Experimental transmission to a calf of an isolate of Spanish classical scrapie

Rosa Bolea,1*† Carlos Hedman,1† Óscar López-Pérez,1 Belén Marín,1 Enric Vidal,2 Martí Pumarola,2 Fabien Corbière,3 Antonio Romero,4 Bernardino Moreno,1 Inmaculada Martín-Burriel,1 Olivier Andréoletti3 and Juan José Badiola1

Abstract

Multiple theories exist regarding the origin of bovine spongiform encephalopathy (BSE). An early and prominent theory proposed that BSE was the result of the adaptation of sheep scrapie to cattle. The reports to date indicate that the distribution of the pathological prion protein (PrPSc) in experimental bovine scrapie is largely restricted to the central nervous system (CNS). Here, we describe pathological findings in a calf intracerebrally inoculated with a Spanish classical scrapie isolate. While clinical disease was observed 30 months after inoculation and PrPSc was detected in the CNS, the corresponding phenotype differed from that of BSE. Immunohistochemistry and PMCA also revealed the presence of PrPSc in the peripheral nerves, lymphoid tissues, skeletal muscle and gastrointestinal tract, suggesting centrifugal spread of the scrapie agent from the brain. To the best of our knowledge, this is the first report describing the detection of PrPSc in tissues other than the CNS after experimental transmission of scrapie to cattle.

From a public health perspective, bovine spongiform encephalopathy (BSE) is the most important transmissible spongiform encephalopathy (TSE). BSE and variant Creutzfeldt–Jakob disease (vCJD) in humans share the same causative agent [1, 2]. Several theories have been proposed regarding the origin of BSE. For example, epidemiological data suggest that BSE originated following the exposure of cattle to a scrapie-like agent in ruminant-derived feed [3]. Studies of the adaptation of scrapie to cattle have shown that oral inoculation of cattle with scrapie does not result in disease transmission [4, 5]. While cattle are susceptible to different classical scrapie isolates when inoculated intracerebrally [6, 7], in all cases the corresponding clinical presentation and neuropathological features [5, 6, 8, 9] can be distinguished from those of BSE, even after a second passage [8, 10]. Moreover, neither the molecular signature nor strain typing by mouse bioassay indicate any evidence of classical [9] or atypical [11] BSE.

Depending on the affected species, the strain of the TSE agent, the prion protein genotype of the recipient and other factors, pathological prion protein (PrPSc) can be distributed in organs outside of the central nervous system (CNS). PrPSc has been detected in the lymphoreticular system (LRS), enteric nervous system (ENS), skeletal muscle, adrenal gland, pancreas and other organs in sheep naturally infected with classical scrapie [12–15]. In naturally and experimentally BSE infected cattle, PrPSc is primarily distributed in the brain, spinal cord, retina and distal ileum [16–20]. Moreover, ultrasensitive techniques such as protein misfolding cyclic amplification (PMCA) [21] have enabled the detection of minuscule amounts of PrPSc in mesenteric lymph nodes and other organs from cattle experimentally infected with the BSE agent [22]. Although PrPSc has been detected in the CNS of cattle intracerebrally inoculated with the classical scrapie agent, a single study in which the mesenteric lymph node and spleen were examined by

*Correspondence: Rosa Bolea, rbolea@unizar.es

Keywords: ovine; scrapie; bovine; BSE; peripheral tissues; prion.

Abbreviations: BG, basal ganglia; BSE, bovine spongiform encephalopathy; CNS, central nervous system; Cbl, cerebellum; CSC, cervical spinal cord; ENS, enteric nervous system; FC, frontal cortex; GIT, gastrointestinal tract; Ht, hypothalamus; LSC, lumbar spinal cord; LRS, lymphoreticular system; MD, medulla oblongata; p.i., post-inoculation; OC, occipital cortex; OD, optical density; PrPSc, pathological prion protein; PNS, peripheral nervous system; PrPSc, protease-resistant prion protein; PMCA, protein misfolding cyclic amplification; TPC, temporal/parietal cortex; T, thalamus; TSC, thoracic spinal cord; TBM, tingible body macrophages; TSE, transmissible spongiform encephalopathy; vCJD, variant of Creutzfeldt–Jakob disease; WB, Westerm blot; GBC, basal ganglia cortex; O, obex.

†These authors contributed equally to this work.
immunohistochemistry (IHC) found no evidence of peripheral distribution of PrP\textsuperscript{Sc} [6].

Here, we describe the clinical signs, neuropathological features and molecular signature of a heifer experimentally infected with a classical scrapie isolate derived from a field-infected sheep of Spanish origin. The objective of this study was to determine whether a single isolate from a natural scrapie-infected sheep (ARQ/ARQ) could induce BSE-like disease in cattle. Our findings describe for the first time the distribution of PrP\textsuperscript{Sc} in the peripheral nerves, lymphoid tissues, skeletal muscle and gastrointestinal tract of a cow infected with classical scrapie.

A 10-month-old Pyrenean breed calf was used. The inoculum consisted of 1 ml of a 10% solution of homogenized oesophagus from a sheep naturally infected with scrapie (ARQ/ARQ) and diagnosed by the Reference Laboratory for TSE products as part of the active TSE surveillance programme of Aragón, Spain.

The calf was placed under general anaesthesia. The trephine was positioned paramedially in the mid-frontal region of the head, at an angle of 90° with respect to the rostro-caudal slope of the front of the skull. The inoculum was injected into the frontal cortex via a 9-cm-long needle (gauge, 22).

The animal was monitored daily by animal husbandry staff. Clinical assessments were carried out once per week until the development of clinical signs, and once daily thereafter. The animal was euthanized by exsanguination after intravenous injection of sodium pentobarbital. A systematic post-mortem examination was conducted and a range of samples were collected from the CNS, peripheral nervous system (PNS), LRS, gastrointestinal tract (GIT), skeletal muscle (extraorbital) and other tissues. The samples were collected in duplicate: one was fixed in a solution of 10% formaldehyde in saline and the other one was frozen at −80 °C for subsequent molecular analyses.

Formalin-fixed tissues were trimmed and processed according to standard histopathological procedures. A 4-μm-thick tissue section from each sample was stained with haematoxylin and eosin. Adjacent sections were immunolabelled using the monoclonal antibody (mAb) L42 for detection of PrP\textsuperscript{Sc} deposition by IHC, as described previously [23]. Corresponding tissues from a healthy heifer were used as control samples for IHC.

Samples of frontal cortex (FC), thalamus (T), cerebellum (Cbl), oesophagus (MO) and lumbar spinal cord (LSC) were analysed by Western blot (WB) using the mAb Sha31 (Bio-Rad Laboratories). Homogenates (10% in saline solution) were prepared using tissue from the brain, sciatic nerve, brachial nerve, mesenteric lymph node, extraorbital muscle, ileum and spleen. These inocula were then subjected to three rounds (48 h each) of PMCA, as previously described [24]. Tg338 mouse brain expressing the high susceptibility allele (VRQ) of the ovine PrP gene [25] was used as the substrate. The reaction was performed on a microplate (Axygen, Union City, CA, USA) into which a single ceramic bead was placed [26]. The Dawson scrapie strain [27] and healthy bovine brain homogenate were used as positive and negative controls, respectively. PMCA products were analysed by Western blot (WB) using the mAb Sha31 (Bio-Rad Laboratories).

At 30 months post-inoculation (p.i.), the heifer stood motionless, with its head lowered or resting against nearby objects when undisturbed, and did not overreact to external stimuli. Ataxia and severe lethargy were observed. Two weeks later, the animal showed abdominal distension and discomfort, which resulted in prostration. The animal was then euthanized. No macroscopic abnormalities were observed during necropsy, except for distension and the presence of abundant gravel in the small intestine.

IDEXX analysis revealed PrP\textsuperscript{Sc} positivity in the CNS. The optical density (OD) values for BSE conjugate varied among CNS samples: the highest OD values were obtained for the frontal cortex (3.575), followed by the oesophagus (3.507); intermediate values were obtained for the LSC (2.506) and cerebellum (2.480); and the lowest values were obtained for the thalamus (2.227). Despite these findings, IDEXX failed to detect PrP\textsuperscript{Sc} in PNS, LRS, GIT, or skeletal muscle samples.

Histopathological examination of CNS samples revealed no morphological alterations. Immunohistochemistry revealed the distribution of PrP\textsuperscript{Sc} throughout multiple neuroanatomical areas (Fig. 1a). Three distinct PrP\textsuperscript{Sc} deposition patterns were identified: a granular cytoplasmic pattern in neuronal perikarya; an intra-glial pattern (Fig. 1b, c); and a less frequently observed particulate neuropil-associated pattern (Fig. 1d). PrP\textsuperscript{Sc} was not observed in control tissues.

No histopathological alterations were observed in tissues outside the CNS. However, small deposits of PrP\textsuperscript{Sc} were distributed throughout several peripheral tissues. In the PNS, periaxonal immunolabelling was observed in transverse sections of the sciatic nerve (Fig. 2a). In the LRS, PrP\textsuperscript{Sc} was detected in tingle body macrophages (TBMs) present in the mesenteric lymph nodes (Fig. 2b). This same pattern was observed outside the lymphoid follicles in the tonsils and the submandibular and prescapular lymph nodes. No clear PrP\textsuperscript{Sc} staining was detected in the spleen. In the GIT, immunoreactivity was observed in intracytoplasmic granules within the neurons of the ENS in the duodenum, jejunum, ileum (Fig. 2c) and caecum. PrP\textsuperscript{Sc} immunoreactivity was also detected in the muscle spindles of the extraorbital muscle (Fig. 2e).
While protease-resistant prion protein (PrP\textsubscript{res}) was detected in the CNS by WB, the molecular profile differed from that of the original scrapie inoculum, and that of classical BSE. The mAb 6H4 revealed an unglycosylated band of a lower molecular mass than that of natural scrapie. This profile was similar to that observed for the BSE sample, but contained monoglycosylated and diglycosylated bands of lower masses than those observed for BSE. The mAb P4 failed to detect PrP\textsubscript{res} in either the bovine scrapie samples or the control BSE samples, but did detect the classical scrapie strain (Fig. 3a). WB using the mAb Sha 31 failed to detect the presence of PrP\textsubscript{res} in peripheral tissues (Fig. 3b).

A third round of PMCA at a dilution of 10\textsuperscript{-2} succeeded in amplifying PrP\textsubscript{res} in the brain, sciatic nerve, mesenteric lymph node, extraorbital muscle and ileum samples, all of which were positive by IHC. A signal was detected in spleen samples by PMCA alone (Fig. 3c). Furthermore, amplification of bovine scrapie in this substrate restored the original scrapie glycosylation pattern, revealing an unglycosylated band of a molecular mass higher than that detected for bovine scrapie. No PrP\textsuperscript{Sc} was amplified in brachial nerve or negative control homogenates (Fig. 3d).

This study presents a thorough neuropathological characterization, achieved using IHC, molecular tests and \textit{in vitro} PrP\textsubscript{res} amplification assays, of a calf intracerebrally inoculated with a single classical scrapie isolate. The animal developed clinical signs suggestive of a TSE at 30 months p.i., in accordance with previous scrapie infection studies in cattle describing incubation periods of between 14 [6] and 48 months [7]. Furthermore, the clinical presentation was characterized by general listlessness (weakness and lethargy), in line with the ‘dull form’ previously described in sheep scrapie-infected cattle [9]. While previous studies have described a clinical course of between 6 and 10 weeks, in the present case the animal presented severe abdominal discomfort and was killed 2 weeks after the clinical signs.

\textbf{Fig. 1.} PrP\textsuperscript{Sc} distribution and deposition patterns in different areas of the CNS in a scrapie-infected calf. (a) FC, frontal cortex; BG, basal ganglia; GBC, basal ganglia cortex; T, thalamus; Ht, hypothalamus; TPC, temporal/parietal cortex; OC, occipital cortex; Cbl, cerebellum; P, pons; O, obex; CSC, cervical spinal cord; TSC, thoracic spinal cord; and LSC, lumbar spinal cord. (b) Thalamus. Immunolabelling is predominantly intracellular (intraneuronal and intraglial). (c) Cervical spinal cord. Immunolabelling is predominantly intraneuronal. (d) Basal ganglia caudate nucleus. Immunolabelling is predominantly detected in the neuropil.
associated with the scrapie infection started. The absence of spongiform changes in the brain was in agreement with previous reports [6], but contrasted with the results obtained in natural and experimental BSE infections [28]. The pattern of PrP\textsuperscript{Sc} distribution also closely resembled that described in previous studies of intracerebral transmission of scrapie agent to cattle, consisting of a predominantly intraneuronal pattern [6] in the trigeminal nuclei, olive nucleus and reticular area. No involvement of the dorsal nuclei of the vagus nerve (data not shown) or the solitary tract was observed, in contrast to natural cases of BSE in cattle [29]. No PrP\textsuperscript{Sc} amyloid plaques were observed in any of the brain regions analysed, in contrast to findings reported in L-type BSE [30].

Analysis of bovine scrapie samples by discriminatory WB using mAb P4, which binds ovine PrP in the N-terminal of the PrP\textsuperscript{res}, detected no signal. This lack of detection could have some resemblance to the BSE agent. Similarly, Konold and coworkers [9] reported two distinct prion disease phenotypes in cattle inoculated with two different scrapie pools. One of the phenotypes was positive to mAb P4 and one was negative. However, in agreement with our findings, the authors found that the mAb 6H4 (which binds both bovine and ovine PrP) in the second phenotype, revealed diglycosylated and monoglycosylated bands of molecular masses lower than the corresponding bands detected in BSE samples, indicating that the glycosylation pattern of bovine scrapie differed from that of BSE. Subsequent characterization of the same two scrapie pools in a mouse bioassay revealed no evidence of classical or atypical BSE [11].

In contrast to these studies, our calf was inoculated with a single scrapie isolate. This approach ruled out the possibility that competition between coexisting prions strains could interfere with the development of a BSE-like disease. However, the replication of the classical scrapie isolate in a heifer used in the present experiment failed to produce BSE.

While IDEXX failed to detect the presence of PrP\textsuperscript{Sc} in peripheral tissues, it should be noted that rapid tests may be less sensitive than IHC in cases in which there is minimal PrP\textsuperscript{Sc} accumulation [31]. IHC revealed for the first time the presence of PrP\textsuperscript{Sc} in organs outside the CNS, such as PNS, LRS, extraorbital muscle and GIT from a heifer inoculated with the classical scrapie agent. In a previous similar study, no PrP\textsuperscript{Sc} was detected in either the mesenteric lymph node or the spleen [6]. The accumulation and distribution of PrP\textsuperscript{Sc} in peripheral tissues may be influenced by several factors. Gonzalez and coworkers reported that in sheep orally infected with scrapie both the PrP genotype of the recipient and that of the infecting source may contribute to the resulting neuropathological phenotype [32]. The route of 

**Fig. 2.** Immunohistochemical findings in peripheral tissues in a scrapie-infected calf. The arrows indicate PrP\textsuperscript{Sc} immunolabelling (mAb L42) in the (a) sciatic nerve, (b) mesenteric lymph node, (c) myenteric plexus of the ileum and (d) muscular spindle of the extraorbital muscle.
Fig. 3. Western blot analysis. (a) BSE and bovine scrapie were detected by discriminatory Western blot using the 6H4 but not the P4 antibody. Western blot using Sha31 antibody before (b) and after (c) PMCA assay in scrapie-infected heifer and control (d): brain (1), sciatic nerve (2), brachial nerve (3), mesenteric lymph node (4), extraorbital muscle (5), ileum (6) and spleen (7). MM, molecular mass markers: 20.1, 29.1 and 35.8 kD.
infection may also significantly affect the pathogenesis of the TSE. PrP\textsubscript{Sc} has been detected in the PNS in natural BSE [33, 34], and in the palatine tonsils in cattle orally infected with BSE [18]. We only detected PrP\textsubscript{Sc} in individual cells in some ganglia of the ENS, as previously described in cattle orally infected with BSE [35], and observed no clear involvement of the lymphoid follicles of the Peyer’s patches. Our findings demonstrate that the IDEXX test is capable of detecting scrapie prions in cattle. However, the lack of detection of PrP\textsubscript{Sc} in peripheral tissues by IDEXX and WB may have resulted from very low levels of PrP\textsubscript{Sc} in the different tissue samples analysed [20].

Highly sensitive techniques such as PMCA allow the detection of minute amounts of PrP\textsubscript{res} [21]. In our scrapie-inoculated heifer, PMCA amplified PrP\textsubscript{res} from the sciatic nerve, mesenteric lymph-node and ileum, all of which showed mild positivity by IHC, and in spleen samples. These findings are supported by previous studies in which PMCA was used to amplify PrP\textsubscript{res} in spleen [36], mesenteric lymph-node and ileum samples [22] from cows experimentally infected with BSE.

Our findings describe the centrifugal spread of PrP\textsubscript{res} following intracerebral inoculation from the CNS to a wide range of peripheral tissues. This spread probably occurs via the nervous system, as suggested by immunohistochemical detection of PrP\textsubscript{res} in the sciatic nerve and ileum, although other pathways such as the haematogenous route may also be implicated, as previously described in scrapie-infected sheep [37]. Further analyses using ultrasensitive techniques (e.g. PMCA or mouse bioassay) could provide a better understanding of the peripheral distribution of scrapie in cattle. To the best of our knowledge, this is the first report demonstrating the presence of PrP\textsubscript{Sc} in peripheral tissues in a heifer experimentally infected with scrapie agent. These findings thus describe a novel pathological feature of intracerebral scrapie infection in cattle.

References


