Bovine spongiform encephalopathy agent in spleen from an ARR/ARR orally exposed sheep

Olivier Andréoletti,1 Nathalie Morel,2 Caroline Lacroux,1 Virginie Rouillon,1 Céline Barc,3 Guillaume Tabouret,1 Pierre Sarradin,3 Patricia Berthon,3 Philippe Bernardet,3 Jacinthe Mathey,1 Sérénine Lugan,1 Pierrette Costes,1 Fabien Corbière,1 Juan-Carlos Espinosa,4 Juan Maria Torres,4 Jacques Grassi,2 François Schelcher1 and Frédéric Lantier3

Correspondence
Olivier Andréoletti
o.andreoletti@envt.fr

1UMR INRA ENV Toulouse, Interactions Hôte-Agent Pathogènes, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles, 31076 Toulouse, France
2CEA, Service de Pharmacologie et d’Immunologie, CEA/Saclay, 91191 Gif sur Yvette cedex, France
3INRA, Pathologie Infectieuse et Immunologie, INRA Nouzilly, 37380 Nouzilly, France
4CISA, Instituto National de Investigacion y Tecnologia Agraria y Alimentaria, 28130 Valdeolmos, Spain

Oral contamination with bovine spongiform encephalopathy (BSE) agent in susceptible PRNP genotype sheep results in widespread distribution of prion in the host. Because ARR homozygous sheep are considered to be resistant to transmissible spongiform encephalopathies, they have been selected to eradicate scrapie from sheep flocks and to protect the human food chain from small ruminant BSE risk. However, results presented here show that several months after an oral challenge with BSE agent, healthy ARR/ARR sheep can accumulate significant amounts of PrPSc in the spleen.

Several lines of evidence have shown that the variant Creutzfeldt–Jakob disease epidemic in humans is very probably due to the consumption of bovine spongiform encephalopathy (BSE) agent-contaminated bovine products (Bruce et al., 1997). The possible presence of BSE agent in sheep has already led countries like Great Britain (National Scrapie Plan), France and The Netherlands to promote, through nationally-funded programmes and regulations, the breeding of ARR/ARR sheep (Dawson et al., 2003), which are considered to be resistant to transmissible spongiform encephalopathies (TSEs). Recently, the first ‘natural’ case of BSE was reported in a goat (Eloit et al., 2005).

TSE-free New Zealand Suffolk lambs from ARR/ARR and ARQ/ARQ ewes, kindly provided by H. Simmons (VLA, ADAS DEFRA, UK), were used for oral inoculation. All animals used in these experiments were treated according to EEC recommendations for animal welfare and under the supervision of the local INRA Ethics Committee. The PRNP genotypes at codons 136, 154 and 171 were routinely checked for each animal using RFLP-PCR (PII Laboratory, INRA-Tours, France). The infectious material was derived from ARQ/ARQ sheep intracerebrally (IC) inoculated with cattle BSE agent. Twenty-four-hour-old ARQ/ARQ (n=8) and ARR/ARR (n=10) lambs were exposed to a dose of 2·5 g infected brain through natural suckling. A second and similar inoculation of those animals was performed at 14 days of age. Animals were housed in high-confinement (level 3) facilities dedicated to BSE in sheep studies, to avoid any risk of cross-contamination with scrapie. Animal groups (n=3) from each genotype were killed at 4 and 10 months post-challenge (p.c.). At 19 months, the remaining ARQ/ARQ (n=2) animals were clinically affected and euthanized. The remaining ARR/ARR (n=4) animals were still healthy at 29 months p.c.

PrPSc detection was carried out in collected samples using immunohistochemistry (IHC), ELISA and Western blot analyses. PrPSc detection was performed as described previously (Andréoletti et al., 2002) using mAb BAR221 (1/3000 diluted) antibody raised against recombinant ovine protein, which recognizes amino acid sequence 141–152. ELISA measurements were performed using the TeseE sheep/goat kit (Bio-Rad), a test that has been recently validated by the European Community for the post-mortem diagnosis of TSE in small ruminants. Western blot analysis, preceded by immunopurification, was performed as previously described (Andréoletti et al., 2004) using mAb Sha31, which binds to the 145–152 sequence of PrP (YEDRYYRE).

In ARQ/ARQ animals, PrPSc was detected in various lymphoid tissues as early as 4 months p.c. and in the
central nervous system (obex and medulla) from 10 months p.c. At 10 and 19 months p.c., there was a high level of accumulation in secondary lymphoid organs. These findings are consistent with published data (Bellworthy et al., 2005).

Strikingly, in one challenged ARR/ARR animal, a strong PrPSc ELISA-positive signal was observed in the spleen at 10 months p.c. This result was reproduced with 10 random samples taken from the same tissue. Following this finding, PRNP full open reading frame (ORF) sequences of all killed animals were checked by sequencing both strands of two overlapping PCR fragments covering the complete ovine PRNP ORF (Primer1F, GTGGGCATTTGATGCTGAC; Primer1R, TGGTTGGGTACACGATG; Primer2F, TCAGCCCATGCTGATG; Primer2R, CTGCAAGTGAGACTCCCTCC). Sequencing analysis of the full PRNP ORF confirmed the ARR/ARR sample genotype and the absence of any other mutation in the PRNP gene.

Using these techniques, no PrPSc was detected in any other tissue from this animal or in any other challenged ARR/ARR animal. However, since it has been clearly established in rodents that consistent infectivity levels can accumulate in the absence of detectable PrPSc (Lasmezas et al., 1997; Race et al., 2001), a systematic bioassay in RIII mice of a tissue panel from these animals is ongoing.

Using Western blotting and Sha31 antibody, the observed apparent molecular mass of the non-glycosylated band was similar in spleen samples from BSE agent-exposed ARR/ARR and BSE agent-positive ARQ/ARQ animals, but clearly differed (lower molecular mass) from a control spleen from an ARQ/ARQ animal with ‘natural’ scrapie (Fig. 1a). As previously described (Race et al., 1998), in both scrapie and BSE, a shift in the apparent molecular mass of proteinase K (PK)-resistant PrP was observed between brain and spleen (Fig. 1a).

Both Sha31 and 2A11 antibodies enabled detection of PrPSc in spleen and brain from scrapie- or BSE-affected ARQ/ARQ sheep (Fig. 1b, c). However, 2A11 (even after overexposure) was unable to detect abnormal PrP in brain tissue from an IC BSE agent-inoculated ARR/ARR sheep in the terminal stage of the disease, whereas strong labelling was observed with Sha31 (Fig. 1b, c; lane 3). Similarly, the PrPSc ELISA-positive ARR/ARR spleen was positive using Sha31, whereas no signal was observed using 2A11 (Fig. 1b, c; lane 5). Taken together, these data confirm unequivocally that PrPSc accumulating in this particular spleen sample is composed entirely of the ARR allelic variant. Finally, accumulated PrPSc can be considered to be the product of prion replication and not the consequence of a passive accumulation of the inoculum.

ELISA and Western blotting results showed that the total amounts of PrPSc in the ARR/ARR spleen samples were 5- to 10-fold lower than those in spleens of 10-month-old ARQ/ARQ BSE-infected sheep. Using IHC, abnormal PrP was observed in about 40 % of the ARR/ARR spleen lymphoid formations, whereas in ARQ/ARQ sheep about 90–100 % were positive (Fig. 2a, b).

To confirm definitively the PK-resistance of the IHC-labelled PrP and characterize the distribution of abnormal prion protein, the paraffin-embedded tissue (PET) blot technique was used (Schulz-Schaeffer et al., 2000) (Fig. 2c, d). Briefly, sections were collected on 0.45 µm nitrocellulose membranes, before drying and deparaffinization. PK (Roche) digestion (250 µg ml⁻¹, 2 h, 55 °C) was performed before denaturation in guanidium isothiocyanate solution (3 M, 10 min, at room temperature). Immunodetection was carried out using mAb Sha31 (4 µg ml⁻¹), followed by
application of an alkaline phosphatase-coupled secondary antibody (diluted 1/500; Dako). Binding of secondary antibody was revealed using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate chromogen.

In ARQ/ARQ animals, strong labelling was observed in spleen B-cell follicle germinal centres and marginal zones, which demarcate the white from the red pulp (Fig. 2a–c). In ARR/ARR tissue samples, PrPSc was not observed in B-cell germinal centres, but was found only in the marginal zone (Fig. 2b–d). In this area, PrPSc-positive cells were distributed in a rim shape (Fig. 2c, d). To further investigate the nature of these cells, PrPSc/CD68 double-labelling was performed as previously described (Andreoletti et al., 2002) using R521-7 rabbit anti-ovine PrP protein serum (1/1000 diluted; kindly provided by L. Van Keulen, CIDC, Lelystad, The Netherlands) and a mouse anti-CD68 antibody (1/150 diluted; Serotec). A strong positive double-labelling (Fig. 2e, f) was observed and demonstrated the ‘phagocyte’ nature of the PrPSc-positive cells (Holness et al., 1993).

The marginal zone in the spleen is a highly efficient system specialized in blood-borne antigen trapping (Aichele et al., 2003). Strong involvement of the CD68-positive cells in the marginal zone could thus suggest that blood circulation has occurred in ARR/ARR animals orally exposed to the BSE agent.

Successful transmission of BSE has been reported after IC challenge in ARR/ARR sheep (Houston et al., 2003). The IC route is considered to be of low relevance to natural exposure, and PrPSc or infectivity have never been previously reported in tissue from ARR/ARR animals orally challenged with BSE (Bellworthy et al., 2005; Jeffrey et al., 2001).
However, the total number of animals orally inoculated with BSE included in both these published studies was small (n=14 for animals challenged and investigated post-mortem). Space constraints in high-security-level animal facilities is the probable explanation for the use of such a low number of animals. In our study, similar space constraints limited the size of the ARR/ARR animal group to 10. To date, from the six challenged and killed animals, only one was found to be positive, which indicates a low transmission rate of BSE agent in animals bearing the ARR/ARR genotype. Such a low transmission rate is a possible explanation for the negative results previously reported. Another point is that in both published studies, ARR/ARR animals were orally dosed at 6 months of age (Bellworthy et al., 2005; Jeffrey et al., 2001). In our study, lambs were challenged first at birth and then at 2 weeks of age. In lambs, the efficiency of infection is considered to decrease with age (Detwiler & Baylis, 2003) and, in nature, TSE infection in sheep seems to occur very soon after birth (Andreletti et al., 2005; Jeffrey et al., 2001). In this study, due to early exposure, the infectious challenge may have been more efficient than that shown in previous experiments. Moreover, it is worth noting that the challenge dose used in the present study was similar (5 g) to the dose used in previously published experiments, although sheep-derived BSE agent was used in this study instead of cattle BSE agent (Bellworthy et al., 2005; Jeffrey et al., 2001). A possible adaptation of BSE to the sheep could also be an explanation for its improved ability to infect ARR/ARR animals.

Our findings clearly demonstrate that ARR/ARR sheep orally exposed to BSE agent can replicate the agent in their spleen. It also suggests that ARR/ARR sheep are not absolutely resistant to a natural oral challenge of BSE. However, due to the low efficiency of transmission observed in our experimental conditions, even though highly infectious doses and sheep-adapted BSE were used, the BSE transmission risk in ARR/ARR sheep should be considered to be marginal.

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