

PrP^{Sc} accumulation in myocytes from sheep incubating natural scrapie

O Andréoletti¹, S Simon², C Lacroux¹, N Morel², G Tabouret¹, A Chabert¹, S Lugan¹, F Corbière¹, P Ferré³, G. Foucras¹, H. Laude⁴, F. Eychenne⁵, J Grassi² & F Schelcher¹

Because variant Creutzfeldt–Jakob disease (vCJD) in humans probably results from consumption of products contaminated with tissue from animals with bovine spongiform encephalopathy, whether infectious prion protein is present in ruminant muscles is a crucial question. Here we show that experimentally and naturally scrapie-affected sheep accumulate the prion protein PrP^{Sc} in a myocyte subset. In naturally infected sheep, PrP^{Sc} is detectable in muscle several months before clinical disease onset. The relative amounts of PrP^{Sc} suggest a 5,000-fold lower infectivity for muscle as compared to brain.

PrP^{Sc} has been recently detected in the muscle of rodents and humans terminally affected with transmissible spongiform encephalopathies

(TSEs)^{1–3}, the class of disease that includes bovine spongiform encephalopathy (BSE). However, PrP^{Sc} accumulation in muscle from animals entering the human food chain has never been demonstrated and muscle structures containing PrP^{Sc} have not been identified^{1,2}.

PrP^{Sc} in muscle was initially observed after intracerebral (IC) challenge in a mouse model of scrapie². To look for PrP^{Sc} in sheep muscle, we first used a similar IC inoculation route. Six sheep homozygous for an allele encoding the susceptible A₁₃₆R₁₅₄Q₁₇₁ (ARQ) variant of PRP received 0.05 g equivalent of scrapie-positive brain (Langlade isolate). Clinical scrapie occurred at 332 ± 13 d; affected sheep were killed and their tissues sampled. (All animals used in these experiments were treated in accordance with European Economic Community recommendations for animal welfare and under the supervision of the local Institut National de la Recherche Agronomique Ethics Committee.) We compared data from ARQ sheep with those from seven ARR/ARR sheep 20 months of age that had been naturally exposed to scrapie (negative controls).

In four of six susceptible sheep, PrP^{Sc} accumulated in sciatic and brachial nerves as determined by ELISA and immunohistochemistry (IHC). In three of the four, we detected small but consistent amounts of PrP^{Sc} in psoas major, supraspinal (forelimb) or semimembranous (hind limb) muscles. We did not detect PrP^{Sc} in any ARQ/ARQ control sheep (Fig. 1a–c).

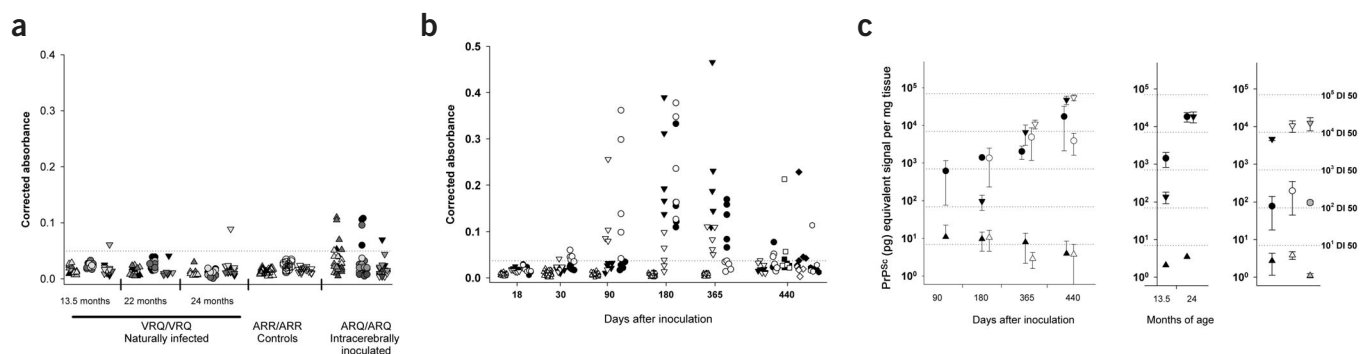


Figure 1 ELISA assessment of PrP^{Sc} accumulation in muscle of sheep naturally infected or inoculated IC or orally with scrapie. **(a)** PrP^{Sc} in semimembranous (▽), psoas (△) and supraspinal (○) muscles from 6 ARQ/ARQ sheep inoculated IC (clinical stage), 7 naturally exposed ARR/ARR sheep (controls) and 12 naturally exposed VRQ/VRQ sheep (4 aliquots each). VRQ/VRQ sheep were killed and their tissue sampled at the preclinical stage (13.5 months), first clinical onset (22 months) and advanced clinical stage (24 months) of disease. **(b)** PrP^{Sc} in ARR/ARR and VRQ/VRQ lambs exposed orally to 5 g scrapie-positive brain 12 h after birth. In VRQ/VRQ sheep, clinical disease onset occurred 440 d.p.e. At six time points after exposure, four ARR/ARR (▽) and two VRQ/VRQ sheep were killed and samples obtained for testing from semimembranous (▽) and supraspinal (○) muscles, plus, in clinically affected animals, tongue (□), diaphragm (◇) and brachial triceps (●). **(c)** Comparative amounts of PrP^{Sc} (pg PrP per mg fresh tissue) determined by reference to a standard curve established with recombinant VRQ PrP using a sandwich immunoassay. In each three-infection model, only animals with PrP^{Sc}-positive muscles were included. Values were determined for muscle (▲) as the mean of all positive samples from one sheep, and for mesenteric lymph node (●) and brainstem (▼) as the mean of five tissue samples per animal. For comparison, amounts of PrP^{Sc} in successive dilutions (dotted lines) of an equivalent brain isolate were determined. Infectious titer in this brain isolate (DI, infective dose) was determined in transgenic mice expressing VRQ ovine PrP (Tg338). Animals are differentiated using a gray color scale.

¹UMR INRA ENVT 1225, Interactions Hôte Agent Pathogène, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles, 31076 Toulouse, France. ²CEA, Service de Pharmacologie et d'Immunologie, DRM, CEA Saclay, 91191 Gif sur Yvette Cedex, France. ³UMR INRA ENVT 181, Pharmacologie Toxicologie Expérimentale, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles, 31076 Toulouse, France. ⁴INRA, Virologie Immunologie Moléculaires, INRA Domaine de Vilvert, 78350 Jouy-en-Josas, France. ⁵INRA Domaine de Langlade, 31450 Pompertuzat, France. Correspondence should be addressed to O.A. (o.andreoletti@envt.fr).

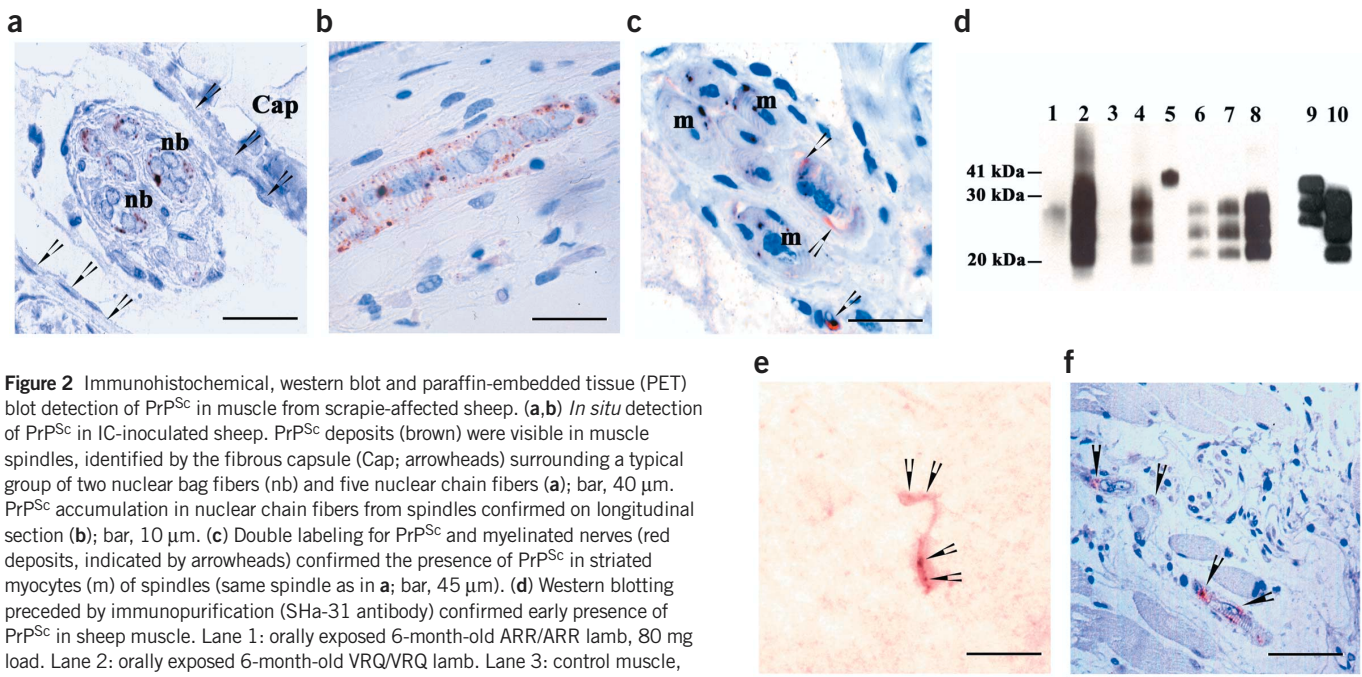


Figure 2 Immunohistochemical, western blot and paraffin-embedded tissue (PET) blot detection of PrP^{Sc} in muscle from scrapie-affected sheep. **(a,b)** *In situ* detection of PrP^{Sc} in IC-inoculated sheep. PrP^{Sc} deposits (brown) were visible in muscle spindles, identified by the fibrous capsule (Cap; arrowheads) surrounding a typical group of two nuclear bag fibers (nb) and five nuclear chain fibers **(a)**; bar, 40 μ m. PrP^{Sc} accumulation in nuclear chain fibers from spindles confirmed on longitudinal section **(b)**; bar, 10 μ m. **(c)** Double labeling for PrP^{Sc} and myelinated nerves (red deposits, indicated by arrowheads) confirmed the presence of PrP^{Sc} in striated myocytes (m) of spindles (same spindle as in **a**; bar, 45 μ m). **(d)** Western blotting preceded by immunopurification (SHA-31 antibody) confirmed early presence of PrP^{Sc} in sheep muscle. Lane 1: orally exposed 6-month-old ARR/ARR lamb, 80 mg load. Lane 2: orally exposed 6-month-old VRQ/VRQ lamb. Lane 3: control muscle, 16 mg. Lane 4: as in lane 2 but 16 mg. Lane 5: molecular weight markers. Lanes 6–8: PrP^{Sc}-positive sheep brain, 0.02, 0.04 and 0.06 mg, respectively. Lane 9: Proteinase K-untreated brain from a control ARR/ARR animal. Lane 10: PK-treated, scrapie-infected brain from a VRQ/VRQ animal, 0.06 mg. **(e,f)** PET blot **(e)**; SHA-31 antibody; bar, 150 μ m) and IHC detection of PrP^{Sc} **(f)**; 8G8 antibody; bar, 75 μ m) in two adjacent sections of tongue from an orally inoculated 12-month-old VRQ lamb. PrP^{Sc} deposits in muscle spindle seen on IHC had a shape similar to that on the PET blot membrane. Coarse granules (arrowheads) identified by IHC were also visible on the PET blot.

We investigated structures accumulating PrP^{Sc} in muscle by IHC⁴. Unexpectedly, we observed PrP^{Sc} deposits in specialized striated myocytes (Fig. 2a–c), which are part of structures known as muscle spindles. We obtained similar images with different antibodies (see **Supplementary Methods** online) and did not observe labeling in any control. Spindles were identified by their typical architecture: 2 nuclear-bag fibers and 4–8 nuclear-chain fibers surrounded by a fibrous capsule (Fig. 2a). In the equatorial region of the spindle, nuclear-bag fibers show an accumulation of nuclei (Fig. 2a), whereas nuclear chain fibers show a typical row of central nuclei (Fig. 2b). In mammals, spindles are highly innervated structures (Fig. 2c) that ensure muscle proprioception. PrP^{Sc} deposition frequency in spindles was roughly correlated with the ELISA results. In ELISA-positive samples, the proportion of positive spindles varied from zero of two to seven of seven structures assessed. In one case, one of four spindles was PrP^{Sc} positive, whereas ELISA gave a negative result. These data, as well as variations in ELISA results (between sample aliquots; Fig. 1a,b), are likely to reflect the heterogeneous distribution of PrP^{Sc}-containing spindles and the paucity of muscle spindles⁵.

IC inoculation has low relevance with regard to natural infection, which probably occurs by the oral route¹. To assess the presence of PrP^{Sc} in muscles from naturally scrapie-infected sheep, we investigated sheep from the Langlade flock (see **Supplementary Methods**) homozygous for the gene encoding the susceptible VRQ variant of PRP. Groups of four infected sheep were killed at 13.5 months (preclinical stage), 22 months (first clinical signs) and 24 months (advanced clinical signs) and their tissues sampled. We detected PrP^{Sc} in both lymphoid and CNS tissues of all VRQ/VRQ sheep we investigated.

In rodent scrapie models, PrP^{Sc} is detected in muscle only in terminally affected animals^{1,2}. In naturally infected sheep, two samples of semimembranous muscle were PrP^{Sc} positive, one at 24 months (after development of clinical disease) but the other at 13.5 months, 8

months before clinical onset (Figs. 1a and 2b). Sciatic nerves and muscle spindles from these sheep showed PrP^{Sc} accumulation. The frequency of PrP^{Sc} deposition in spindles was higher in muscle from clinically affected sheep than from preclinically affected sheep (respectively, five of five and one of four spindles assessed).

The low frequency of PrP^{Sc}-positive samples after natural infection, as compared to IC inoculation, suggests that PrP^{Sc} spreads to muscles less efficiently under natural conditions. However, prion spread to muscle might depend on the infection pressure. To mimic high infection pressure, we exposed sheep orally to a massive dose of prions at birth (see **Supplementary Methods**). Groups of two VRQ/VRQ and four ARR/ARR lambs were sequentially killed at 18, 30, 90, 180 and 365 days post-exposure (d.p.e.) and their tissues sampled. At 440 d.p.e., clinical signs were evident in the last four VRQ/VRQ lambs. In naturally infected VRQ/VRQ sheep from the Langlade flock, clinical onset typically occurs around 680 d. The marked reduction of the incubation period (240 d) reflects fast prion dissemination kinetics in overexposed animals. In the 20 ARR/ARR animals, we did not detect PrP^{Sc} in any tissue by ELISA or observe any labeling in muscle spindles. In VRQ/VRQ lambs, we detected PrP^{Sc} in muscles (Fig. 1b) and nerves by ELISA as early as 90 d.p.e. The early presence of PrP^{Sc} presence in muscle was confirmed by immunoprecipitation and western blotting (180 d) (Fig. 2d). At this age, IHC showed PrP^{Sc}-positive nerves, including intramuscular roots, but no positive spindles. We found positive spindles in 12-month-old sheep (preclinically infected) and in clinically infected VRQ/VRQ sheep. In the latter, PrP^{Sc} was detected by ELISA (Fig. 1b), paraffin-embedded tissue blotting (Fig. 2e) and IHC (Fig. 2f) not only in psoas, supraspinal and semimembranous muscles, but also in tongue and diaphragm—consistent with extensive spreading in muscle tissues.

To estimate infectious titer in sheep with PrP^{Sc}-positive muscle, we measured their PrP^{Sc} content relative to that of an infected brain sam-

ple previously titrated by IC inoculation in Tg338 ovine transgenic mice⁶ (see **Supplementary Methods**). All PrP^{Sc} levels in muscle from naturally infected sheep were <4 pg (equivalent of recombinant PrP) per mg, as compared to >2 × 10⁴ pg per mg of brain or lymph node. When related to the brain infectivity titer, this suggests a 5,000-fold difference in infectivity level (**Fig. 1c**).

This is the first evidence for the presence of PrP^{Sc} in muscle from TSE-incubating animals of a species that enters the human food chain. Dietary exposure to scrapie is currently considered nonhazardous to humans^{7,8}. However, the presence of PrP^{Sc} in muscles from sheep naturally infected with scrapie calls for a review of this question with particular attention to the case of BSE in sheep^{9,10}. The small amounts of PrP^{Sc} in sheep muscle do not significantly alter the human transmission risk, as it is known that lymph nodes present in muscles can bear a significantly higher PrP^{Sc} load¹¹. In addition, our data obtained in sheep cannot be extrapolated to BSE in cattle, which is characterized by very low PrP^{Sc} and infectivity in peripheral tissues.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

The authors thank the INRA domain of Langlade & VLA Weybridge for

producing and breeding the animals used in this study, Bio-Rad for providing the TeSeE sheep-goat kits and G. Hunsmann (German Primate Center, Göttingen, Germany) for providing mouse monoclonal antibody 8G8. This work was supported financially by 'GIS infections à prion' (French research ministry), the European Union (QLK3-CT-2002-01309) and the Midi-Pyrénées Region, France.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 20 February; accepted 5 May 2004

Published online at <http://www.nature.com/naturemedicine/>

1. Thomzig, A., Kratzel, C., Lenz, G., Kruger, D. & Beekes, M. *EMBO Rep.* **4**, 530–533 (2003).
2. Bosque, P.J. *et al. Proc. Natl. Acad. Sci. USA* **99**, 3812–3817 (2002).
3. Glatzel, M., Abela, E., Maissen, M. & Aguzzi, A. *N. Engl. J. Med.* **349**, 1812–1820 (2003).
4. Andreoletti, O. *et al. J. Histochem. Cytochem.* **50**, 1357–1370 (2002).
5. Watanabe, K. & Suzuki, A. *Okajimas Folia Anat. Jpn.* **76**, 203–2019 (1999).
6. Vilotte, J.L. *et al. J. Virol.* **75**, 5977–5984 (2001).
7. Brown, P., Cathala, F., Raubertas, R.F., Gajdusek, D.C. & Castaigne, P. *Neurology* **37**, 895–904 (1987).
8. van Duyn, C.M. *et al. Lancet* **351**, 1081–1085 (1998).
9. Kao, R.R. *et al. Science* **295**, 332–335 (2002).
10. Ferguson, N.M., Ghani, A.C., Donnelly, C.A., Hagenaars, T.J. & Anderson, R.M. *Nature* **415**, 420–424 (2002).
11. van Keulen, L.J. *et al. J. Clin. Microbiol.* **34**, 1228–1231 (1996).