

Distribution and accumulation of PrP in gut-associated and peripheral lymphoid tissue of scrapie-affected Suffolk sheep

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The distribution of disease-associated prion protein (PrP) was investigated in eight animals (20–24 months of age) from a flock of Suffolk sheep that had experienced frequent cases of natural scrapie over a period of several years. Tissue from the central nervous system (CNS), alimentary tract, peripheral nervous system and lymphoreticular system was examined by histopathology and immunohistochemistry. The lymphoid tissues were subjected further to histoblot and immunofluorescence examination. The four clinically affected PrP^{ARQ/ARQ} sheep had widespread accumulations of disease-associated PrP in the CNS, lymphoreticular system and peripheral ganglia. In the two PrP^{ARQ/ARQ} sheep that did not show clinical signs of scrapie, only limited vacuolation and PrP accumulation were detected in the brain, but the results from the lymphoreticular system and peripheral nervous system were comparable with the clinically affected animals. The remaining PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep did not show proteinase K-resistant PrP accumulations in the lymphoid tissues examined and immunohistochemistry did not reveal the presence of disease-associated PrP. In lymphoid tissues of the PrP^{ARQ/ARQ} sheep, the dominant localization of disease-associated PrP was in lymphoid nodules and double immunofluorescence labelling for PrP and CD21 provided further support for the role of follicular dendritic cells in scrapie in sheep. A striking finding in the present study was the large accumulations of disease-associated PrP in the lymphoid nodules of the alimentary tract at the late sub-clinical and clinical stage of the infection. The study also identified disease-associated PrP in extra-nodular sites of lymphoid tissues, such as the marginal zone of the spleen, and these observations were used to argue that cells of the mononuclear phagocyte system of sheep may be involved in the uptake, transport, elimination and shedding of the scrapie agent.

Introduction

The accumulation of an abnormally folded, partially proteinase-resistant form of the prion protein (PrP) in nervous and lymphoid tissues occurs during the pathogenesis of scrapie, which is a transmissible spongiform encephalopathy (TSE) causing a fatal neurodegenerative disorder in sheep and goats. The localization of the abnormal isoform of PrP in lymphoid nodules of secondary lymphoid tissues, such as lymph nodes and spleen, is a distinctive feature of lymphoreticular involvement in scrapie (van Keulen *et al.*, 1996). Given the close relationship between the TSE agent and the abnormal isoform

of PrP in scrapie and other prion diseases (Bolton *et al.*, 1982; Prusiner, 1982), this PrP is often described as disease-associated PrP (Bruce *et al.*, 2000; Jeffrey *et al.*, 2000).

The early accumulation of disease-associated PrP in lymphoid tissues has been shown to facilitate neuroinvasion (Lasmézas *et al.*, 1996), although it has also been shown that neuroinvasion can occur without the involvement of lymphoid tissues (Kimberlin *et al.*, 1983; Baldauf *et al.*, 1997). In scrapie, clinical disease is restricted to certain susceptible PrP genotypes within the various breeds of sheep (Westaway *et al.*, 1994; Hunter, 1997; Hunter *et al.*, 1997a). However, some susceptible PrP genotypes of sheep, such as PrP^{VRQ}/PrP^{ARR} Texel sheep, do not show this characteristic distribution of disease-associated PrP in lymphoid nodules and the disease appears to occur in these sheep without lymphoreticular involvement

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(van Keulen *et al.*, 2000). Thus, the role of the lymphoreticular involvement in scrapie remains unclear.

Scrapie affects adult sheep of any age but the modal age of occurrence of clinical signs is 42 months and clinical cases are rare in sheep under 1 year of age (Bradley, 1997). The accumulation of disease-associated PrP in lymphoid tissues precedes the appearance of clinical signs by many months and has been detected in sheep as young as 5 months of age exposed to natural scrapie (van Keulen *et al.*, 2000). Heggebo *et al.* (2000) recently reported changes in the distribution of PrP in gut-associated lymphoid tissues of lambs as early as 1 week after experimental oral exposure to scrapie-infected material. Furthermore, Andréoletti *et al.* (2000) showed that the distribution of disease-associated PrP in lymphoid tissues increases as the disease progresses and suggested that infection of lymphoid tissues, particularly gut-associated lymphoid tissues, may lead to shedding of scrapie infectivity to the environment and thus indirectly contribute to the horizontal transmission of scrapie observed under natural field conditions. The closer definition of the involvement of lymphoreticular tissue in the pathogenesis of scrapie is relevant to both the early detection of infected animals and the elimination of possible sources of disease transmission.

Follicular dendritic cells (FDCs) have been implicated as the cells of the lymphoreticular system that sustain replication of the agent in TSEs (Brown *et al.*, 1999; Kitamoto *et al.*, 1991; McBride *et al.*, 1992). High levels of PrP are detected on FDCs in both TSE-infected and uninfected mice (Ritchie *et al.*, 2000) and, although the presence of mature B cells has been shown to influence the course of infection in mice (Frigg *et al.*, 1999; Klein *et al.*, 1997), most studies in mice demonstrate that FDCs are the dominant cell type harbouring PrP in the lymphoreticular system (Brown *et al.*, 1999; Mabbott *et al.*, 2000; Montrasio *et al.*, 2000). However, FDCs are a sessile cell population localized to the primary and secondary nodules of lymphoid tissues. Moreover, germinal centres of secondary nodules are poorly innervated (Felten *et al.*, 1985). Thus, while the accumulation and replication of disease-associated PrP in germinal centres may be specific for TSEs, these accumulations may also be a 'dead-end' for the pathogenesis of the disease. Other cell types or processes must be involved in the lymphoreticular phase of TSEs, not only to bring the TSE agent into contact with germinal centre FDCs but also to transport the replicated agent to other sites in the host and eventually to shed the agent to the environment. Recent studies have suggested the involvement of mobile dendritic cells (Bruce *et al.*, 2000), while other investigators have implicated macrophages in the pathogenesis of TSEs (Andréoletti *et al.*, 2000; Beringue *et al.*, 2000). The distribution of disease-associated PrP in the lymphoid tissues of sheep approaching or within the early stages of clinical disease would be expected to provide an insight into the processes and cell populations involved in the lymphoreticular phase of scrapie. Accordingly, the present study was undertaken to investigate the dis-

tribution of PrP in the lymphoid tissues of Suffolk sheep in the late sub-clinical and early clinical phase of natural scrapie. Immunohistochemical detection of disease-associated PrP was compared with the distribution of proteinase-resistant PrP (PrP^{RES}) in histoblots and the co-localization of PrP and FDCs was evaluated in a sensitive immunofluorescence procedure.

Methods

■ **Sheep and tissue collection.** The pathology, epidemiology and genetics of scrapie infection in this flock have been described previously (Hunter *et al.*, 1997b; Jeffrey *et al.*, 2000). As described in other flocks of Suffolk sheep (O'Rourke *et al.*, 1997; Westaway *et al.*, 1994), clinical disease occurs in PrP^{ARQ/ARQ} homozygotes. Prior to 1997, cases of scrapie in this flock had been confirmed on the basis of histopathological changes in brain tissue.

In the present study, eight sheep were studied (Table 1). Two PrP^{ARQ/ARQ}, one PrP^{PARR/ARQ} and one PrP^{PARR/ARR} sheep were killed at 20 months of age. None of these sheep had any clinical evidence of disease. A further four sheep were killed at 23–24 months of age having had a short period with clinical signs consistent with scrapie.

From each sheep, tissue samples were collected for histological, immunohistochemical and histoblot analysis from the brain, spinal cord, lymphoid tissues and alimentary tract. The following lymphoid tissues were collected into 10% buffered formalin: retropharyngeal, sub-mandibular, superficial cervical, mesenteric and mediastinal lymph nodes; tonsil, spleen and thymus and the third eyelid containing conjunctival lymphoid aggregates were collected from some sheep. From the alimentary tract, the following sites were identified and tissue samples were fixed in 10% neutral buffered formalin or frozen in isopentane chilled in liquid nitrogen and stored at -70°C until use: rumen (two sites), omasal-reticulum junction, abomasums (pylorus, greater and lesser curvature), duodenum, jejunum (several sites containing grossly identified Peyer's patches), ileal Peyer's patch near the insertion of the ileo-caecal fold and colon (adjacent to the ileo-caeco-colic junction and at the descending colon near to the spiral colon).

■ **Immunohistochemistry in paraffin-embedded tissues.** Avidin–biotin complex and peroxidase–anti-peroxidase immunohistochemical staining for disease-associated accumulations of PrP was conducted using modifications of methods published previously (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 Jan Langeveld (IDLO, Lelystad, The Netherlands) (van Keulen *et al.*, 1996) and several other monoclonal and polyclonal anti-PrP sera, including P4 and L42 (Hardt *et al.*, 2000), R145, R476, R482 and R486 (kindly provided by R. Jackman; VLA Weybridge, Surrey, UK), 505, 532 and 523.7/524 (van Keulen *et al.*, 1996), 1A8 (Langeveld *et al.*, 1993), 1B3 (Farquhar *et al.*, 1989; Garssen *et al.*, 2000), R24 (Caughy *et al.*, 1991) and 1B4, BG4 and FH11 (unpublished). The control sections included the use of either pre-bleed antiserum (1A8 and 1B3) or anti-isotype control sera as the primary antibody.

■ **Histoblot.** For histoblots, frozen sections (9 μm in thickness) were mounted on nitrocellulose (0.45 μm pore size; Sigma) and treated in a standard manner (Taraboulos *et al.*, 1992), with some minor modifications.

Briefly, the tissues were subjected to proteolysis with 400 $\mu\text{g}/\text{ml}$ proteinase K (Serva Electrophoresis) at 55°C for 4 h. After rinsing in TBS, the membrane was incubated for 20 min with 3 mM PMSF followed by denaturation for 10 min in 3 M guanidine SCN (Sigma). To block non-specific binding, 5% non-fat milk was added to the membrane for 1 h. The

Table 1. Histopathological and immunochemical examination of sub-clinically and clinically scrapie-affected Suffolk sheep

Genotype	Age (months)	Clinical disease	CNS		Lymph node PrP (no. positive/no. tested)
			Vacuolation	PrP	
ARQ/ARQ	24	Yes	+++	+++	9/9
ARQ/ARQ	24	Yes	+++	+++	9/9
ARQ/ARQ	23	Yes	+++	+++	9/9
ARQ/ARQ	23	Yes	+++	+++	9/9
ARQ/ARQ	20	No	+	+	9/9
ARQ/ARQ	20	No	+	++	9/9
ARQ/ARR	20	No	-	-	0/9
ARR/ARR	20	No	-	-	0/9

membrane was then incubated with the anti-PrP monoclonal antibody L42 overnight at 4 °C. After rinsing in TBS, 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.

Immunofluorescence in frozen tissues. Sections from frozen tissues were cut 7 µm in thickness and fixed in 10% formol-calcium for 20 min. An indirect double immunofluorescence technique was used to detect the co-localization of an anti-PrP antibody (L42; isotype IgG₁) and an anti-CD21 antibody (Du2-74-25; isotype IgG_{2b}). A Coumarine kit (NEN Life Science Products) was used to enhance the detection of PrP. Previous studies have shown that enhancement procedures increase the sensitivity of PrP detection in frozen sections from lymphoid tissues in sheep (Heggebo *et al.*, 2000). Following fixation, the blocking reagent from the Coumarine kit was added to the sections for 15 min. The two primary antibodies were added simultaneously to the sections and the sections were incubated overnight at 4 °C. Coumarine- and FITC-labelled isotype-specific secondary antibodies were used to detect the anti-PrP and anti-CD21 antibodies, respectively. The sections were coverslipped with polyvinyl alcohol and examined with a Leica DMRXA microscope equipped for fluorescence (Leica Microsystems). Dark field fluorescence digital images were collected with a SPOT RT Slider digital camera (Diagnostic Instruments) using FITC and Coumarine filters. The control sections included the omission of one or both primary antibodies and/or one or both secondary antibodies. These control sections showed that non-specific staining was present in the crypt epithelium and lamina propria of the alimentary tissue sections and in connective tissue trabeculae in lymph nodes and spleen tissue sections.

Results

Histopathology and immunohistochemistry

Central nervous system (CNS). The results of histopathological and immunohistochemical examination of the CNS of the eight sheep in the present study are shown in Table 1. The four PrP^{ARQ/ARQ} animals with clinical signs that were consistent

with scrapie showed vacuolation and disease-associated PrP accumulation in the CNS (data not shown). In the two PrP^{ARQ/ARQ} sheep that did not show clinical signs, only limited vacuolation and PrP accumulation were detected in the brain. Moderate or sparse vacuolation was present in the dorsal vagal nucleus of both sheep. In one of these sheep, there was widespread accumulation of PrP in the brainstem and in the thoracic spinal cord. In the other sheep, disease-associated PrP was confined to the dorsal vagal nucleus in the brainstem and to the intermediolateral columns of the thoracic spinal cord. Accordingly, the two PrP^{ARQ/ARQ} sheep that did not show clinical signs were assumed to be in the late sub-clinical phase of the disease (Table 1). No histopathological evidence of vacuolation was present in the tissue sections from the brain of the PrP^{ARR/ARR} or PrP^{ARR/ARQ} sheep and brain tissue from these two animals contained no disease-associated accumulations of PrP.

Lymphoid tissue. Histopathological lesions were not detected in any of the lymphoid tissues examined. As described previously for lymphoid tissues from clinical scrapie in Swifter and Texel sheep (van Keulen *et al.*, 1996), the clinical cases of scrapie in PrP^{ARQ/ARQ} Suffolk sheep showed generalized PrP accumulation in most germinal centres of all lymph nodes examined (Table 1). PrP accumulations were also present in the two animals assumed to be in the late sub-clinical phase of scrapie. In the immunohistochemical examination of lymphoid tissues, disease-associated accumulations of PrP were labelled with a varying intensity of each of the anti-PrP sera. Secondary nodules of lymph nodes showed a diffuse extracellular pattern of staining within the light zone of the germinal centres, corresponding to the localization of FDCs (Jeffrey *et al.*, 2000). A more intense and dense immunostaining was seen throughout the germinal centres, including the dark zone and in the mantle. This pattern corresponded to intracytoplasmic staining of tingible body macrophages (Jeffrey *et al.*, 2000). To a

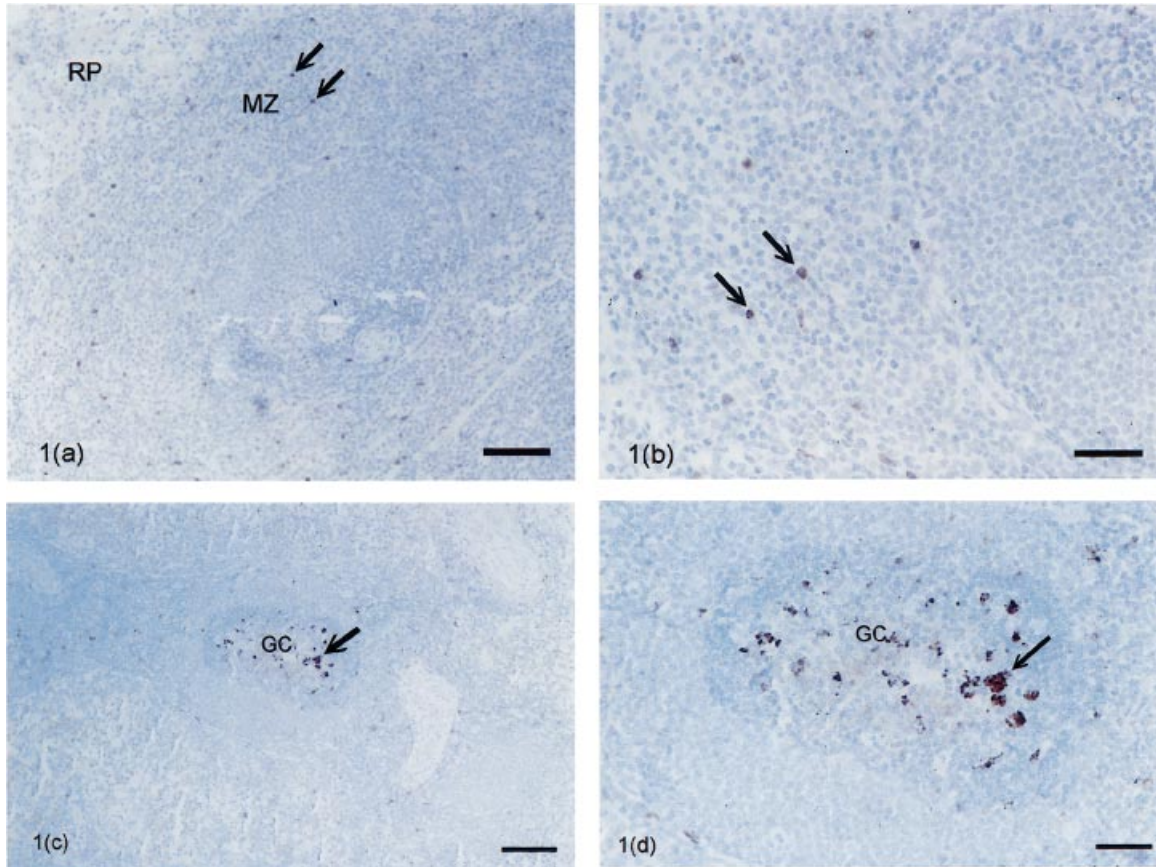


Fig. 1. Immunohistochemistry of the spleen. (a) Staining for PrP is present in positive cells (arrows) in the marginal zone (MZ) in an ARR/ARR sheep. RP, red pulp. Bar, 40 μ m. (b) Higher magnification of (a) rotated 90 degrees. Bar, 12 μ m. (c) Staining is also present in the germinal centre (GC) of an ARQ/ARQ sheep; note the large accumulation of granular-type staining in some cells (arrow). Bar, 65 μ m. (d) Higher magnification of (c). Bar, 26 μ m. Stained with anti-PrP sera P4.

variable extent, all the anti-PrP sera used in the present study gave weak or moderate staining of sinusoidal macrophages of mesenteric lymph nodes and cells of the paracortex, including the mesenteric lymph nodes in both the PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep. In the spleens of the clinically and subclinically affected Suffolk sheep, there was marked immunolabelling of tingible body macrophages in germinal centres and some nodules showed immunolabelling of FDCs in the light zone (Fig. 1). Most of the anti-PrP sera gave virtually no labelling in the red pulp but in the marginal zone and in periarteriolar lymphocyte sheath of the white pulp: the cytoplasm of a variable number of mononuclear cells showed immunolabelling for PrP (Fig. 1). These antibodies also produced virtually no staining in the red pulp of the PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep. However, in all sheep, including the PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep, the P4 antibody produced diffuse immunolabelling of the red pulp. In the PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep, discrete intense immunolabelling of a sub-population of mononuclear cells within the marginal zone was present. This pattern of marginal zone labelling was also present in the clinically and sub-

clinically affected sheep. As with the other antibodies, the P4 antibody also produced immunolabelling of mononuclear cells in the periarteriolar lymphocyte sheaths and in germinal centres where there was prominent labelling of FDCs and tingible body macrophages. Weak staining of some connective tissue, including the capsule of secondary nodules, was also observed with the P4 and R486 antibodies.

Gut-associated lymphoid tissue. In the clinically and subclinically affected sheep, there were disease-associated accumulations of PrP in all nodules of Peyer's patches in the jejunum and ileum and in the lymphoid aggregates of the colon. Nodules in the tonsils of these animals also showed disease-associated accumulations of PrP. In the Peyer's patches and colonic aggregates of all sheep, including the PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep, immunostaining was detected in a small proportion of mononuclear cells of the dome, submucosal internodular areas and lamina propria with some of the antibodies used (Fig. 2). The extent of this staining was most prominent with the R486 antibody. Other antibodies, such as R482, which is also a polyclonal antibody recognizing

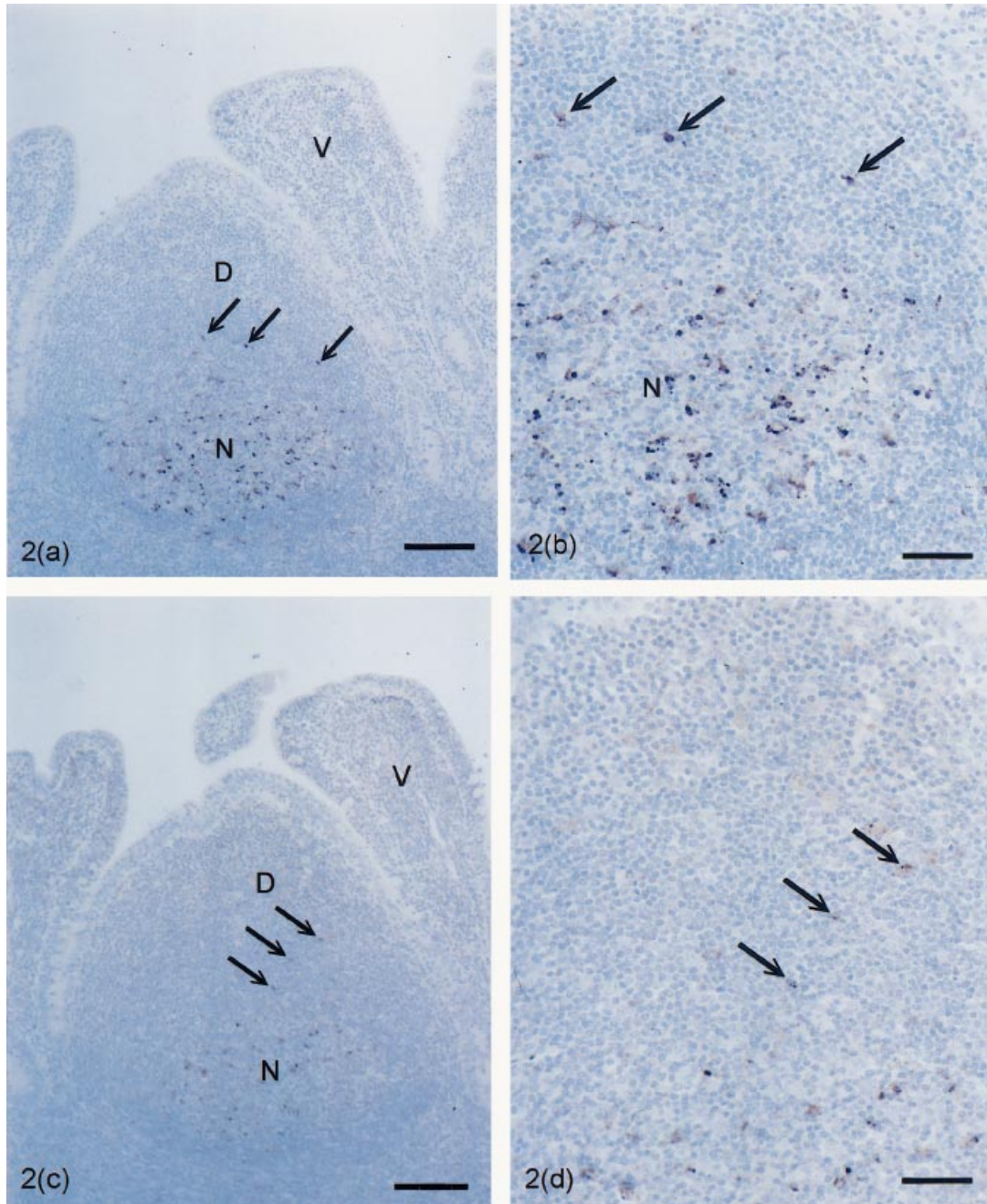


Fig. 2. Immunohistochemistry of the gut-associated lymphoid tissue. (a) Peyer's patch from an ARQ/ARQ sheep; note the presence of staining in the nodule (N) and also in cells (arrows) of the dome (D). Section has been stained using anti-PrP antibody R145. V, villi. Bar, 55 μ m. (b) Higher magnification of (a). Bar, 35 μ m. (c) Same area as seen in (a) stained with anti-PrP sera P4. Bar, 55 μ m. (d) Higher magnification of (c). Bar, 35 μ m.

the same peptide sequence as R486, gave much less immunostaining of cells in the lamina propria. There were no disease-associated accumulations of PrP in the germinal centres of gut-associated lymphoid tissues of the PrP^{ARR/ARR} or PrP^{ARR/ARQ} sheep.

In the abomasum of some clinically affected sheep, several foci of inflammation were present in the mucosa, which closely

resembled those induced by parasites in grazing sheep. These foci consisted of mainly lymphocytic and macrophage infiltrates, which sometimes formed nodules within the mucosa. Where nodules were present, immunohistochemical staining for PrP was also found (Fig. 2). Similar accumulations of PrP were detected in acquired inflammatory foci containing lymphoid nodules in the duodenum and large intestine.

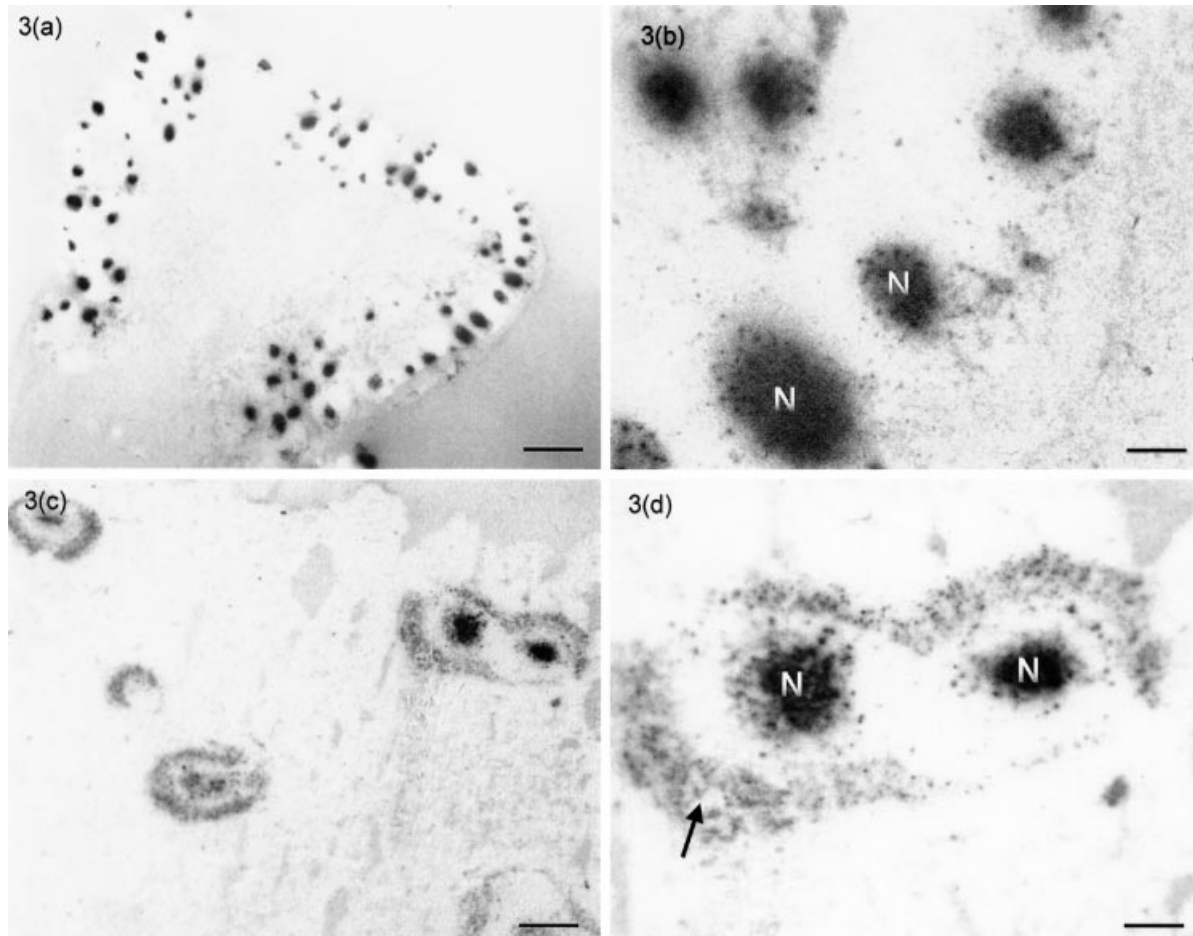


Fig. 3. Histoblot of the lymph node and spleen. Histoblot analysis of PrP^{RES} accumulation in lymphoid tissue from an ARQ/ARQ sheep exposed to the scrapie agent. (a) Retropharyngeal lymph node. Bar, 1600 μ m. (b) Higher magnification of the same lymph node in (a) showing dense accumulation of staining in nodules (N). Bar, 200 μ m. (c) Histoblot analysis of PrP^{RES} in the spleen. Bar, 500 μ m. (d) Higher magnification of (c) showing two nodules with intense staining (N). A region of less intense staining (arrow), corresponding to the marginal zone, is clearly present. Bar, 180 μ m.

Histoblots

In tissues from clinically and sub-clinically affected sheep, the histoblot findings were relatively consistent in all six sheep. Proteinase K-resistant PrP (PrP^{RES}) was detected in all of the organs examined, including ileal and jejunal Peyer's patches, lymphoid aggregates adjacent to the ileo-caeco-colic junction in colon, spleen, distal jejunal lymph node, superficial cervical lymph node and retropharyngeal lymph node.

The distribution of PrP^{RES} was similar in the three lymph nodes examined. In the six PrP^{ARQ/ARQ} sheep, staining was most prominent in the nodules in the lymph node cortex, although scattered foci of staining were present in the paracortex (Fig. 3). Staining for PrP^{RES} was also prominent in the nodules of the spleen. An area of weaker staining that was distinct from the nodular staining was detected in the spleen (Fig. 3). This area corresponded to the region of the marginal zone.

In the gut-associated lymphoid tissues of the six PrP^{ARQ/ARQ} sheep, staining for PrP^{RES} was detected in lymphoid nodules. The pattern of staining for PrP^{RES} in lymphoid nodules differed between the three sites examined in the alimentary tract. In the persisting lymphoid nodules of the ileal Peyer's patch, PrP^{RES} was localized mainly to the light central zone and neck region of the nodules and the dark peripheral zone tended to show little or no staining (Fig. 4). In the nodules of the jejunal Peyer's patch, staining for PrP^{RES} tended to be distributed through the whole nodule, while the lymphoid nodules of the colon showed a distinct central belt of staining (Fig. 4). At all three alimentary sites, nodules showed scattered foci of strong staining, presumably corresponding to tingibile body macrophages, and there were some scattered foci of staining in the internodular areas (Fig. 4). Staining for PrP^{RES} was also detected in the capsule of lymphoid nodules at the three sites, which was not evident in the nodules of lymph nodes or the spleen (Figs 3 and 4).

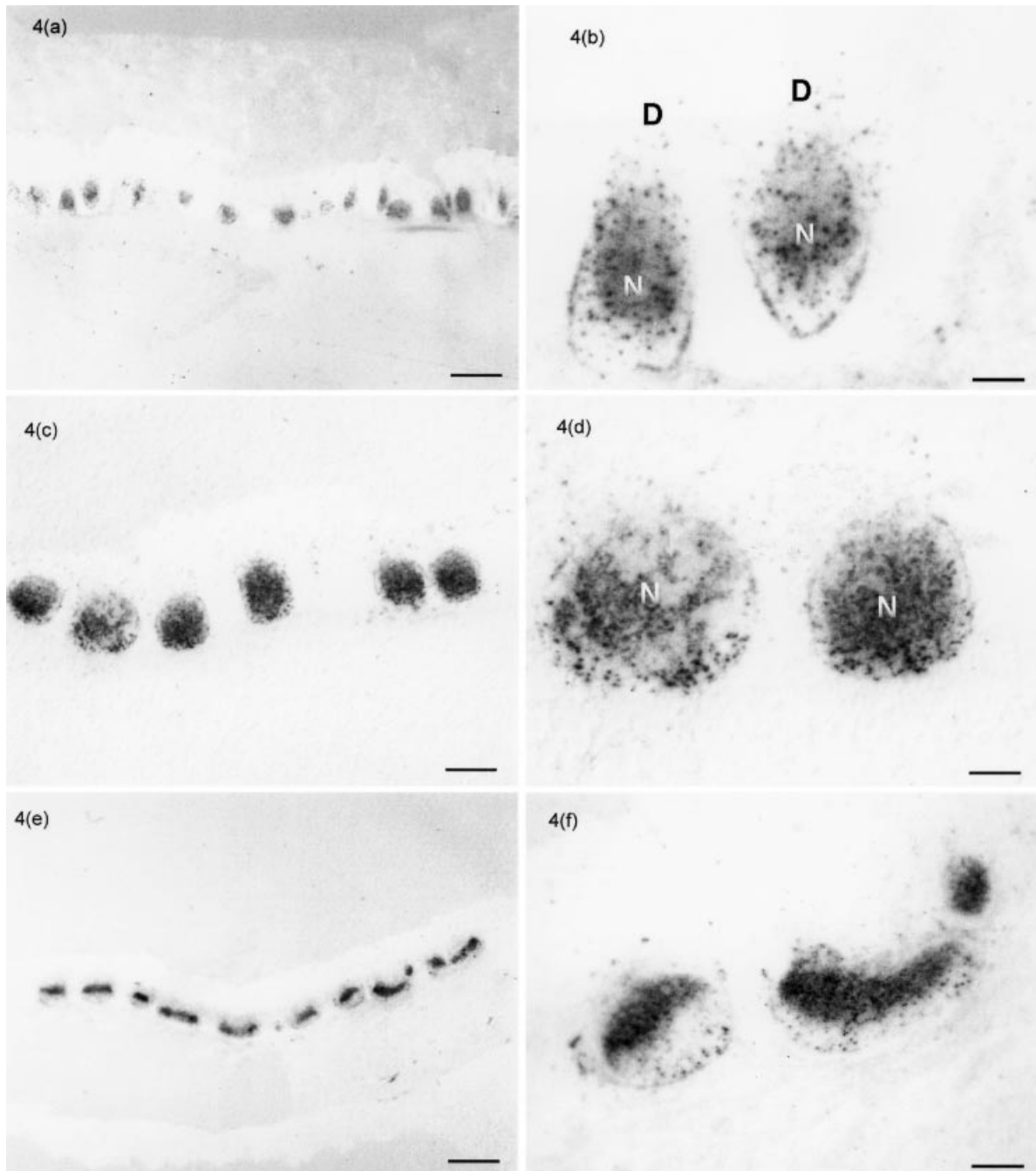


Fig. 4. Histoblot of the gut-associated lymphoid tissue (GALT). Histoblots were prepared from GALT from sheep with the genotype ARQ/ARQ. (a) Ileal Peyer's patch. Bar, 1000 μm . (b) A higher magnification showing two of the nodules from (a); note the staining present in a region corresponding to the dome (D) and nodular staining (N). Bar, 200 μm . (c) Jejunal Peyer's patch. Bar, 500 μm . (d) Higher magnification of (c) showing nodular staining (N). Bar, 150 μm . (e) Lymphoid aggregates in the colon. Bar, 1200 μm . (f) A higher magnification showing two nodules from (e). Bar, 200 μm .

In the tissues examined from the PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep, no PrP^{RES} was detected.

Immunofluorescence

A double immunofluorescence technique was used on frozen tissue to examine the co-localization of PrP and CD21-

positive cells in distal jejunal, superficial cervical and retropharyngeal lymph nodes, spleen and jejunal and ileal Peyer's patch. The distribution of staining for CD21 was similar in all eight sheep. In sheep, the main cellular reactivity of CD21 (complement receptor 2) is FDCs and a sub-population of B cells (Hein *et al.*, 1998; Young *et al.*, 1997). The immunofluores-

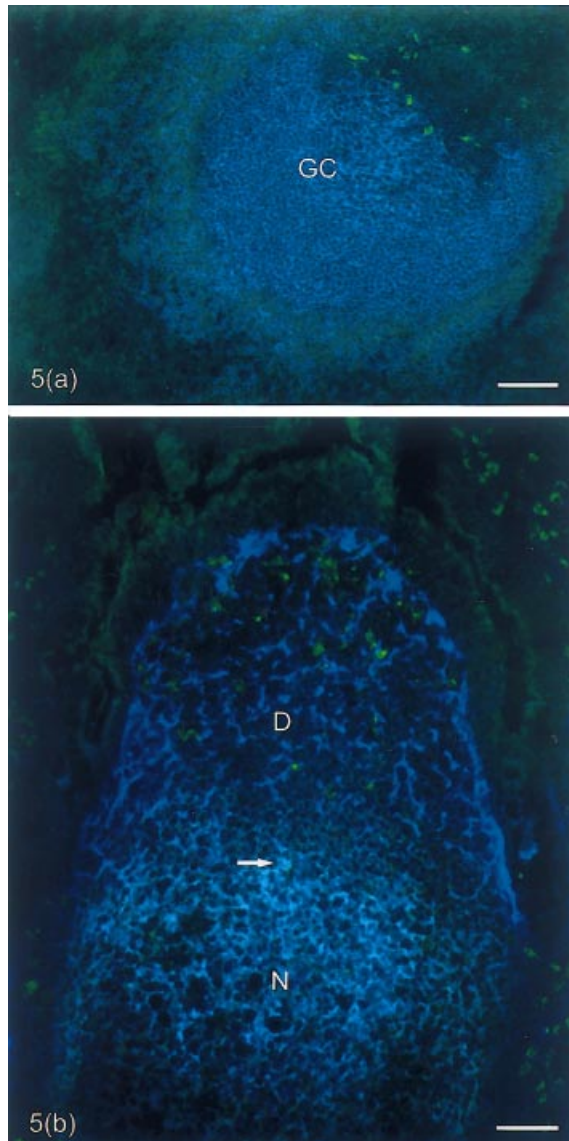


Fig. 5. Immunofluorescent staining of the spleen and ileal Peyer's patch. Frozen sections showing immunolabelling for CD21 and PrP from an ARQ/ARQ sheep. Double-stained cells appear turquoise, while single-stained CD21-positive cells are green and PrP-positive cells appear blue. (a) Spleen section showing a germinal centre (GC). Bar, 38 μ m. (b) Ileal Peyer's patch section of a lymphoid nodule (N) and dome (D); note the double positive cells (arrow). Bar, 19 μ m.

cence technique used to detect PrP did not distinguish between PrP^C and the disease-associated isoform of the protein. The level of staining for PrP that was detected in the two PrP^{ARR/ARR} and PrP^{ARR/ARQ} sheep was less in comparison with the level detected in the six PrP^{ARQ/ARQ} sheep (Fig. 5).

The pattern of double staining detected in the lymph nodes and spleen of the PrP^{ARQ/ARQ} sheep was consistent in the six animals. Double staining for PrP and CD21 was prominent in the germinal centres of lymph node and splenic nodules. Scattered foci of double staining were also present in the

paracortex of lymph nodes and periarteriolar lymphocyte sheaths of the spleen (Fig. 5). In the jejunal and ileal Peyer's patches and the lymphoid aggregates of the colon of the PrP^{ARQ/ARQ} sheep, double staining for PrP and CD21 was prominent in the light central zone of the nodules but only single staining for PrP or CD21 was present in the dome.

Discussion

The present study investigated the distribution of disease-associated accumulations of PrP in the lymphoid tissues of Suffolk sheep in the late sub-clinical and early clinical phase of natural scrapie and showed that disease-associated PrP was prominent throughout the lymphoid tissues examined in these sheep. The widespread presence of disease-associated PrP in the lymphoid tissues of both sub-clinically and clinically affected animals emphasizes the importance of identifying affected animals as early as possible in the course of the disease. A striking finding in the present study was the large accumulations of disease-associated PrP in the lymphoid follicles of the alimentary tract. Andréoletti *et al.* (2000) commented that the progressive accumulation of disease-associated PrP in the gut-associated lymphoid tissues may lead to shedding of scrapie infectivity and contribute to transmission of the disease. However, the localization of disease-associated PrP in lymphoid nodules in the gut raises the question of the movement of the scrapie agent to the gut lumen and the more general issue of the transport of the scrapie agent throughout the lymphoid tissues of the body during the lymphoreticular involvement in the disease.

The present study showed a clear association between disease-associated PrP and lymphoid nodules. The pattern of distribution of disease-associated PrP in germinal centres and the co-localization of immunofluorescence staining for PrP and CD21 provide further documentation that FDCs are a significant site of accumulation of PrP in sheep. Previous studies have described the accumulation of PrP in lymphoid tissues of sheep (Andréoletti *et al.*, 2000; van Keulen *et al.*, 1996, 1999) and ultrastructural studies in scrapie-affected mice (Jeffrey *et al.*, 2000) have reported the accumulation of disease-associated PrP in association with FDCs in secondary follicles but similar ultrastructural studies remain to be performed in sheep. Comprehensive studies in mice have shown that PrP-expressing FDCs are needed for disease (Brown *et al.*, 1999). However, the involvement of FDCs in the pathogenesis of TSEs is not without its complications. FDCs are a sessile cell population and are located in structures that are poorly innervated. Thus, other processes or cell populations must be involved in transporting the agent into the nodules and, presumably, away from the nodules. It should be noted that the origin of FDCs is still a matter of controversy. While it is widely accepted that FDCs are autochthonous cells originating from follicular mesenchymal cells (Rademakers, 1991) or reticular stromal cells (Dijkstra *et al.*, 1984), evidence has been

presented that FDCs derive from bone marrow-produced precursors that migrate to secondary lymphoid organs (Kapasi *et al.*, 1998; Pasparakis *et al.*, 2000). However, the involvement of any mobile antigen-bearing cell population, including dendritic cells (Bruce *et al.*, 2000), in the transport of disease-associated PrP to lymphoid nodules remains to be documented.

Tingible body macrophages also showed a clear association with disease-associated PrP in lymphoid nodules of PrP^{ARQ/ARQ} sheep. Tingible body macrophages are present throughout the lymphoid nodule and ingest apoptotic B cells and are thought to scavenge the ends of FDC processes (Ritchie *et al.*, 2000; Szakal *et al.*, 1988). Jeffrey *et al.* (2000) found accumulations of PrP within lysosomes of tingible body macrophages and concluded that tingible body macrophages ingest excess or abnormal PrP from entire degenerate FDCs or their processes or by scavenging in the extracellular space. The other major cell population present in lymphoid nodules is the B cell. In the present study, a clear association of disease-associated PrP with B cells was not demonstrated. While B cells leave lymphoid nodules in large numbers and some studies have shown that the presence of B cells affects the course of disease (Klein *et al.*, 1997), the weight of evidence now suggests that the effect of B cells on the pathogenesis of TSE is through their influence on FDC maturation (Bruce *et al.*, 2000).

The present study used three methods and a broad range of anti-PrP antibodies to detect the presence of PrP in the PrP^{ARQ/ARQ} sheep. While all three methods showed that the large majority of PrP was localized to lymphoid nodules in the tissues examined, the methods also showed consistently that PrP was to be detected away from nodular structures. Immunohistochemical staining for PrP was present in mononuclear cells in extra-nodular tissues, including the paracortex of lymph nodes and the dome of Peyer's patches. The histoblot examination also detected scattered foci of PrP^{RES} away from the dense accumulations, which characterized lymphoid nodules. While some of this staining may represent nerve tissue, particularly in the gut, the involvement of leukocyte populations should be considered. The double immunofluorescence study found little or no double staining for PrP and CD21 outside the region of the germinal centres. In sheep, CD21 (complement receptor 2) is an integral membrane protein expressed on FDCs as well as on a sub-population of B cells (Hein *et al.*, 1998). In further research, it would be useful to use other markers to examine leukocyte populations, including macrophage and dendritic cell lineage cells in extra-nodular tissue, for the presence of PrP.

In the spleens of the clinically and sub-clinically affected PrP^{ARQ/ARQ} sheep, immunolabelling for PrP was detected in the cells of the marginal zone and periarteriolar lymphocyte sheath. This immunolabelling was present with such different anti-PrP antibodies as the monoclonal antibodies P4 and R145 and the polyclonal antibody R486, suggesting that the staining was not an artefact. The involvement of the marginal zone in

the lymphoreticular phase of scrapie was implicated further by histoblot examination of these animals, which showed a distinct presence of PrP^{RES} away from the dense staining of nodules (Fig. 3). The marginal zone lies between the white and red pulp of the spleen and is usually described as a layer surrounding the periarteriolar lymphocyte sheaths and B cell nodules. It is an area that contains distinctive lymphoid and non-lymphoid cell populations and is a site of the initial filtration and phagocytosis of antigens from blood and of leukocyte emigration (Kraal, 1992). It is tempting to speculate that the disease-associated PrP in the marginal zone and possibly also in the periarteriolar lymphocyte sheath and other extra-nodular sites represents the on-going uptake, transport and elimination of disease-associated PrP by the mononuclear phagocyte system of the sheep. Studies in macrophage-depleted mice (Beringue *et al.*, 2000) have shown that macrophages influence the course of disease and *in vitro* studies show that peritoneal macrophages are associated with scrapie infectivity, which decreases with time after exposure to the scrapie agent (Carp & Callahan, 1982).

In natural scrapie, Suffolk sheep with PrP^{ARQ/ARQ} genotype are susceptible to infection and sheep with PrP^{ARR/ARR} or PrP^{ARR/ARQ} genotypes are largely resistant to scrapie, although occasional rare cases have occurred in the PrP^{ARR/ARQ} genotype (Hunter *et al.*, 1997b; O'Rourke *et al.*, 1997; Westaway *et al.*, 1994). The two PrP^{ARR/ARR} or PrP^{ARR/ARQ} sheep in the present study did not show disease-associated accumulations of PrP in lymphoid nodules. However, tissues sections from these animals did show some immunohistochemical staining. Staining of paracortical and sinusoidal macrophages and some staining of dome cells were detected to a variable degree with all of the antibodies used in these control sheep. However, this staining was present to a greater extent in scrapie-affected sheep. The significance of this staining is presently uncertain. It may represent a non-specific binding feature of all antibodies to macrophages (which may perhaps be increased during infection) or may represent a specific up-regulation of PrP^C bearing cells in disease, as suggested possibly by the higher levels of immunofluorescence staining for PrP detected in the PrP^{ARQ/ARQ} sheep in the present study. PrP^{RES} was not detected in the PrP^{ARR/ARR} or PrP^{ARR/ARQ} sheep, which would argue for the immunohistochemical detection of PrP^C in these sheep.

In conclusion, this study shows that the presence of disease-associated PrP is similar in late sub-clinical and clinically affected PrP^{ARQ/ARQ} Suffolk sheep. The dominant localization of PrP was in the lymphoid nodules but the consistent presence of PrP in extra-nodular sites, such as the dome of Peyer's patches, paracortex of lymph nodes and the marginal zone of the spleen, suggests the involvement of the mononuclear phagocyte system in the lymphoreticular phase of scrapie in sheep. The participation of extra-nodular cell populations in the uptake, transport and elimination of disease-associated PrP may provide the 'missing link' that allows the dense

accumulations of disease-associated PrP in lymphoid nodules to facilitate neuroinvasion and to promote shedding of the scrapie agent to the environment.

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