Pathogenesis of bovine spongiform encephalopathy in sheep

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Abstract The pathogenesis of bovine spongiform encephalopathy (BSE) in sheep was studied by immunohistochemical detection of scrapie-associated prion protein (PrPSc) in the gastrointestinal, lymphoid and neural tissues following oral inoculation with BSE brain homogenate. First accumulation of PrPSc was detected after 6 months in the tonsil and the ileal Peyer’s patches. At 9 months postinfection, PrPSc accumulation involved all gut-associated lymphoid tissues and lymph nodes as well as the spleen. At this time point, PrPSc accumulation in the peripheral neural tissues was first seen in the enteric nervous system of the caudal jejunum and ileum and in the coeliac-mesenteric ganglion. In the central nervous system, PrPSc was first detected in the dorsal motor nucleus of the nervus Vagus in the medulla oblongata and in the intermediolateral column in the spinal cord segments T7–L1. At subsequent time points, PrPSc was seen to spread within the lymphoid system to also involve all non-gut-associated lymphoid tissues. In the enteric nervous system, further spread of PrPSc involved the neural plexi along the entire gastrointestinal tract and in the CNS the complete neuraxis. These findings indicate a spread of the BSE agent in sheep from the enteric nervous system through parasympathetic and sympathetic nerves to the medulla oblongata and the spinal cord.

Introduction

Since the beginning of the epidemic of bovine spongiform encephalopathy (BSE) in cattle in the UK in 1985, there have been concerns about the possible spread of BSE to other animal species including humans. These concerns were later shown to be (at least partially) justified as BSE has spread to wild and domestic cats and humans through the consumption of meat or byproducts derived from BSE-infected cattle [3, 4, 17]. In addition, BSE has spread to wild herbivores and domestic goats, probably through the ingestion of feed containing BSE-infected meat and bone meal [3, 7].

There have been no reports of naturally occurring BSE so far in sheep, although sheep are experimentally susceptible to BSE [8]. Naturally occurring BSE in sheep could present a great danger to both humans and sheep. Meat and byproducts from BSE-infected sheep would present a greater danger to humans than products derived from BSE-infected cattle because, in sheep, BSE infectivity is widespread through the whole lymphoid system (and thereby the whole sheep carcass) [2, 9], in contrast to cattle, where BSE infectivity is primarily limited to the CNS [6, 9, 10]. Furthermore, BSE could theoretically become endemic in the sheep population as has been demonstrated in experimentally infected sheep flocks, where BSE was seen to spread from ewe to lamb either in utero or at birth [1]. This would mean that BSE in sheep would be as difficult to eradicate as natural scrapie, where all attempts at eradication so far have had limited effects. Breeding programmes to select for sheep with PrP genotypes associated with relative resistance to scrapie (and BSE) have decreased the incidence of scrapie in affected flocks, but it is still uncertain if these programmes will eventually lead to the eradication of scrapie. The latter has
been questioned by the appearance of atypical scrapie and the successful intracerebral transmission of BSE to sheep previously thought to be resistant to transmissible spongiform encephalopathies (TSEs) [5, 13].

Recently, the EU has stepped up the surveillance of TSEs in small ruminants. Both slaughtered sheep or goats and fallen stock are sampled for testing by one of the EU-accredited TSE tests for small ruminants. In the case of a positive result, further tests are done to discriminate BSE from scrapie-infected animals. The small ruminant TSE tests are all based on the detection of the scrapie-associated prion protein (PrP Sc) in the obex of an infected sheep or goat. The selection of the obex as sampling site is largely based on our knowledge of the pathogenesis of scrapie in sheep [20]. Whether the obex is as important in the pathogenesis of BSE in sheep is unknown. Previous studies by Bellworthy and others using both immunohistochemistry and bio-assay in mice, have shown PrP Sc and infectivity to be widespread in viscera, lymphoid and nervous tissues but did not clarify the pathogenesis of BSE in sheep [2].

In the present study, we have followed up on our previous studies of natural scrapie in sheep. We have used a similar approach combining a sequential time point study with extensive histopathological and immunohistochemical analysis of selected tissues to study the pathogenesis of BSE in sheep. Although the immunohistochemical detection of scrapie-associated prion protein or PrP Sc may not be as sensitive as the detection of BSE infectivity through mouse bio-assay, it does offer the great advantage of identifying the cell types and neuro-anatomical pathway(s) involved in the pathogenesis of BSE.

Materials and methods

Inoculation of sheep with BSE brain homogenate

The experimental infection of sheep with BSE brain homogenate has been described previously (Table 1) [19]. In short, scrapie-free Texel or Texel cross ewes were bred to produce offspring of the ARQ/ARQ and ARR/ARR genotype (genotypes are expressed as the single-letter amino acid code present at codons 136, 154 and 171 of the PrP gene). The PrP genotype of the lambs was checked by full-length DNA sequencing of the open reading frame of the PrP gene and expressed as the single-letter amino acid code present at codon 136, 154 and 171. The BSE inoculum was administered as 25 ml of a 20% BSE brain homogenate in the back of the mouth. The infectivity of the inoculum, as determined by titration in RIII mice, was $10^{3.97}$ log10 mouse intracerebral/intraperitoneal LD50/g of tissue [2]. Sheep with clinical signs of BSE, consisting of weight loss, pruritus and tremor/ataxia.

Table 1 PrP genotypes of the sheep and the intervals at which they were euthanised after oral dosing with BSE brain homogenate

| Sheep number | PrP genotype | Oral dosing with 5 g of BSE brain | Killed at (months postinfection) |
|--------------|--------------|---------------------------------|---------------------------------|
| 1            | ARQ/ARQ      | +                               | 6                               |
| 2            | ARQ/ARQ      | +                               | 9                               |
| 3            | ARQ/ARQ      | +                               | 12                              |
| 4            | ARQ/ARQ      | +                               | 13                              |
| 5            | ARQ/ARQ      | +                               | 15                              |
| 6            | ARQ/ARQ      | +                               | 17                              |
| 7            | ARQ/ARQ      | +                               | 19                              |
| 8            | ARQ/ARQ      | +                               | 20                              |
| 9            | ARQ/ARQ      | +                               | 21                              |
| 10           | ARQ/ARQ      | +                               | 23                              |
| 11           | ARQ/ARQ      | +                               | 24                              |
| 12           | ARQ/ARQ      | –                               | 72                              |
| 13           | ARR/ARR      | +                               | 72                              |

a PrP genotype was determined by full-length DNA sequencing of the open reading frame of the PrP gene and expressed as the single-letter amino acid code present at codons 136, 154 and 171.

b The BSE inoculum was administered as 25 ml of a 20% BSE brain homogenate in the back of the mouth. The infectivity of the inoculum, as determined by titration in RIII mice, was $10^{3.97}$ log10 mouse intracerebral/intraperitoneal LD50/g of tissue [2].

c Sheep with clinical signs of BSE, consisting of weight loss, pruritus and tremor/ataxia.

The BSE inoculum was kindly provided by Dr. Martin Jeffrey and was also used in previous BSE experiments in sheep in the UK. The infectivity of the inoculum, as determined by titration in RIII mice, was $10^{3.97}$ log10 mouse intracerebral/intraperitoneal LD50/g of tissue [2, 15]. The sheep were housed in Biocontainment Level 3 facilities to constrain any BSE infectivity spreading through the faeces and to avoid any risk of cross-contamination with scrapie. One ARQ/ARQ sheep was killed at 6, 9, 12, 13, 15, 17 and 19 months postinfection (mpi). Four sheep were left to develop clinical disease and were killed for welfare reasons once signs of ataxia were noticed. As negative controls, one ARR/ARR lamb was orally infected with the same dose of the BSE brain homogenate and one ARQ/ARQ sheep was left uninoculated and housed separately from the inoculated sheep.

Tissue sampling

Sheep were killed by intravenous injection of sodium pentobarbital followed by exsanguination. Tissues were sampled in duplicate (brain divided sagitally) with the emphasis on the gastro-intestinal tract, the lymphoid tissues including all gut-associated lymphoid tissues (GALT), the brain, the spinal cord (C1–L6) and the autonomic ganglia.
One set of samples was fixed in phosphate-buffered formalin (pH 7) for 24 h, trimmed and fixed for another 24 h before routine dehydration and processing into paraffin. The other set of samples was stored at −70°C for future biochemical analysis/bio-assay.

PrPSc immunohistochemistry

Tissue sections (4 μm) were deparaffinized in xylene and decreasing gradients of ethanol while the endogenous peroxidase activity was abolished with hydrogen peroxide in methanol. Pretreatment of tissue sections consisted of immersion for 30 min in formic acid followed by autoclaving for 5 min in citrate solution (pH 6). Immunostaining for the presence of PrPSc was performed with the monoclonal 2G11 (Institut Pourquier, Montpellier, France), which was raised against a synthetic peptide based on the ovine PrP sequence 146–182. After extensive rinsing, sections were incubated with Mouse EnVision™ HRP (DakoCytomation, Glostrup, Denmark), followed by incubation with diaminobenzidine containing 0.1 M imidazole. Finally, sections were counterstained with haematoxylin, dehydrated and permanently mounted. When PrPSc was detected in a tissue for the first time, the entire tissue from the animal of the previous kill was screened for the presence of PrPSc. For this purpose, the tissue block was completely trimmed up into serial sections of 5 μm, and multiple sections at 50-μm intervals were immunostained for PrPSc.

Double immunohistochemistry

Double immunostaining was performed on lymphoid tissues to identify PrPSc in cells of the macrophage/monocyte lineage. PrPSc immunostaining was performed as described above with the exception that Powervision AP (Immunovision technologies, Brisbane, CA, USA) was used as secondary antibody and 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT DakoCytomation, Glostrup, Denmark) were used as substrate to develop an insoluble blue reaction product. After a second autoclaving in citrate solution (pH 6) to destroy mouse immunoglobulins from the first immunostaining, sections were incubated with mouse anti-human CD68 (Clone Ki-M6, Serotec, Oxford, England), which recognizes an intracytoplasmic lysosomal-associated epitope expressed by monocytes and macrophages. After rinsing, sections were incubated with Mouse EnVision™ HRP (DakoCytomation, Glostrup, Denmark) and NovaRED™ (Vector Laboratories, Burlingame, CA, USA) to develop an insoluble red reaction product.

Results

One ARQ/ARQ sheep was sacrificed at each of the following time points after oral BSE inoculation: 6, 9, 12, 13, 15, 17 and 19 mpi. Four sheep were left to develop clinical disease consisting of pruritus, weight loss, head tremor and ataxia and were killed for welfare reasons once signs of ataxia were noticed. Incubation times to terminal disease in these four clinical BSE-infected sheep were 20, 21,23 and 24 mpi. Figure 1 schematically represents the progression of the BSE infection in the ARQ/ARQ sheep as followed by the immunohistochemical detection of PrPSc.

At 6 mpi (Fig. 1a), granules of PrPSc were detected in single tingible body macrophages (TBMs) in the gut-associated lymphoid tissues (GALT) of the palatine tonsil (Fig. 2a) and the ileum (Fig. 2b). In the Peyer’s patches of the ileum, PrPSc accumulation was very sparse and only detected after thoroughly screening the entire tissue by going through the entire paraffin block and examining multiple sections every 50 μm. The same approach was applied to other GALT tissues, the gastro-intestinal tissues, the brain stem and the thoracic spinal cord, but no further PrPSc accumulations were found.

At 9 mpi (Fig. 1b), PrPSc was readily detectable in a number of GALT and GALT-draining tissues including the tonsil, retropharyngeal lymph node, Peyer’s patches (jejenum, ileum and caecum), mesenteric lymph nodes and ileo-caecal lymph node, and also in the spleen. The immunostaining was consistent with the previously described accumulation of PrPSc at the follicular dendritic cells (FDCs) and in TBMs (Fig. 3a). In addition, PrPSc was found in the coeliac-mesenteric ganglion and in the submucosal and myenteric plexus of the enteric nervous system (ENS) at the level of the caudal jejenum and ileum (Fig. 3b). In the CNS, PrPSc accumulation was present in the intermediolateral column (IMLC) of the spinal cord at the level T7–L1 (Fig. 3c) and in the brain stem in the ventral part of the dorsal motor nucleus of the nervus Vagus (DMNV) at the level of the obex (Fig. 3d).

At 12–13 mpi (Fig. 1c), all lymphoid tissues including the non-GALT tissues were positive for PrPSc (Fig. 4a). In addition to the staining of FDCs and TBMs, single cells within the cortical and subcortical sinuses of the lymph nodes and in the marginal zone of the spleen were positive for PrPSc. Double immunostaining for CD68 showed co-localization of PrPSc in CD68-positive cells, indicating the mononuclear phagocytic nature of these cells (Fig. 4b). In the ENS, PrPSc accumulation was present from the level of the mid-jejenum to the colon in the 12 months kill and in the caudal jejenum and ileum in the 13 months kill. PrPSc in the IMLC of the spinal cord had spread from segments T2 to L4 and from segments T4 to T13, respectively (Fig. 4c). In the DMNV, PrPSc was present throughout the
entire length of the nucleus, with additional positive staining in the area postrema in the sheep killed at 12 mpi (Fig. 4d).

At 15 mpi (Fig. 1d), PrP<sup>Sc</sup> in the ENS involved the forestomachs at the level of the omasum and abomasum to the colon. All segments of the spinal cord and most of the

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spinal ganglia were positive for PrPSc. In the brain, PrPSc accumulation was seen throughout the medulla oblongata and was sparsely seen in some neurons in the pons.

At 17 mpi (Fig. 1e), PrPSc was detected in the ENS from the omasum to the colon. In the CNS, PrPSc was seen in the entire brain stem and in the hypothalamic and thalamic area. All spinal ganglia from the cervical, thoracic and lumbar spinal cord segments were positive for PrPSc at 17 mpi. Prominent vacuolation was present both in the neuropil and within neurons of the DMNV at the obex.

From 19 mpi and in the four clinically affected sheep (Fig. 1f), PrPSc had spread to involve the entire ENS from
oesophagus to rectum. PrP<sup>Sc</sup> was detected at all levels in the CNS including cerebellum, basal ganglia and cerebral cortex and in the spinal cord.

The BSE-challenged ARR/ARR sheep and the negative control ARQ/ARQ sheep showed no clinical signs of BSE during the course of the experiment and were euthanised at 72 mpi (Table 1). No PrP<sup>Sc</sup> was detected by immunohistochemistry or Western blotting in the tissues collected from these sheep.

**Discussion**

In this study, we investigated the pathogenesis of a BSE infection in sheep by following the accumulation of PrP<sup>Sc</sup> in sheep killed at various time points during the incubation period of BSE. The first tissues to accumulate PrP<sup>Sc</sup> were the lymphoid tissues of the GALT, followed by the GALT-draining lymph nodes and the spleen and, at a later stage, the non-GALT lymph nodes. The temporal spread and pattern of PrP<sup>Sc</sup> deposition in the lymphoid tissues were similar to those described previously for natural scrapie [21, 22]. In the spleen of the BSE-infected sheep, however, a more abundant accumulation of PrP<sup>Sc</sup> was seen in macrophages of the marginal zone. This probably reflects an active trapping and phagocytosis of PrP<sup>Sc</sup> during a systemic circulation of the BSE agent. This is in agreement with the studies of Houston et al., who demonstrated the presence of infectivity in the blood of orally BSE-infected sheep, both in the preclinical and clinical phase, by the transmission of BSE to TSE-free recipients through whole-blood transfusion [12, 14]. The question remains which fraction of the blood contains BSE infectivity and thus whether the BSE agent is present in blood plasma or whether it is associated with a cellular component of the blood. Preliminary results from transfusion with blood fractions have shown infectivity in the buffy coat but could not exclude infectivity in plasma and/or red cell fractions [14].

The first peripheral nervous tissues to accumulate PrP<sup>Sc</sup> in the pathogenesis of BSE in sheep were the post-ganglionic parasympathetic motor neurons in the submucosal and myenteric plexi of the ENS and the post-ganglionic sympathetic motor neurons in the coeliac-mesenteric ganglion. The first accumulations of PrP<sup>Sc</sup> in the CNS were detected in the pre-ganglionic parasympathetic motor neurons of the DMNV in the brain stem and in the pre-ganglionic sympathetic motor neurons of the IMLC in the spinal cord. From these initial sites of PrP<sup>Sc</sup> accumulation in the peripheral and central nervous system, PrP<sup>Sc</sup> subsequently spread to involve the entire ENS and CNS. This pathogenetic route of the BSE agent from the ENS through the efferent motor fibers of the autonomic nervous system to the CNS is similar to the pathogenesis of natural scrapie in sheep [20]. In scrapie- and BSE-infected sheep, the preceding infection of the Peyer’s patches could possibly play a role in facilitating the infection of the post-ganglionic motor neurons of the ENS. In cattle orally exposed to BSE, infectivity has also been found in the Peyer’s patches of the ileum [23, 24]. Preliminary observations on the pathogenesis of BSE in cattle indicate a similar spread of the BSE agent from the gut through the efferent autonomic nervous system to the CNS [11]. However, the role of the ENS in the pathogenesis of BSE in cattle is still obscure as PrP<sup>Sc</sup> was not found in the ENS during the early stages of a BSE infection and only sparsely in the myenteric plexus of cattle in the end stage of BSE [18].

The results of the present study can be compared with previous time course studies on BSE in sheep in which the same dose and the same BSE brain homogenate were used for oral inoculation of ARQ/ARQ Romney sheep. In these studies, infectivity was detected by bio-assay in RIII mice.
in the Peyer’s patches at 4 mpi [2]. In contrast, at this time point, PrPSc was detected immunohistochemically only in the retropharyngeal lymph node and not in the Peyer’s patches [2, 15]. In our study of ARQ/ARQ Texel sheep, we detected sparse deposits of PrPSc at 6 mpi in both the tonsil and Peyer’s patches that were only detected after thorough
screening of the entire tissue by examining sections every 50 µm until the blocks were exhausted. This might explain the difference with the immunohistochemical studies in the Romney sheep where PrPSc was only detected in the retropharyngeal lymph node, but it is in general agreement with the bio-assy, where infectivity was found in the Peyer’s patches at 4 mpi (but not in the tonsil). In the Texel sheep killed at 9 mpi, all GALT tissues, the spleen, ENS, IMLC and DMNV were positive for PrPSc, whereas in the Romney sheep at 10 mpi, PrPSc was only detected in the retropharyngeal lymph node, and infectivity was only detected in the spleen. This indicates a more rapid spread of the BSE infection in the Texel sheep compared to the Romney sheep, since both the dose and the inoculum were the same in the two studies. Because the age at inoculation and the PrP genotypes of the animals in both studies were also the same, the differences in the temporal spread of the BSE agent between the two breeds must be related to other genetic factors outside the PrP region, or to other unknown breed factors. In the Romney sheep, the BSE agent only became widespread at 16 mpi when PrPSc and infectivity were detected in a wide range of nervous tissues, lymphoreticular tissues and viscera including the liver. PrPSc in the liver was reported to be present within Kupffer cells at several stages of the BSE infection (M. Jeffrey, personal communication). In the Texel sheep however, no PrPSc was detected in Kupffer cells or hepatocytes at any stage during the BSE infection.

At present, active surveillance of TSE in sheep and goats has been increased in the EU (EU regulation 214/2005) because of the finding of a BSE-infected goat in France [7] and possibly in the UK (confirmation by strain typing is still pending) [16]. Several rapid tests for TSEs in small ruminants are now available (EU regulation 260/2005), all of which use the medulla oblongata, and in particular, the obex region as source material for testing. In the present study, we detected PrPSc in the DMNV at the obex of BSE-infected sheep from 9 mpi. In a previous study of natural scrapie in sheep, PrPSc was detected in the DMNV in sheep from 10 months of age [20]. Therefore, preclinically TSE-infected animals are likely to be identified through rapid testing as long as the DMNV region in the obex is excised and used for rapid testing. Further biochemical and/or immunohistochemical tests can then be used to differentiate BSE from scrapie infection, while final confirmation of the TSE strain will still require bio-assay in mice.

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