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PrP^d accumulation in organs of ARQ/ARQ sheep experimentally infected with BSE by peripheral routes

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To study the pathogenesis of bovine spongiform encephalopathy infection in small ruminants, two Lacaune sheep with the AA136RR154QQ171 and one with the AA136RR154RR171 genotype for the prion protein, were inoculated with a brain homogenate from a French cattle BSE case by peripheral routes. Sheep with the ARQ/ARQ genotype are considered as susceptible to prion diseases contrary to those with the ARR/ARR genotype. The accumulation of disease-associated prion protein (PrP^d) was analysed by biochemical and immunohistochemical methods. No PrP^d accumulation was detected in samples from the ARR/ARR sheep 2 years post inoculation. In the two ARQ/ARQ sheep that had scrapie-like clinical symptoms, PrP^d was found in the central, sympathetic and enteric nervous systems and in lymphoid organs. Remarkably, PrP^d was also detected in some muscle types as well as in all peripheral nerves that had not been reported previously thus revealing a widespread distribution of BSE-associated PrP^d in sheep tissues.

Keywords: ovine BSE, prion, scrapie, sheep

INTRODUCTION

The bovine spongiform encephalopathy (BSE) agent, linked to the variant Creutzfeldt-Jakob disease in humans (Bruce *et al.*, 1997), has been experimentally transmitted to sheep (Foster *et al.*, 1993), without clinical distinction from natural scrapie. The possible presence of this agent within sheep flocks used for human consumption is therefore of considerable concern. It has recently been demonstrated in France that a major risk factor for introduction of transmissible spongiform encephalopathies (TSE) in a flock is linked to the use of proprietary concentrates and milk replacers (Philippe *et al.*, 2005), which have been implicated in the BSE epidemic in cattle (Wilesmith *et al.*, 1992). Furthermore, the BSE agent was recently identified in a naturally infected French goat (Eloit *et al.*, 2005) and this reinforces the possible presence of the BSE agent in the sheep and goat flocks.

In sheep, the development of prion disease is complex and depends on several factors such as the genotype and breed of animals as well as the nature of the infectious agent. Thus, polymorphism of the prion protein PrP gene that predominantly determines scrapie or BSE susceptibility are linked to variations of codons 136, 154 and 171. The $V_{136}R_{154}Q_{171}/V_{136}R_{154}Q_{171}$ genotypes are associated with a very high susceptibility to scrapie (Hunter, 1997) whereas the ARR/ARR animals are more resistant to scrapie (Hunter, 2003) even though cases of scrapie have been reported in this genotype (Ikeda *et al.*, 1995; Buschmann *et al.*, 2004). The ARQ/ARQ genotype, largely represented in flocks, and associated with scrapie susceptibility, would lead to an increased susceptibility to BSE (Houston & Gravenor, 2003).

To study the pathogenesis of BSE in small ruminants, Lacaune sheep were inoculated with the BSE agent. The accumulation of the disease-associ-

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Abbreviations: BSE, bovine spongiform encephalopathy; CNS, central nervous system; DAB, diaminobenzidine; dpi, days post infection; IHC, immunohistochemistry; mAb, monoclonal antibody; PrPd, disease-associated prion protein; recPrP, recombinant prion protein; TSE, transmissible spongiform encephalopathy.

ated prion protein, PrP^d, the most specific marker of the disease (Bolton *et al.*, 1982), was investigated using three complementary methods that allowed us to obtain both qualitative and quantitative results (Madec *et al.*, 2004) allowing the identification of the tissues that may represent a risk for consumption.

MATERIAL AND METHODS

Animals. In our study, two ARQ/ARQ Lacaune sheep were inoculated either by intra-peritoneal (SB1 sheep) or intra-splenic (SB3 sheep) routes with brain homogenate from a French BSE-affected cow. One sheep naturally died at 672 days post inoculation (dpi) and the other was euthanized 1444 dpi. Both sheep had clinical signs of neurological disorders and had molecular characteristics of PrP^d consistent with BSE infection (Houston & Gravenor, 2003; Lezmi *et al.*, 2004). The other Lacaune sheep with the ARR/ARR genotype (SB2) was inoculated by the intra-peritoneal route and was sacrificed at 673 dpi.

Samples from healthy sheep 2 years of age having the ARR/ARR and ARR/ARQ genotypes were used as negative controls. The negative status of these animals was confirmed by checking the absence of PrP^d in the central nervous system (CNS) and tonsils.

Immunohistochemistry (IHC). IHC allowing the identification of PrP^d at the cellular level with high sensitivity was performed as described in previous studies (Lezmi *et al.*, 2003); PrP^d deposits appeared *in situ* as brown or black deposits using DAB alone or intensified with $NiCl_2$.

Western-blot (WB). Following a WB procedure previously described (Biacabe *et al.*, 2004), PrP^d detection was performed using anti-PrP mAbs Bar233 and peroxidase-conjugated anti-mouse IgG (Southern Biotechnology Associates). This method has the advantage of identifying with high specificity the proteinase K resistant form of PrP^d. However, the Western Blot analysis is less sensitive than the ELISA method, and as all samples were not in sufficient quantities for both biochemical analysis, samples were tested by ELISA in priority, as this method allows quantification of PrP^d.

ELISA. ELISA was performed with the extraction kit 'Platelia BSE Bio-Rad' currently used for TSE diagnosis (Grassi *et al.*, 2001). For each plate an internal standard was used, i.e. ovine recombinant prion (^{rec}PrP) purified as previously described (Betemps & Baron, 2001). ELISA detection was performed using SAF34 anti-PrP mAbs as capture antibodies and acetylcholinesterase-conjugated Bar224 anti-PrP mAbs as a tracer.

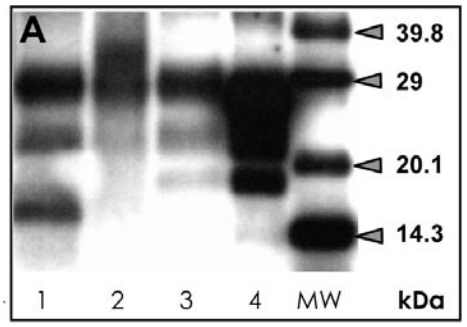

Figure 1. Detection of PrP^d by Western blot — ARQ/ARQ sheep.
Representative pattern of PrP^d observed in different tissues. Lanes 1 and 2: mandibular and iliac-medial lymph nodes, respectively; lane 3: adrenal gland; lane 4: cortex. All homogenates were prepared from sheep SB3. MW, molecular mass.

RESULTS AND DISCUSSION

For all samples analysed from the ARR/ARR sheep, no PrP^d was detected by any of the three PrP^d detection methods used (Table 1). This result correlates with the higher genetic resistance to TSE associated with this genotype naturally affected with scrapie (Elsen *et al.*, 1999) or orally infected with the BSE agent (Jeffrey *et al.*, 2001). However, resistance of the ARR/ARR sheep challenged with TSE infection is not considered complete since natural scrapie cases have been reported in sheep with this genotype (Ikeda *et al.*, 1995; Buschmann *et al.*, 2004; French surveillance program, unpublished data). Furthermore, BSE has been transmitted to ARR/ARR sheep by the intra-cerebral route (Houston *et al.*, 2003).

In both ARQ/ARQ sheep, the CNS (including retina), the lymphoid system and the autonomous nervous system were identified by each method as major sites of PrP^d accumulation (Table 1, Fig. 1) and were also described earlier by other groups in experimentally BSE affected sheep (Foster *et al.*, 2001; Jeffrey *et al.*, 2001) as well as in naturally scrapie-affected sheep (van Keulen *et al.*, 1999; Jeffrey *et al.*, 2001). In the CNS, the quantities of abnormal PrP, expressed as equivalent in ^{rec}PrP, were estimated by ELISA at up to 13000 ng/g of brainstem tissue. Comparatively, the levels found in 13 ARQ/ARQ or ARQ/VRQ sheep clinically affected with natural scrapie averaged 40000 ± 20000 ng of PrP^d/g of CNS tissues. Lymphoid organs accumulated lower levels of PrP^d and large quantities of material were required to detect a signal by Westren blot in the mandibular or iliac medial lymph nodes (LN) of SB3 (Fig. 1). In the spleen of SB1 and SB3, 46 and 2 ng equivalent of PrP^d/g of tissue were detected, respectively. In the ileum, 232 ng equivalent of PrP^d/g of tissue was detected and correlated with a higher number and size of germinal centres when compared to spleen or iliac lymph nodes. The mean quantity of PrP^d in the CNS was 187- and

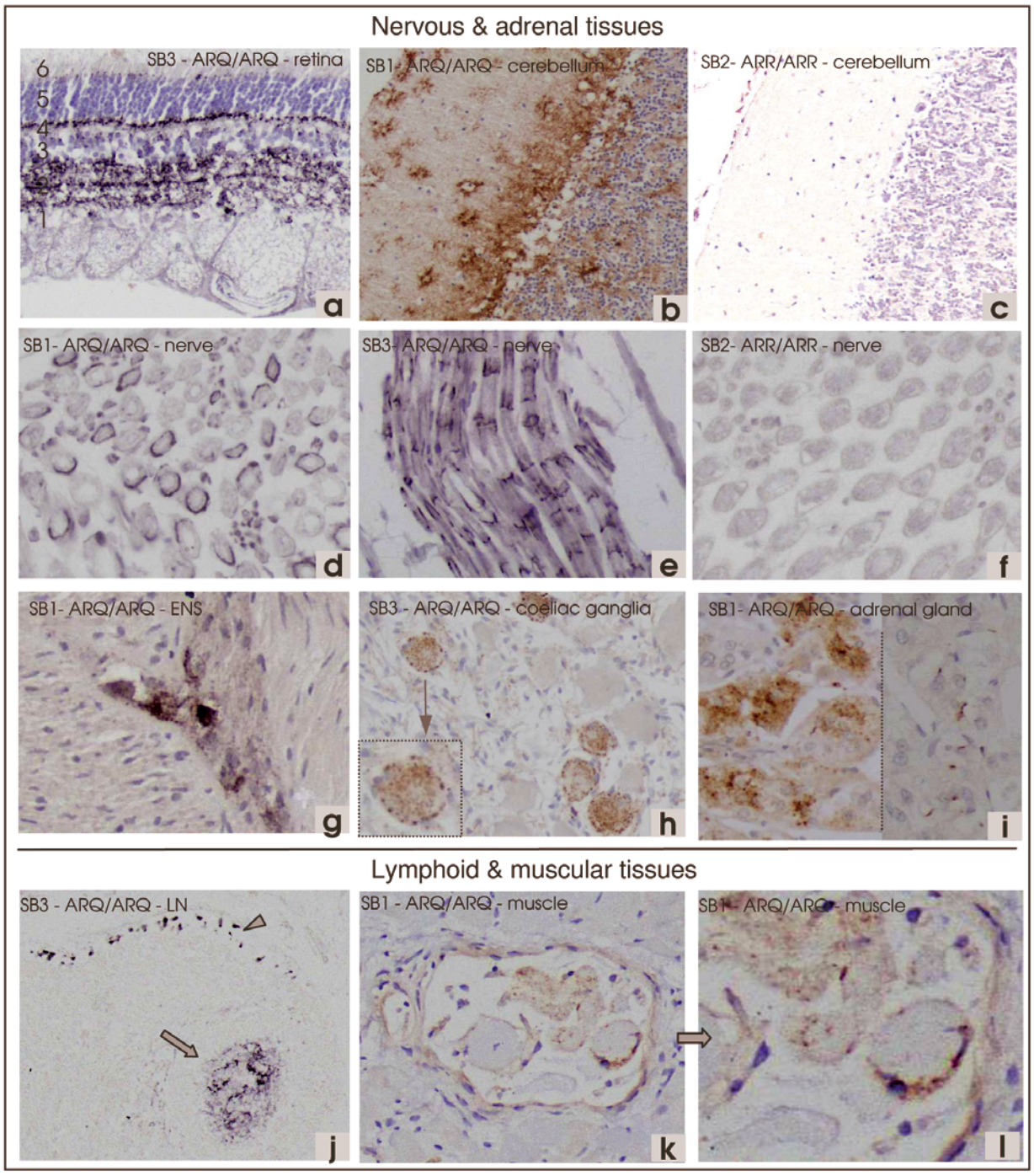

Figure 2. Immunodetection of PrP^d in various tissues.
PrP^d accumulation was detected in the retina (a, black deposits, ×200) and in the cerebellum of both ARQ/ARQ sheep (b, brown deposits) but not in the ARR/ARR sheep (c). PrP^d was also detected in the ARQ/ARQ sheep in sciatic nerve (d–e, black deposits, ×400, ×200) but neither in the ARR/ARR sheep nor in healthy controls (f, ×200). In the enteric nervous system of ARQ/ARQ sheep, PrP^d was detected associated to neurons (g, black deposits, ×200) as well as in the coeliac ganglia in which intra and peri-neuronal PrP^d deposits were visualized (h, brown deposits, ×200; higher magnification in the inset). In the adrenal gland, two types of PrP^d accumulation were observed, dense intracellular and synaptic-like (i, brown deposits, ×400). In lymph nodes, the PrP^d accumulation was mainly detected in germinal centers (arrow) (j, black deposits, ×100). In the muscle PrP^d accumulation was observed associated to neuro-muscular spindle (k–l, brown deposits, ×100 and ×400).

36-fold greater than the quantities determined respectively in spleen and in the intestine.

Qualitatively, different types of PrP^d deposits in the brain were identified from the frontal cortex to the lumbar spinal cord. These PrP^d deposits were mainly identical to those previously identified in scrapie- or BSE-affected sheep (Ryder *et al.*, 2001; Gonzalez *et al.*, 2002). In the retina, PrP^d accumula-

Table 1. Detection of PrP^d by immunohistochemistry (IHC), ELISA and Western blot (WB).

For ELISA measurements, samples were stated positive or negative by reference to a cut-off value calculated as the mean of the measurements made on the negative controls plus 3-fold the value of the standard deviation calculated on negative controls. A "grey area" was defined between the value of the cut off and the mean of the negative controls plus 2-fold the value of the standard variation (Table 1, '+/–' results). PrP^d accumulation was also quantified (values in brackets, ng/g of tissue). For each plate, at least three negative controls chosen in function of the studied tissue were deposited in duplicate.

Samples	SB1 ARQ/ARQ				SB3 ARQ/ARQ				SB2 ARR/ARR			
	IHC	ELISA	WB	Con.	IHC	ELISA	WB	Con.	IHC	ELISA	WB	Con.
CNS												
Frontal cortex	+++	+ (4526)	++++	++++	+++	+	++++	++++	–	–	nt	–
Cerebellum	+++	+	++++	++++	+++	+ (5372)	++++	++++	–	–	nt	–
Brain stem	++++	+	++++	++++	++++	+ (13266)	++++	++++	–	–	nt	–
Cervical sp. cord	+++	+ (2959)	++++	++++	+++	+ (2081)	++++	++++	–	–	nt	–
Thoracic sp. cord	+++	+ (1172)	++++	++++	+++	+ (2439)	++++	++++	–	–	nt	–
Lumbar sp. cord	+++	+ (4819)	++++	++++	+++	+ (4002)	++++	++++	–	–	nt	–
Lymphoid organs												
Tonsils	++	na	++	++	++	+ (107)	na	++	–	na	–	–
Soft palate	na	na	na		++	na	na	++	na	na	na	
Retropharyngeal LN	++	na	na	++	+ *	–	nt	+	–	na	na	–
Mandibular LN	+	+/–	–	+	+ *	+ (2 ± 1)	+	+	–	–	nt	–
Thoracic LN	+*	na	na	+	na	na	na		na	na	na	
Mediastinal LN	+	–	nt	+	+	–	nt	+	–	–	nt	–
Ruminal LN	na	na	na		+ *	na	na	+	na	na	na	
Ileal LN	+	na	na	+	–	+ (5)	nt	+	–	na	na	–
Caecal LN	+	na	na	+	+	–	–	+	–	–	nt	–
Spiral colon LN	+	+ (1± 0)	nt	+	–	+/–	nt	+/–	na	–	nt	–
Ilio–femoral LN	+	+/–	nt	+	+ *	na	na	+	–	–	nt	–
Iliac–medial LN	–	–	nt	–	+ *	+ (12)	+/–	+	–	–	nt	–
Spleen	+/++	+ (46)	+	++	+	+ (2)	–	+	–	–	nt	–
Prescapular LN	+	+ (12)	+	+	+ *	na	na	+	–	–	nt	–
Precrural LN	na	na	na		+ *	+ (5)	+/–	+	–	na	na	–
Popliteal LN	+ *	+ (2 ± 1)	–	+	+ *	+ (25)	+/–	+	–	–	nt	–
Digestive tract												
Oesophagus ENS	na	na	na		–	–	nt	–	na	na	na	
Rumen ENS	na	na	na		–	–	nt	–	na	na	na	
Reticulum ENS	na	na	na		–	–	nt	–	na	na	na	
Omasum ENS	na	na	na		–	–	nt	–	na	na	na	
Abomasum ENS	na	na	na		+	–	nt	+	na	na	na	
Duodenum ENS	+	–	–	+	–	–	nt	–	–	–	nt	–
Jejunum ENS	na	na	na		–	–	nt	+	–	na	na	–
Ileum ENS	+	+ (232)	+	+	+	–	nt	+	nt	nt	nt	
Ileum PP	++			++	+			+				
Ileo-caecum ENS	+	na	na	+	+	na	na	+	–	na	na	–
Ileo-caecum PP	++			++	++			++	–			
Caecum ENS	+	+/–	+/–	+	–	–	nt	–	nt	nt	nt	
Spiral colon ENS	+	+ (12)	+/–	+	–	–	nt	–	–	nt	nt	–
Spiral colon PP	+				na				–			
Rectum ENS	–	na	na	–	–	–	nt	–	na	na	na	
Per. Nerv. Syst.												
Cervical.Cra.gg	++	na	na	++	+	na	na	+	–	na	na	–
Coeliac ganglia	++	na	na	++	+	na	na	+	–	na	na	–
Sympathic gg	na	na	na		+/–	na	na	+/–	–	na	na	–
Vagus nerve	+/–	–	–	+/–	+/–	–	nt	+/–	–	na	na	–
Splancnic nerve	+/–	na	na	+/–	+/–	na	na	+/–	–	na	na	–
Sciatic nerve	+	–	–	+	+	–	nt	+	–	–	nt	–
Brachial plexus	+	–	nt	+	+	–	nt	+	–	–	nt	–
Lingual nerve	na	na	na		+	+ (2)	nt	+	na	na	na	
Phrenic nerve	na	na	na		na	na	na		–	na	na	–

Eyes												
Aqueous humour	na	+ (35)	nt	+	na	na	na		na	–	nt	–
Cornea	–	–	nt	–	–	+/–	nt	+/–	–	na	na	–
Lens	–	–	–	–	–	–	–	–	–	na	na	–
Iris	–	–	nt	–	–	+/–	–	+/–	–	na	na	–
Optic nerve	–	–	nt	–	–	–	nt	–	–	na	na	–
Retina	+++	+ (7)	nt	+++	+++	+ (15)	–	+++	–	na	na	–
Vitreous humour	na	+ (29)	–	+	na	–	–	–	na	–	nt	–
Other samples												
Adrenal gland	++	+ (111)	+	++	+	+ (53)	+	+	–	–	nt	–
Urinary bladder	na	na	na		–	–	nt	–	na	na	na	
Carotid	na	na	na		–	–	nt	–	na	na	na	
Gallbladder	na	na	na		–	na	na	–	na	na	na	
Heart, auricle	–	na	na	–	–	na	na	–	na	na	na	
Heart, ventricle	–	–	–	–	–	–	nt	–	–	na	na	–
Kidney	–	–	nt	–	–	–	nt	–	–	–	nt	–
Liver	–	–	–	–	–	–	nt	–	–	–	nt	–
Lung	–	na	na	–	–	–	nt	–	–	na	na	–
Mouth mucosa	na	na	na		–	na	na	–	na	na	na	
Ovary	na	na	na		–	–	nt	–	–	na	na	–
Pancreas	na	na	na		–	–	nt	–	na	na	na	
Salivary gland	–	na	na	–	–	–	nt	–	–	na	na	–
Skin	–	na	na	–	–	–	nt	–	na	na	na	
Skin lesion	–	na	na	–	na	na	na		na	na	na	
Striated muscles	+	–	nt	+	+	–	nt	+	nt	nt	nt	
Thyroid	na	na	na		–	–	nt	–	na	na	na	
Tongue	+	na	na	+	+	–	nt	+	na	na	na	
Trachea	na	na	na		–	+ (21)	nt	+	na	na	na	
Uterus	na	na	na		–	–	nt	–	–	na	na	–

The intensity of PrP^d immunolabelling was estimated for IHC and Western blot (WB) according to the scoring: – no labelling; +/– trace of light labelling; + light labelling; ++ moderate labelling; +++ intense labelling; and ++++ very strong labelling. Basing on the results obtained with the different methods, a final conclusion (con.) was proposed for each tissue on the presence of PrP^d. CNS, central nervous system; ENS, enteric nervous system; PP, Peyer's patches; gg, ganglia; sp. cord, spinal cord; nt, not tested; na, not available; +*, PrP^d accumulation in under-capsular area in some lymph nodes (LN).

tion was mainly detected in the ganglionar layer (1), intern (2) and extern (4) plexiform layers (numbers corresponding to the different layers in the retina, Fig. 2a). Interestingly, in the enteric nervous system of ARQ/ARQ sheep, PrP^d was detected associated with neurons (Fig. 2g) as well as in the coeliac ganglia in which intra- and peri-neuronal PrP^d deposits were visualized (Fig. 2h). In the adrenal gland, two types of PrP^d accumulation were observed as dense intracellular or synaptic-like deposits (Fig. 2i).

In lymphoid organs, PrP^d was detected in germinal centres of secondary lymphoid follicles, in follicular dendritic cells and in tingible body macrophages (Fig. 2j). PrP^d was also detected in cells with a morphology consistent with macrophages in the subcapsular sinus of some lymph nodes (Fig. 2j, arrowhead, Table 1*). These observations are in agreement with previous results obtained both in sheep naturally affected with scrapie (Jeffrey *et al.*, 2000; Lezmi *et al.*, 2001; Ersdal *et al.*, 2005) and in experimentally BSE-infected sheep (Lezmi *et al.*, 2001; Jeffrey *et al.*, 2001). Interestingly, not all germinal centres were labelled for PrP^d; this partial absence of labelling in germinal centres (as in tonsils) was not observed in samples from 13 natural scrapie-infected sheep in which all lymphoid germinal centres were positively labelled for PrP^d. This agreed with data describing an early and systematic immune system involvement in lambs affected with scrapie (Andreoletti *et al.*, 2000) which was not a feature of BSE agent infection in sheep during the first passage (Jeffrey *et al.*, 2001; Martin *et al.*, 2005).

In our study, as opposed to previous published results, in both ARQ/ARQ sheep, PrP^d was detected by IHC in all motor nerves and associated with Schwann's cells (Fig. 2d, e). PrP^d deposits were similarly detected in all other tissue samples containing peripheral nerves, most notably in nerves in muscle samples. This observation was not reported in other studies with sheep BSE (Foster *et al.*, 2001; Jeffrey *et al.*, 2001). However, we observed the same type of deposits in two other sheep (ARQ/VRQ) naturally affected with scrapie (not shown) and two previous articles report similar data in sheep with natural scrapie (Groschup *et al.*, 1999; Archer *et al.*, 2004).

PrP^d presence was also identified in striated muscles for both ARQ/ARQ sheep. These deposits were associated with neuromuscular spindles that are highly innervated structures made of groups

of myocytes surrounded by a thin fibrous capsule (Fig. 2k, l) and are a specialized subset of myocytes implicated in proprioception. In the tongue of sheep, the accumulation of PrP^d in these structures was less evident. Only one study reported the PrP^d presence in the muscle of sheep affected with scrapie using IHC and ELISA (Andreoletti *et al.*, 2004). Here, sampling and analysis of different muscles were not systematic and thus the ELISA/IHC results were not correlated. However, the accumulation in muscle tissue of PrP^d in sheep affected with scrapie is not systematic (Andreoletti *et al.*, 2004). Recently, pathological prion protein was detected in muscles of hamsters and mice infected with rodent-adapted BSE or vCJD (Thomzig *et al.*, 2006). Previously, other studies failed to detect prion in nerves and muscles of BSE- or scrapie-infected sheep (Foster *et al.*, 2001; Hamir *et al.*, 2004) possibly relying on the use of different pre-treatments and antibodies.

In conclusion, we have shown that the inoculation of the BSE agent of French origin by peripheral routes to Lacaune sheep lead to the development of the clinical disease only in ARQ/ARQ sheep. The distribution of PrP^d in ARQ/ARQ sheep infected with BSE was very similar to that described in natural scrapie. Overall, we demonstrated for the first time the presence of PrP^d in muscles and nerves of sheep infected experimentally with BSE agent, which stresses the potential risk for humans related to consumption of sheep products from sheep naturally infected with BSE.

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