulates in lymphoid organs, and the follicular dendritic cell (FDC) in germinal centres has been shown to be the cell of the lymphoid system that sustains replication (Brown *et al.*, 1999; Kitamoto *et al.*, 1991; McBride *et al.*, 1992). After natural infection of scrapie in sheep, PrP^d has been detected in lymphoid nodules of the Peyer's patches of the gut as early as 5 months after oral infection (Andréoletti *et al.*, 2000; van Keulen *et al.*, 2000). The subsequent presence of PrP^d in tissues of the enteric nervous system (ENS) in the gut wall has supported the suggestion that the ENS is the site of initial neuroinvasion for the scrapie agent. However, the innervation of lymphoid tissue of the Peyer's patches in sheep is not well documented.

The ENS is a complex system of intrinsic enteric neurons, nerves and supporting cells and extrinsic nerve processes of the sympathetic and parasympathetic nervous systems that are embedded in the wall of the gut and extend from the pharynx to the anal sphincter and into the pancreas and gall bladder (Costa *et al.*, 1987). Within the ENS, two types of nerve meshworks can be distinguished, the ganglionated plexi and the sparse meshworks of aganglionic nerve strands. The major ganglionated plexi are the myenteric (Auerbach's) plexus, located between the circular and longitudinal muscle layers of the gut wall, and the submucosal (Meissner's) plexus (Furness & Costa, 1980; Timmermans *et al.*, 1992). In large mammals, including sheep, the submucosal plexus is divided into outer and inner plexi, located close to the inner circular muscle layer and lamina muscularis mucosae, respectively (Balemba *et al.*, 1999; Timmermans *et al.*, 1992). However, only limited histochemical and immunocytochemical studies of the innervation of Peyer's patches in ruminants have been undertaken. In cattle, a topographic and structural study of the

ENS in jejunum and ileum was performed and demonstrated overall similarities with other species (Balemba *et al.*, 1999), although there were some differences to the reported organization in the pig (Krammer & Kühnel, 1993).

Germinal centres are poorly innervated (Felten *et al.*, 1985), and transfer of the scrapie agent from the accumulations of PrP^d at these sites to nerve endings of the peripheral nervous system is therefore difficult to explain. Access to peripheral nerves is facilitated if myelination of the nerves is reduced or absent (Kimberlin *et al.*, 1983). Therefore, the mantle zone of lymphoid follicles that are innervated by terminal unmyelinated nerve fibres has been proposed as the entry point of the scrapie agent into the peripheral nervous system, as this is a region where FDC processes come in close contact with nerve fibres (Glatzel *et al.*, 2000). The aim of the present study was to investigate the localization of the ENS in the Peyer's patches of sheep and to describe the distribution of PrP^d within the ENS of scrapie-affected Suffolk sheep.

METHODS

Sheep and tissue collection. Young animals from a closed flock of Suffolk sheep were examined. The pathology, epidemiology and genetics of scrapie infection in this flock have been described previously (Hunter *et al.*, 1997). As described in other Suffolk flocks (Hunter *et al.*, 1997; Westaway *et al.*, 1994), clinical disease occurs almost exclusively in PrP^{ARQ/ARQ} homozygotes.

In the present study, eight sheep (20- to 24-month-old) were studied from the above-mentioned flock which had experienced frequent cases of natural scrapie over a period of several years. At 20 months old, two PrP^{ARQ/ARQ} sheep, one PrP^{ARR/ARQ} sheep and one PrP^{ARR/ARR} sheep were placed in deep anaesthesia and killed by exsanguination. None of these sheep had any clinical evidence of disease. A further four sheep were killed at 23 to 24 months old, having had a short period with clinical signs consistent with scrapie.

From each sheep, tissues from the brain, spinal cord, lymphoid tissues and alimentary tract were collected for histological and immunohistochemical analysis. The results of brain and lymphoid tissue histology and immunohistochemistry have been reported elsewhere (Heggebø *et al.*, 2002). From the alimentary tract of each sheep, the following sites were identified and placed in 10 % neutral-buffered formalin or were quenched in isopentane cooled with liquid nitrogen and subsequently retained in a -70 °C freezer until use: rumen (two sites), omasal-reticulum junction, abomasum (pylorus, greater and lesser curvature), duodenum, jejunum (several sites containing grossly identified jejunal Peyer's patches), ileum at the ileocaecal fold and at the ileo-colic junction, and colon (adjacent to the ileocaecocolic junction and

at the descending colon near to the spiral colon).

Electron microscopy. The tissues collected from the gut for electron microscopy were immersion-fixed in 2 % periodate-lysine-paraformaldehyde/0.5 % paraformaldehyde for 24 h at 4 °C. Tissues from the gut were cut into 1 mm cubes, post-fixed in 2 % osmium tetroxide, dehydrated and embedded in Araldite. Thick sections were stained by toluidine blue. Mesas were trimmed and 65 nm thick sections were taken from blocks identified as containing representation of the mucosal and Peyer's patches or of the muscularis with ENS ganglia. These sections were placed on 300 mesh nickel grids and post-fixed with 2.5 % glutaraldehyde in PBS. Grids were counterstained with 1 % uranyl acetate and lead citrate.

Immunohistochemistry in paraffin-embedded tissues. Avidin–biotin complex and peroxidase–anti-peroxidase immunohistochemical staining for disease-associated accumulations of PrP was done using modifications of previously published methods (Haritani *et al.*, 1994). Tissues were subjected to formic acid pre-treatment and hydrated autoclaving. Immunohistochemistry for the detection of PrP was performed using the R521.7 antibody, which was kindly provided by J. Langeveld, IDLO, Lelystad, The Netherlands (van Keulen *et al.*, 1996), and several other monoclonal and polyclonal anti-PrP sera including P4 and L42 (kindly provided by M. H. Groschup, Germany) (Hardt *et al.*, 2000) and R486 (kindly provided by R. Jackman, VLA Weybridge, Surrey). The control sections included the use of isotype control sera as the primary antibody.

To enhance the sensitivity of the immunohistochemical method for the detection of PrP in paraffin-embedded tissues, the above procedure was modified to incorporate an additional enhancement procedure, as previously used on frozen tissue (Heggebø *et al.*, 2000). Briefly, following incubation with the peroxidase-conjugated avidin–biotin complex and washing, the sections were incubated with biotinyl tyramide (TSA-Indirect) for 5 min, washed and then incubated with streptavidin–horseradish peroxidase for 30 min. Peroxidase activity was detected using 3-amino-9-ethyl carbazole (Sigma) for 10 min. The reaction was stopped by washing the sections in distilled water. The primary antibody used was L42 and an isotype control serum was used for the control sections. With this enhancement procedure, some weak staining for PrP was detected in the myenteric plexus of formalin-fixed intestines from the PrP^{ARR/ARR} sheep and the PrP^{ARR/ARQ} sheep. Parallel sections from these resistant sheep that were stained using L42 but without the enhancement steps showed no staining from PrP.

Immunohistochemistry for the detection of cells and nerve fibres of the ENS was performed using polyclonal antibodies against protein gene product 9.5 (PgP 9.5), neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP), all supplied by Dako. For PgP 9.5, an antigen retrieval procedure was used, involving the autoclaving of tissue sections in 0.2 % citrate buffer at 121 °C for 20 min. The

primary antibody was incubated overnight at room temperature and immunolabelling was detected using an avidin–biotin complex procedure (ABC kit, Vector Elite) with the substrate chromogen 3,3'-diaminobenzidine and enhancement in 0.5 % aqueous copper sulphate for 3 min.

Histoblot. For histoblot, frozen sections (12 µm in thickness) were mounted on nitrocellulose (Sigma nitrocellulose membrane, 0.45 µm pore size) and performed in a standard manner (Taraboulos *et al.*, 1992), with some minor modifications (Heggebø *et al.*, 2002).

Briefly, the tissues were subjected to proteolysis with proteinase K (400 µg ml⁻¹; Serva Electrophoresis) at 55 °C for 4 h. After several pre-treatment steps, the membrane was incubated with the anti-PrP monoclonal antibody L42 overnight at 4 °C. The membrane was incubated with a secondary anti-mouse antibody (Vectastain ABC kit, Vector Laboratories) for 30 min, followed by incubation with streptavidin–alkaline phosphatase conjugate (Amersham Pharmacia) for 30 min. A reaction product was produced using BCIP/NBT Pre-mixed Solution (Zymed) for 10 min. The histoblots were examined and images captured using a Leica DC100 digital camera mounted on a Leica MZ 12.5 Stereomicroscope.

RESULTS

Distribution of ENS

(a) Electron microscopy. Electron microscope examination of intestinal tissue identified frequently occurring small-diameter nerve fibres within the core of intestinal villi in the lamina propria and immediately subjacent to villous epithelial cells at all examined intestinal sites (Fig. 1). Nerve fibres were also found within the dome overlying Peyer's patch lymphoid nodules and immediately beneath the follicle-associated epithelium. Frequently, nerve fibres were present adjacent to the endothelium of lacteals within the core of villi, in the lamina propria and in the dome.

Small bundles of nerve fibres, as identified by the presence of typical peptidergic granules, synaptic vesicles and regular arrangement of microtubules, were present in the lymphoid nodules of jejunal and ileal Peyer's patches. Nerve terminals and nerve fibres were identified in the dark peripheral and light central zones of the Peyer's patch nodules (Fig. 2). Nerve processes were usually associated with reticular dendritic cell processes or were present adjacent to blood vessels, although some processes ran between lymphocytes and tingible body macrophages. Sparse nerve fibres were present within the capsule of Peyer's patch nodules. Within the capsule, cells, collagen fibres and intercellular ground substance surrounded the nerve fibres.

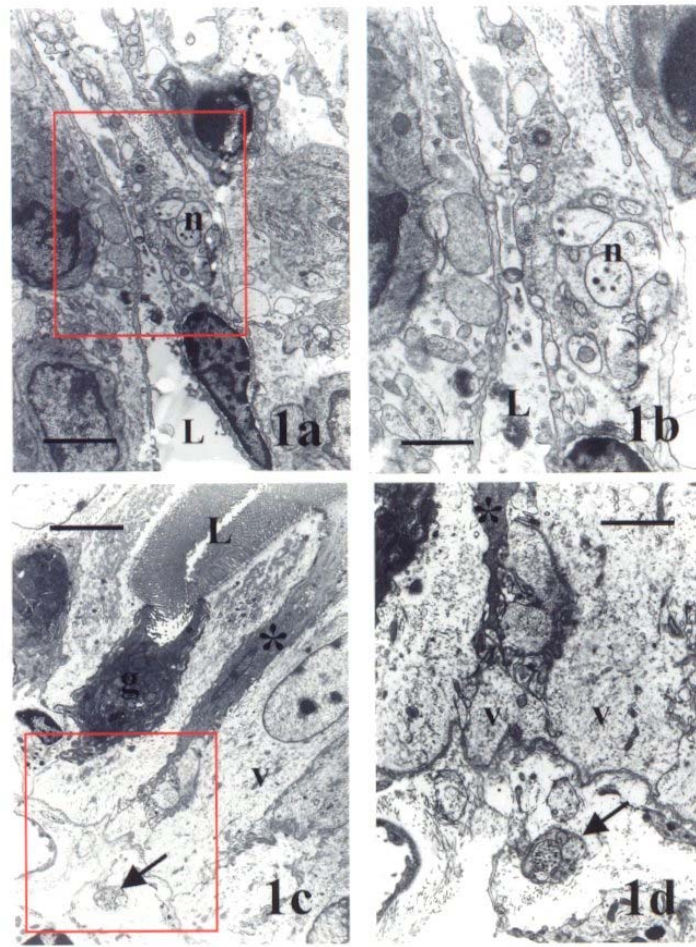


Fig. 1. Electron microscopy of ENS of lamina propria of Suffolk sheep. (a) Lamina propria in the core of a villus. Note proximity of nerve bundles (n) to a lymphatic vessel. L, lumen of lymphatic vessel. Bar, 4.8 μm . (b) Higher magnification of inset in (a). Bar, 2.4 μm . (c) Lamina propria adjacent to epithelium. Nerve bundle (arrow) is in close proximity to an exfoliating villous epithelial cell (*). v, villous epithelial cells; L, gut lumen; g, goblet cell. Bar, 12 μm . (d) Higher magnification of inset in (c). Bar, 6 μm .

(b) Immunohistochemistry. Structures of the intrinsic and extrinsic nervous system of the gut were labelled with antibodies to PgP 9.5 and NSE. In the serosa, bundles of nerve fibres and isolated fibres were present, generally in cross-section. Between the muscle layers of the gut wall, there was strong labelling of the large neurons of the myenteric plexus, with smaller fibres traversing the muscle layers, particularly the inner muscular layer (Fig. 3a). In the submucosa, there was strong labelling of the submucosal plexus (Figs 3a, b and 4a) and longitudinal and transverse sections of nerve fibres were often associated with blood and lymphatic vessels. Numerous short longitudinal and transverse sections of nerve fibres were found between the mucosal crypts and glands (Fig. 3a, b). In the lamina propria, there were prominent thin linear tracts, often showing varicose dilations, which followed the longitudinal axis of the villi and were in close association with the lacteals. These tracts extended to the tip of the villi and appeared to form a network immediately beneath the epithelium (Fig. 3c). Punctate labelling was also present in the lamina propria, presumably corresponding to cross-sections of the linear tracts.

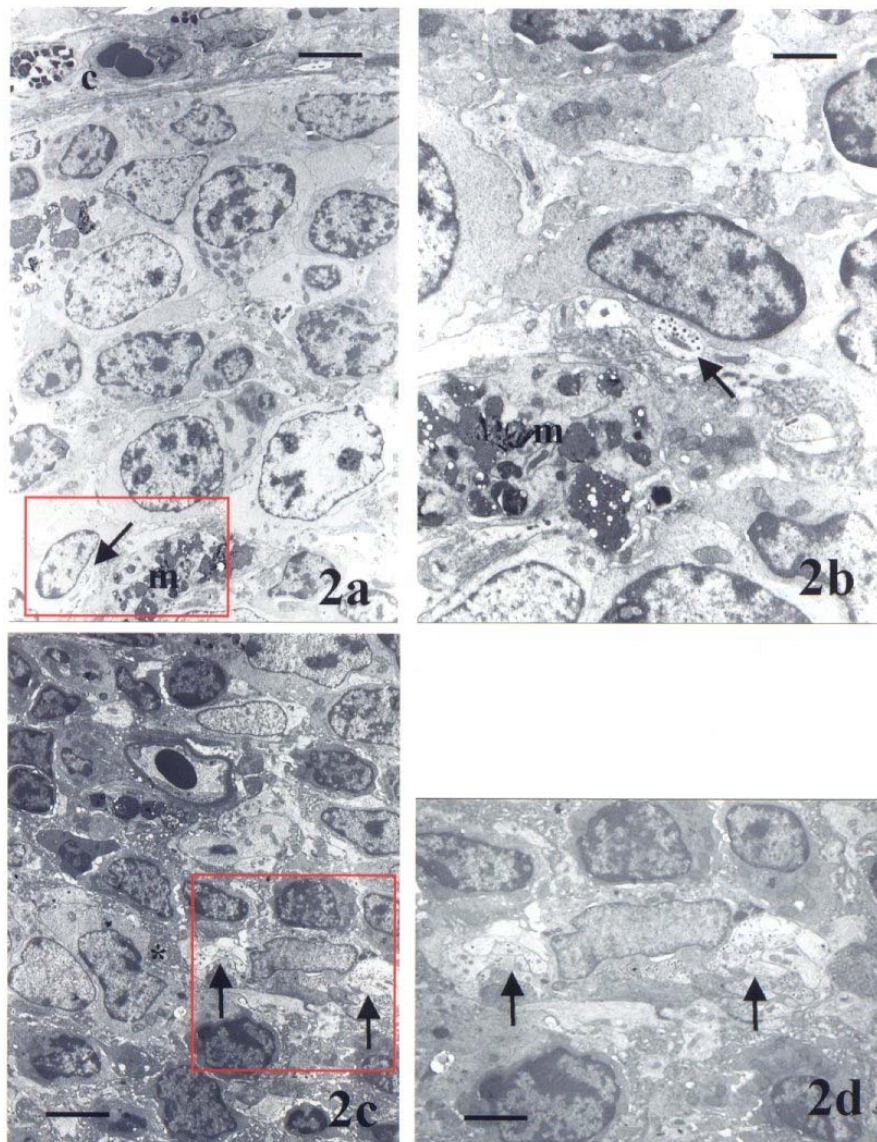


Fig. 2. Electron microscopy of ENS of jejunal Peyer's patch nodules of Suffolk sheep. (a) Dark peripheral zone of a lymphoid nodule showing nerve bundles (arrow) within the nodule and within the surrounding capsule (c). m, tingible body macrophage. Bar, 6.5 μm . (b) Higher magnification of inset in (a) showing a nerve bundle (arrow) in close proximity to a tingible body macrophage (m). Bar, 1.8 μm . (c) Light central zone of a lymphoid nodule showing nerve bundles (arrows) in close proximity to a follicular dendritic cell (*). Bar, 5.5 μm . (d) Higher magnification of (c). Bar, 3 μm .

In the organized lymphoid tissue of the gut, the lymphoid nodules contained short longitudinal nerve-fibre-like or punctate granular (presumably cross-sections of fibres) immunolabelling, which was particularly prominent in the basal (abluminal) part of the nodule (Fig. 3b, d and 4c). In some instances, the fibres could be seen to extend from the capsule of the lymphoid nodule. The number of fibres was low (1–5 per nodule) in most animals but occasionally reached 15–20 fibres per nodule. The proportion of nodules showing innervations varied between animals from 20 to 60 %, with nerves being present in an average of around 40 % of nodules. Longitudinal nerve-fibre-like immunoreactivity was detected in

around 45 % of the capsules that surrounded the lymphoid nodules but was not necessarily concurrent with the presence of immunolabelling within the nodule (Fig. 3e). In the interfollicular T-cell areas, longitudinal and presumably transverse sections of nerve fibres were readily and consistently detected (Fig. 3b); with PgP 9.5, some weak immunolabelling of dendritic-like cells was present in these areas. In the dome areas, short longitudinal nerve fibre-like or punctate granular (presumably cross-sections of fibres) immunolabelling was found in approximately 60 % of domes examined (Fig. 3f). The number of fibre-like structures per dome was low (2–6 per dome) and the fibre-like structures were most often present immediately beneath the follicle-associated epithelium. In addition, a few unidentified cells showed faint cytoplasmic labelling for PgP 9.5.

Clear differences in innervation of the organized lymphoid tissues were not found between different gut areas, but the Peyer's patches consistently showed, with the exception of the T-cell-dependent areas, a much lower degree of innervation than the gut structures not containing organized lymphoid tissue. No difference in nerve distribution or density was observed when comparing the gut tissues from scrapie-affected and unaffected sheep.

The supportive cells of the ENS, which include Schwann cells, satellite cells and enteric glial cells, were labelled with antibodies to GFAP. Immunolabelling for GFAP was predominantly localized to the ganglia of the myenteric and submucosal plexi but was also detected in the meshwork of nerve fibres interconnecting these plexi within the muscle layers of the gut wall and within the submucosa (Fig. 4b). GFAP-positive fibres were also detected within or closely adjacent to the capsule of the lymphoid nodule (Fig. 4d).

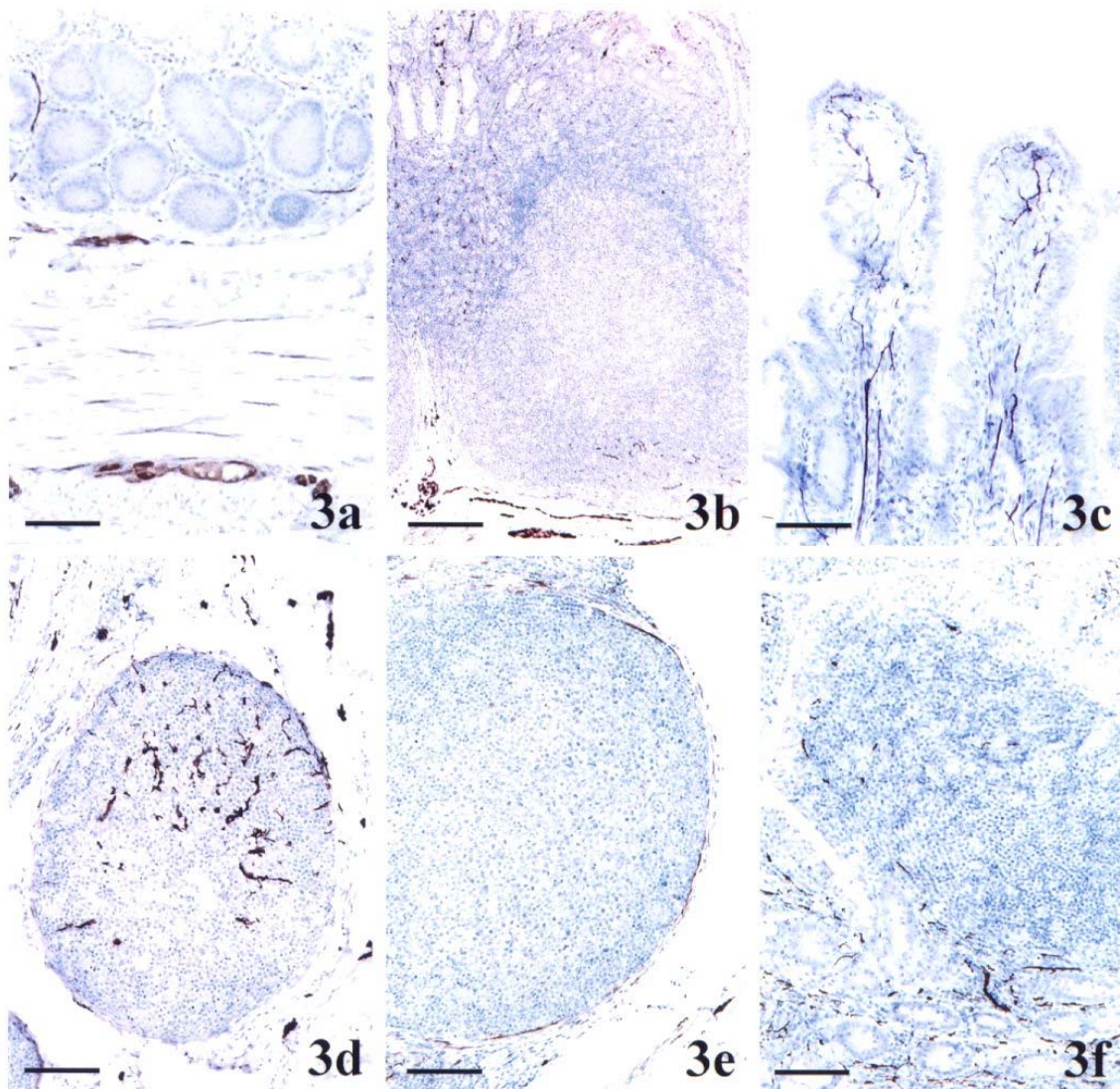


Fig. 3. Immunolabelling for PgP 9.5 in the intestine of scrapie-affected and -unaffected Suffolk sheep. (a) Jejunum of unaffected sheep: strong labelling of ganglion and satellite cells in the myenteric and submucosal plexi; nerve-like fibres in the inner muscular layer and in the lamina propria between the crypts. Bar, 100 μ m. (b) Colon of scrapie-affected sheep: strong reactivity in the submucosal plexus and less so in the T-cell area adjacent to the lymphoid nodule and in the lamina propria between the glands; note also a few nerve-like fibres in the basal part of the nodule. Bar, 200 μ m. (c) Jejunum of unaffected sheep: nerves running along the longitudinal axis of the villi and ending immediately beneath the absorptive epithelium. Bar, 100 μ m. (d) Terminal ileum of scrapie-affected sheep: abundant nerve-like structures in a lymphoid nodule located in the submucosa; note also immunoreactivity in the connective stroma of the vascular arcade. Bar, 100 μ m. (e) Terminal ileum of unaffected sheep: immunolabelling in the capsule of the lymphoid nodule, but not within the nodule. Bar, 100 μ m. (f) Terminal ileum of unaffected sheep: immunolabelled nerve-like fibres underneath the follicle-associated epithelium in the dome and between the crypts of the absorptive epithelium; note lower density of fibres in the dome than in the lamina propria between crypts. Bar, 100 μ m. All samples are counterstained with haematoxylin.

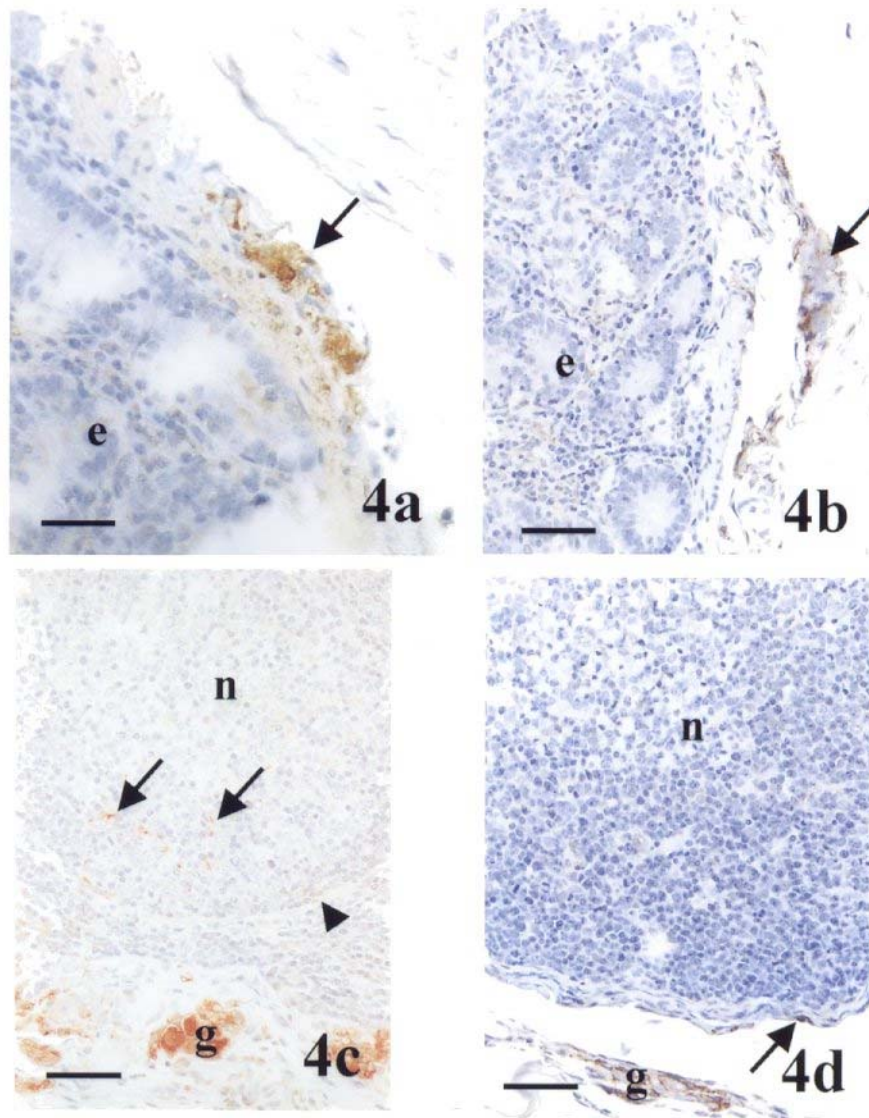


Fig. 4. Immunolabelling for NSE and GFAP in the jejunum of a scrapie-affected Suffolk sheep. (a) Strong labelling for NSE is present in a ganglion (arrow) of the submucosal plexus adjacent to the absorptive epithelium (e). Bar, 50 μm . (b) Labelling for GFAP is present in satellite cells of a ganglion of the submucosal plexus adjacent to the absorptive epithelium (e). Bar, 100 μm . (c) Labelling for NSE is present in ganglia (g) of the submucosal plexus adjacent to a lymphoid nodule (n) of the jejunal Peyer's patch. Note that labelling for NSE (arrows) is present within the lymphoid nodule. Arrowhead indicates capsule. Bar, 100 μm . (d) Labelling for GFAP is present in a ganglion of the submucosal plexus adjacent to a lymphoid nodule (n) of the jejunal Peyer's patch. Note that some labelling is also present in or closely adjacent to the capsule of the lymphoid nodule but not within the nodule. Bar, 100 μm . All samples are counterstained with haematoxylin.

Disease-specific accumulations of PrP within the ENS

Disease-specific patterns of PrP immunolabelling were found in the enteric neurons, both of the submucosal and myenteric plexi, in all scrapie-affected sheep. However, enteric neuron staining was not found in formalin-fixed intestines of the PrP^{ARR/ARR} sheep or of the PrP^{ARR/ARQ} sheep. The accumulation of PrP immunolabelling was therefore presumed to be scrapie specific, as previously suggested (van

Keulen *et al.*, 1996). Disease-specific accumulations of PrP were detected with each of the antibodies tested and in similar sites in each case. Histoblot examination of the ileal Peyer's patches of scrapie-affected sheep demonstrated the presence of proteinase K-resistant PrP (PrP^{RES}) in locations corresponding to the myenteric plexus, which appeared as a continuous, greyish thin line separate from the strong labelling present in lymphoid nodules (Fig. 5).

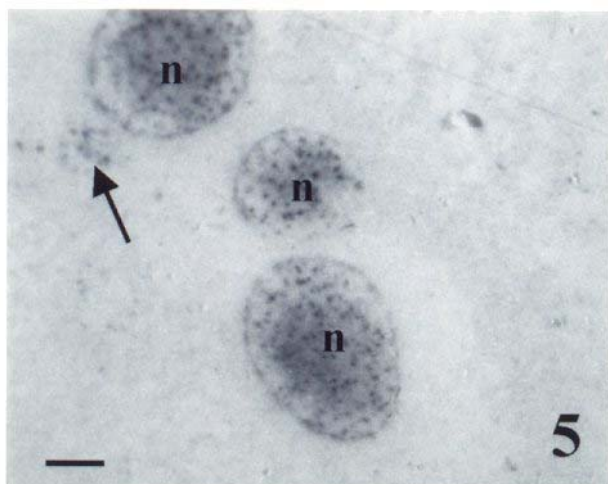


Fig. 5. Histoblot of ileal Peyer's patch of a scrapie-affected Suffolk sheep. Deposits of PrP^{RES} are present in lymphoid nodules (n) and in a location consistent with ganglion of the submucosal plexus (arrow). Bar, 200 μ m.

Intra-neuronal accumulation of PrP and PrP accumulation within satellite cells of both the myenteric and submucosal plexi of the enteric ganglia were found consistently at all sites within the small and large intestine (Fig. 6). The extent of ENS-associated PrP accumulation paralleled that found in lymphoid tissues of the gut wall. Virtually all enteric ganglia had evidence of disease-specific PrP accumulation in intestinal areas where there was abundant disease-specific PrP in adjacent lymphoid tissue of the Peyer's patch. However, only sparse neurons of the abomasum and duodenum showed evidence of PrP^d accumulation. Intra-neuronal disease-specific PrP accumulation was not found in the enteric ganglia of the forestomachs.

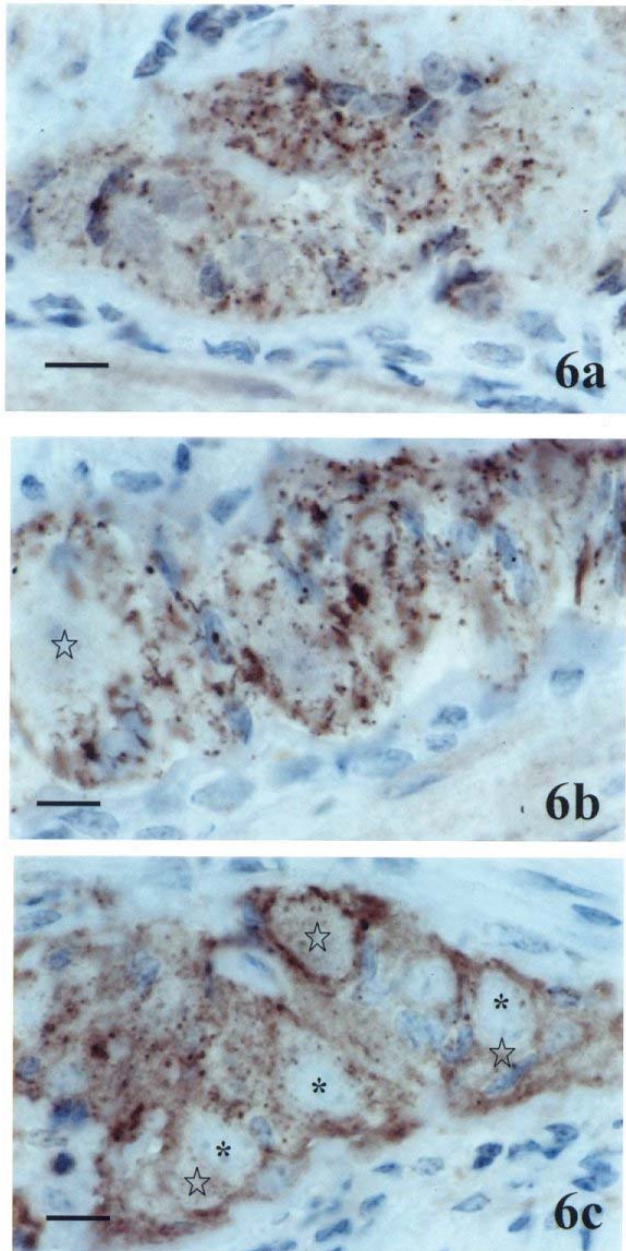


Fig. 6. Immunohistochemistry for PrP^d in ENS of a scrapie-affected Suffolk sheep. Serial sections have been cut from a ganglion in the myenteric plexus and labelled with different anti-PrP antibodies. (a) Rabbit 521 antibody shows intense granular accumulations of PrP^d, most probably in satellite cells and in neuronal perikarya. Bar, 9 μm. (b) Rabbit 486 antibody reveals intense granular labelling, as seen with 521. Neuronal perikarya are only lightly labelled (star) but most satellite nuclei are associated with strong labelling. Bar, 9 μm. (c) Mouse P4 monoclonal antibody shows labelling that is different from antibodies 521 and 486. The labelling is more diffuse and less granular. There is some intraneuronal labelling (perikarya marked by unfilled stars; nuclei marked by asterisks), although the majority of labelling appears to be around neurons rather than within. Bar, 9 μm. All samples are counterstained with haematoxylin.

As patterns of immunolabelling for PrP^d in Peyer's patch nodules following conventional PrP immunocytochemistry did not suggest the accumulation of PrP^d in nerve fibres (Fig. 7a–d), a modified immunohistochemical protocol for the detection of PrP^d, incorporating an enhancement step, was used on tissues from the ileal and jejunal Peyer's patches. The enhancement protocol produced strong staining in the same sites in the lymphoid tissues as described above with the conventional immunohistochemical protocol for the detection of PrP^d (Fig. 7e). There was strong staining in ganglia of myenteric and submucosal plexi and staining was also prominent in nerve fibres in the submucosa in the limited tissue space between lymphoid nodules, particularly in the ileal Peyer's patch. In the dome of lymphoid nodules, PrP appeared to be associated with single cells with a size and morphology consistent with macrophages or dendritic cells. Within the lymphoid nodules, a rim of cells immediately adjacent to the capsule showed the presence of PrP that was not obvious without enhancement but PrP was not detected within the capsule (Fig. 7e).

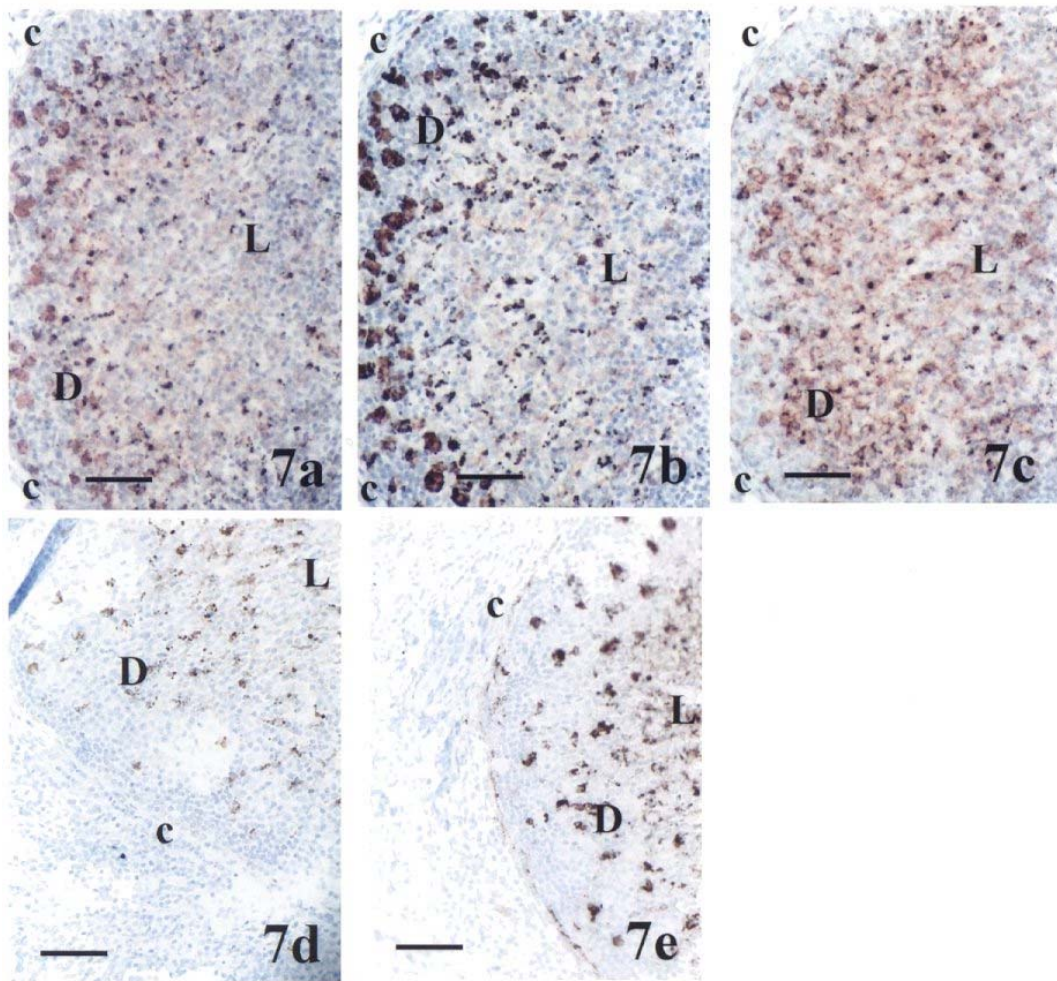


Fig. 7. Immunodetection of PrP in gut-associated lymphoid tissue of scrapie-affected Suffolk sheep. (a–c) Serial sections were cut from the jejunal Peyer's patch and labelled with different anti-PrP antibodies using an immunohistochemical technique. (a) Rabbit 521 antibody shows strong labelling within the lymphoid nodule. D, dark zone; L, light zone; c, capsule. Bar, 35 μ m. (b) Rabbit 486 antibody reveals strong labelling of tingible body macrophages throughout the light (L) and dark (D) zones but the accumulations of PrP^d appear larger and more conspicuous in macrophages of the dark zone. c, capsule. Bar, 35 μ m. (c) Mouse P4 monoclonal antibody shows stronger labelling in the light (L) zone of the lymphoid nodule than the other two antibodies. D, dark zone; c, capsule. Bar, 35 μ m. (d–e) Immunohistochemical labelling of lymphoid nodules in the ileal Peyer's patch. (d) Mouse L42 monoclonal antibody shows labelling in the light (L) and dark (D) zones of a nodule following a conventional immunohistochemical procedure. c, capsule. Bar, 50 μ m. (e) An adjacent section from the ileal Peyer's patch of the same animal as shown in (d) has been labelled with L42 using an immunohistochemical technique incorporating an enhancement procedure. Stronger labelling is present in the same locations in the light (L) and dark (D) zones as seen in (d). Note that labelling of a rim of cells immediately adjacent to the capsule (c) is more apparent than in (d). Bar, 50 μ m. Samples in (a)–(e) are counterstained with haematoxylin.

DISCUSSION

This study shows that in scrapie-affected Suffolk sheep PrP^d is distributed extensively within or in close vicinity to the ENS of the gut. Experimental studies in rodents have provided strong evidence that following oral challenge infection spreads from sites in the gastrointestinal tract via splanchnic and vagus nerves to the spinal cord and brain, respectively (Baldauf *et al.*, 1997; Beekes *et al.*, 1996, 1998; Beekes & McBride, 2000; Race *et al.*, 2000; Glatzel *et al.*, 2001; McBride *et al.*, 2001). Scrapie infectivity has been found in peripheral nerves of a diseased sheep (Groschup *et al.*, 1996) and PrP^d has been detected in the ENS of sheep with natural scrapie (van Keulen *et al.*, 1999). Furthermore, the demonstration in sheep of early depositions of PrP^d in the parasympathetic nucleus of the vagus (Begara-McGorum *et al.*, 2000; van Keulen *et al.*, 2000) and/or in the intermediolateral column of the spinal cord (Jeffrey *et al.*, 2001b; van Keulen *et al.*, 2000) provides strong arguments for the ENS as a site of entry to neural tissues for scrapie and bovine spongiform encephalitis agents and as a pathway for centripetal spread of infection from the gut to the brain after oral uptake. The findings of the present study support the postulated role of ENS in the early pathogenesis of scrapie and focus attention on the contribution of compartments within gut-associated lymphoid tissue to neuroinvasion.

In the present study of sheep in the late subclinical and early clinical phase of scrapie, PrP^d was consistently present in the ENS when there were also abundant deposits of PrP^d in adjacent gut-associated lymphoid tissue. In terminal scrapie in mice (Maignien *et al.*, 1999) and sheep (van Keulen *et al.*, 1999), PrP^d has been detected at sites in the gut extending from the stomach in mice and the rumen in sheep to the colon and rectum. Heggebø *et al.* (2002) examined the distribution of PrP^d in the lymphoid tissues of the present group of sheep and in the gut found that the dominant localization of PrP^d was in the lymphoid nodules of the jejunum, ileum and colon. At other sites such as the abomasum and duodenum, deposits were detected in nodules if these had formed in relation to inflammatory foci. The sparse amounts or absence of PrP^d detected in the ENS at these sites was consistent with the previously reported reduced presence of nodular lymphoid tissue (Heggebø *et al.*, 2002). In a study of natural scrapie in Romanov sheep, Andréoletti *et al.* (2000) also found a reduced presence or an absence of PrP^d (PrP^{Sc}) in the autonomic myenteric nervous plexus at digestive tract sites away from the large lymphoid aggregates of the jejunum and ileum. Drawing support from studies in rodents (Beekes & McBride, 2000), these investigators suggested that the passage of scrapie from lymphoid structures to the nervous system occurred at the level of nerve fibres innervating these lymphoid organs. However, as noted by Heggebø *et al.* (2002), the general perception that mammalian lymphoid nodules are poorly innervated has cast doubt on the likelihood that neuroinvasion of the scrapie agent occurs within the lymphoid nodule.

A major contribution of the present study was the ultrastructural and immunocytochemical demonstration of a network of nerve fibres within the lymphoid nodules and capsule of the ileal and jejunal Peyer's

patches of 20- to 24-month-old Suffolk sheep. This observation indicates that the Peyer's patch nodules provide close contact between nerve fibres and cell populations with abundant PrP^d, including FDCs and tingible body macrophages and may represent an important site for neuroinvasion. A link between PrP^d-rich FDCs and nerve endings has been sought (Bruce *et al.*, 2000; Glatzel & Aguzzi, 2000; Glatzel *et al.*, 2001), but the general observation that mammalian germinal centres are poorly innervated has directed attention to transport of infectivity away from FDCs by dendritic cells and B-cells or the interaction of nerves and FDCs in the mantle zone rather than in the nodule itself. The involvement of noradrenergic neurons in prion neuroinvasion in natural scrapie (Bencsik *et al.*, 2001a, b) has been investigated in splenic lymphoid tissue of sheep. These studies showed a close proximity of noradrenergic endings to PrP-expressing cells or PrP^{Sc}-accumulating cells but the investigators did not show that tyrosine hydroxylase-positive nerves penetrated lymphoid nodules. The present study used the pan-nerve fibre markers, PgP 9.5 and NSE, to map the distribution of the ENS in association with Peyer's patches and identified fibres within lymphoid nodules. Although a marker such as PgP 9.5 has been reported to label non-neuronal cell populations (Hamzeh *et al.*, 2000; Langlois *et al.*, 1994, 1995), the ultrastructural studies confirmed the presence of nerve fibres within Peyer's patch nodules. Indeed, the relatively high frequency of nerve fibres at the ultrastructural level suggested that, in areas such as the dome and lamina propria, the use of some immunocytochemical markers underestimated the presence of ENS. The presence of nerve fibres in these areas may be of relevance for direct neuroinvasion.

Immunocytochemical studies of the innervation of mammalian lymphoid tissue have mostly been done in rodents and have shown that B-cell-dominated germinal centres contain few or no nerve fibres in contrast to the rich network in the surrounding T-cell regions (Felten *et al.*, 1985; Felten & Felten, 1988; Lorton *et al.*, 1991). The present study differs from these earlier neuroanatomical works in many respects, including the species and tissue examined, and the age and disease status of the investigated animals. Few studies have addressed the distribution of nerves and supporting cells in the ENS of Peyer's patches of sheep but studies performed in cattle (Balemba *et al.*, 1999) and pigs (Krammer & Kühnel, 1993) did not report the presence of nerve fibres in nodules, although other studies in cats (Fehér *et al.*, 1992; Ichikawa *et al.*, 1994), pigs (Kulkarni-Narla *et al.*, 1999) and hamsters (Pfoch & Unsicker, 1972) have reported nerve fibres in nodules. A feature of the biology of gut-associated lymphoid tissue in cattle, pigs and sheep is that the large continuous aggregate of lymphoid tissue in the ileum and distal jejunum undergoes involution around the time of sexual maturity (Griebel & Hein, 1996; Reynolds & Morris, 1983). While the studies in cattle and pigs did not consider the influence of involution on innervation, studies in rodents and birds have shown that the innervation of lymphoid organs changes with age (Felten *et al.*, 1987). With involution, tissues such as the thymus (Madden *et al.*, 1998) and bursa of Fabricius (Ciriaco *et al.*, 1995) experience an apparent increase in the density of noradrenergic fibres that may be related to altered immune function. Whether changes occur in the density of nerve fibres in the lymphoid nodules of ageing

sheep needs to be investigated. Furthermore, the disease status of the animals in the present study may have influenced the distribution of the ENS, although nerve fibres were found in lymphoid nodules of Peyer's patches from the gut of both scrapie-affected and -unaffected sheep. While this observation would suggest that the presence of nerve fibres is not a consequence of scrapie infection, the possibility that scrapie infection and the accumulation of PrP^d in lymphoid nodules influences innervation also needs to be studied further. A preliminary study of young sheep (1- to 2-month-old) did not find nerve fibres in gut-associated lymphoid nodules (L. González, unpublished observation).

The patterns of immunolabelling for PrP^d resembling fibre-like structures were not obtained in nodules and have not been reported by others investigating the accumulation of PrP^d in the gut-associated lymphoid tissue of sheep (Andréoletti *et al.*, 2000; Heggebø *et al.*, 2002; van Keulen *et al.*, 1999; Jeffrey *et al.*, 2001a). The use of enhancement protocols in the present study to increase the sensitivity of the immunohistochemical procedure did not result in the identification of PrP^d profiles consistent with nerve fibres. It is proposed that spread of PrP^d along peripheral nerves occurs by established axonal transport mechanisms and that in nerve fibres the scrapie agent is in transit rather than being actively replicated (McBride *et al.*, 2001), which may preclude immunohistochemical detection. Immuno-electron microscopy studies will be important in resolving the role of nervous tissue within gut-associated lymphoid nodules for neuroinvasion in sheep.

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