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## The bank vole (*Myodes glareolus*) as a sensitive bioassay for sheep scrapie

Michele Angelo Di Bari,<sup>1</sup> Francesca Chianini,<sup>2</sup> Gabriele Vaccari,<sup>1</sup> Elena Esposito,<sup>1</sup> Michela Conte,<sup>1</sup> Samantha L. Eaton,<sup>2</sup> Scott Hamilton,<sup>2</sup> Jeanie Finlayson,<sup>2</sup> Philip J. Steele,<sup>2</sup> Mark P. Dagleish,<sup>2</sup> Hugh W. Reid,<sup>2</sup> Moira Bruce,<sup>3</sup> Martin Jeffrey,<sup>4</sup> Umberto Agrimi<sup>1</sup> and Romolo Nonno<sup>1</sup>

### Correspondence

Romolo Nonno  
romolo.nonno@iss.it

<sup>1</sup>Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

<sup>2</sup>Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK

<sup>3</sup>Neuropathogenesis Unit, Roslin Institute, Ougston Building, West Mains Road, Edinburgh EH9 3JF, UK

<sup>4</sup>Veterinary Laboratories Agency (VLA-Lasswade), Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK

Despite intensive studies on sheep scrapie, a number of questions remain unanswered, such as the natural mode of transmission and the amount of infectivity which accumulates in edible tissues at different stages of scrapie infection. Studies using the mouse model proved to be useful for recognizing scrapie strain diversity, but the low sensitivity of mice to some natural scrapie isolates hampered further investigations. To investigate the sensitivity of bank voles (*Myodes glareolus*) to scrapie, we performed end-point titrations from two unrelated scrapie sources. Similar titres [ $10^{5.5}$  ID<sub>50</sub> U g<sup>-1</sup> and  $10^{5.8}$  ID<sub>50</sub> U g<sup>-1</sup>, both intracerebrally (i.c.)] were obtained, showing that voles can detect infectivity up to 3–4 orders of magnitude lower when compared with laboratory mice. We further investigated the relationships between PrP<sup>Sc</sup> molecular characteristics, strain and prion titre in the brain and tonsil of the same scrapie-affected sheep. We found that protease-resistant PrP<sup>Sc</sup> fragments (PrP<sup>res</sup>) from brain and tonsil had different molecular features, but induced identical disease phenotypes in voles. The infectivity titre of the tonsil estimated by incubation time assay was  $10^{4.8}$  i.c. ID<sub>50</sub> U g<sup>-1</sup>, i.e. fivefold less than the brain. This compared well with the relative PrP<sup>res</sup> content, which was 8.8-fold less in tonsil than in brain. Our results suggest that brain and tonsil harboured the same prion strain showing different glycoprofiles in relation to the different cellular/tissue types in which it replicated, and that a PrP<sup>Sc</sup>-based estimate of scrapie infectivity in sheep tissues could be achieved by combining sensitive PrP<sup>res</sup> detection methods and bioassay in voles.

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## INTRODUCTION

Scrapie of sheep and goat is a transmissible spongiform encephalopathy (TSE) or prion disease. The key feature of this group of fatal neurodegenerative diseases is represented by the post-translational modification of the cellular prion protein, PrP<sup>C</sup>, into an abnormal protease-resistant isoform named PrP<sup>Sc</sup>. In TSEs, the accumulation of PrP<sup>Sc</sup> in tissues of infected individuals usually correlates with the presence of infectivity (McKinley *et al.*, 1983; Race *et al.*, 2001).

Although no evidence exists that scrapie transmits to humans, sheep tissues containing the highest levels of

infectivity are excluded from human consumption, as a precautionary measure. The Regulation (EC) 999/2001 (European Parliament, 2001) includes the spleen and ileum of sheep and goats of all ages as specified risk material (SRM), whilst the skull, including brain, eyes, tonsils and spinal cord are also considered SRM in animals over 12 months. This approach is based on available knowledge, but there is an increasing need to refine and optimize it by updating information on infectivity in other tissues such as skeletal muscle and viscera.

Natural sheep scrapie represents the prototype of TSEs and an appropriate model for acquiring knowledge on the infectivity of tissues. The occurrence of natural and experimental scrapie has been linked to polymorphisms

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at codons 136 (A/V), 154 (R/H) and 171 (Q/R) of the PrP gene (Belt *et al.*, 1995). Sheep carrying the V136/R154/Q171 (or VRQ) allele are considered the most susceptible to scrapie, while the ARR allele confers resistance. In sheep breeds, like Suffolk and Sarda sheep, which lack the VRQ allele, the ARQ/ARQ genotype is considered the most susceptible to scrapie (Agrimi *et al.*, 2003; Baylis & Goldmann, 2004).

Early research on tissue distribution of infectivity in sheep affected by scrapie relied on bioassays in small ruminants or mice (reviewed by Detwiler & Baylis, 2003). These studies suggested an early replication of the agent in the lymphoreticular and peripheral nervous tissues before entering the brain, and provided the basis for the categorization of sheep tissues in relation to the levels of infectivity (World Health Organization, 2006). However, it should be noted that these animal models present some critical limitations. Bioassays in sheep are hampered by the impractical use of large animals. On the other hand, data produced by the use of the mouse model also has serious limitations, considering the inefficient or even unsuccessful transmission sometimes observed following experimental inoculation of natural scrapie (Bruce *et al.*, 2002). The transmission of scrapie isolates from ARQ/ARQ sheep to wild-type mice has been reported to be difficult (Bruce *et al.*, 2002; Piening *et al.*, 2006). This also suggests that the actual distribution and quantification of infectivity in tissues of scrapie-affected sheep could be underestimated or biased by the different susceptibility of animal models to the various scrapie strains.

After the discovery of PrP<sup>Sc</sup> as a marker of infection (Bendheim *et al.*, 1984), rapid methods for detecting scrapie infection by Western-blot and ELISA became available. Recent studies conducted by these means reveal a widespread distribution of PrP<sup>Sc</sup>, including skeletal muscles (Andréoletti *et al.*, 2004), tongue (Casalone *et al.*, 2005), placenta (Race *et al.*, 1998), kidneys (Sisó *et al.*, 2006; Ligios *et al.*, 2007), salivary glands (Vascellari *et al.*, 2007) and skin (Thomzig *et al.*, 2007). Although PrP<sup>Sc</sup> is believed to be the most useful marker of TSEs, the relationship between the amount of PrP<sup>Sc</sup> and the level of infectivity of a given tissue is not known, making it difficult for policy makers to derive meaningful information from studies relying solely on PrP<sup>Sc</sup> detection. Bioassays remain the only effective tools for detecting and quantifying TSE agents. Furthermore, the recent discovery of infectivity in biological fluids in which PrP<sup>Sc</sup> has never been reported, such as the blood of scrapie-affected sheep (Hunter *et al.*, 2002) and the saliva of elk with chronic wasting disease (CWD) (Mathiason *et al.*, 2006), emphasizes the need for more sensitive and rapid bioassays for scrapie.

Recent studies on transgenic mice overexpressing PrP homologous to the donor species have demonstrated that such expression can abrogate the species barrier (Scott *et al.*, 1989). These transgenic mouse models offer improved bioassay sensitivity for human, bovine and

cervid TSE infections (Safar *et al.*, 2002, 2005; Buschmann & Groschup, 2005; Angers *et al.*, 2006).

With the aim of improving bioassay sensitivity for sheep scrapie, transgenic mice overexpressing either the ARQ or the VRQ ovine prion protein alleles have recently been generated (Crozet *et al.*, 2001; Vilotte *et al.*, 2001; Cordier *et al.*, 2006). These mouse models were shown to abrogate the species barrier when challenged with scrapie, and allowed the transmission of scrapie isolates with different PrP genotypes, irrespective of the homology with the transgene used in the mouse, in that both ARQ and VRQ transgenic models allowed the transmission of scrapie isolates from either ARQ- or VRQ-bearing sheep (Vilotte *et al.*, 2001; Beringue *et al.*, 2006; Baron & Biacabe, 2007). The efficiency of transmission, however, was reported to be highly variable depending on the isolate, with incubation times ranging from less than 100 days for a VRQ/VRQ isolate (Vilotte *et al.*, 2001) up to 2 years for some ARQ/ARQ isolates (Beringue *et al.*, 2006).

Recently we have used the bank vole (*Myodes glareolus*, formerly *Clethrionomys glareolus*) as a new and efficient model for bioassay of TSEs from different species, including man, sheep, goat, mouse and hamster (Cartoni *et al.*, 2005; Piening *et al.*, 2006; Nonno *et al.*, 2006; Agrimi *et al.*, 2008).

In this study, we report that voles are highly sensitive to ARQ/ARQ and ARQ/VRQ scrapie isolates, one of which has been shown to be refractory to transmission in classical murine strains, thus allowing a relatively fast quantification of infectivity in non-nervous tissues of scrapie-infected sheep. Furthermore, we report the transmission of ARQ/ARQ scrapie isolates from a flock of Suffolk sheep with endemic scrapie, showing that voles are efficiently infected by scrapie isolates which are poorly or not transmissible to conventional and transgenic mouse models.

## METHODS

**Terminology.** Throughout the manuscript we use 'prion' to refer to the infectious agent of TSEs, 'PrP<sup>Sc</sup>' to refer to the abnormal disease-associated PrP isoform and 'PrP<sup>res</sup>' to refer to protease-resistant PrP<sup>Sc</sup> fragments.

**Scrapie samples.** In the present study five different scrapie sources were inoculated in voles (Table 1). Of these, two (SSTB and SST7) originated from the brain and the tonsils of the same sheep; two (SCR6 and SCR1) were brain isolates from individual sheep whose transmissions in mice have been previously reported (Bruce *et al.*, 2002); the last (SSUK6) was a brain pool.

SS7B and SS7T were obtained from an ARQ/ARQ Sarda sheep with clinical scrapie reported in Italy in 1997.

SCR6 and SSUK6 were obtained from the same UK flock of Suffolk sheep with endemic scrapie. SCR6 was from a clinically affected sheep diagnosed in 1992, while SSUK6 originated from a pool of brains collected in 2002 from 10 clinically affected sheep.

Finally, SCR1 was obtained from a scrapie-affected ARQ/VRQ Grayface sheep collected in the UK in 1985.

**Table 1.** Survival times of voles and C57Bl/6 mice infected with natural scrapie isolates

ND, Not determined.

Recipient species	Scrapie source	Primary transmission				Second passage		
		Clin. signs (+)	Pathol. (+)	PrP <sup>Sc</sup> (+)	Survival time (days ± SD)	Transm. rate (%)	(+)/Inoculated	Survival time (days ± SD)
Bank vole	SS7B	14/14	14/14	14/14	188 ± 33	100	10/10	102 ± 6
	SS7T	10/10	10/10	10/10	211 ± 39	100	14/14	101 ± 7
	SCR6	18/18	18/18	18/18	197 ± 19	100	11/11	98 ± 4
	SSUK6	22/22	22/22	22/22	175 ± 18	100	11/11	96 ± 4
	SCR1	18/18	18/18	18/18	145 ± 9	100	ND	ND
C57Bl/6 mice	SS7B	0/6	2/6	4/6	730 ± 10	66	ND	ND
	SS7T	0/7	0/7	1/7	718	14	ND	ND

**Transmission experiments.** The inocula from SS7B, SS7T, SCR1 and SCR6 were prepared as 10% w/v homogenates (10<sup>-1</sup> dilution) in PBS. For SS7B and SCR1, serial tenfold dilutions in PBS (10<sup>-1</sup> – 10<sup>-6</sup>) were prepared and used for end-point titration into voles. For the preparation of tonsil homogenate (SS7T), the tonsil was carefully isolated free of surrounding tissues under a dissecting microscope. The isolated tonsil was cut into small pieces and homogenized in a Teflon-glass potter.

The brain pool SSUK6 was prepared as a 20% homogenate in 0.32 M sucrose solution. Inocula for second passages were prepared as 10% homogenates in PBS from the brain of individual voles culled at the terminal stage of the disease.

The research protocol has been approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health, according to Legislative Decree 116/92 (Decreto Legislativo, 1992), which has implemented in Italy the European Directive 86/609/EEC (Council of the European Communities, 1986) on laboratory animal protection. Bank voles (Istituto Superiore di Sanità breeding colony) and C57Bl/6 mice (Charles River, Como, Italy) were housed in standard cages and treated according to Legislative Decree 116/92 guidelines, and animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal Welfare. All animals were individually identified by a passive integrated transponder.

Groups of 12–22 8-weeks-old voles and C57Bl/6 mice were injected by the intracerebral (i.c.) route (20 µl) into the left cerebral hemisphere under ketamine anaesthesia (0.1 µg ketamine g<sup>-1</sup>). Beginning 1 month after inoculation, animals were examined twice per week until the appearance of neurological signs, and were then examined daily. Diseased animals were culled with carbon dioxide at the terminal stage of disease, but before neurological impairment was such as to compromise welfare and, especially, adequate drinking and feeding. Survival time was calculated as the interval between inoculation and culling. Using the vole model, ID<sub>50</sub> per gram of SS7B and SCR1 was determined by end-point titration according to the Spearman and Kärber method (Hamilton *et al.*, 1977). Survivors were animals surviving to 900 days post-infection (p.i.) (SS7B) or 700 days p.i. (SCR1) with no signs of infection (confirmed by post-mortem analysis of brain by Western blot and immunohistochemical detection). Animals culled for intercurrent disease were analysed for brain PrP<sup>Sc</sup> and excluded from analysis when negative. The infectivity titre of SS7T was estimated by the incubation time assay, using mean survival times derived from SS7B end-point titration, best fitted to an exponential decay curve (GraphPad Prism software).

**Western Blot.** SS7T and SS7B inocula (10% w/v homogenates in PBS) were added with PBS/sarcosyl up to a final 2% sarcosyl (Sigma) concentration. As a reference material (SBH) we prepared serial twofold dilutions of a scrapie brain homogenate (10% w/v homogenate in PBS with 2% sarcosyl from the medulla of an ARQ/ARQ sheep with clinical scrapie) in 10% normal brain homogenate. All samples were incubated for 20 min at room temperature and then digested with proteinase K (200 µg ml<sup>-1</sup>) for 1 h at 37 °C with gentle shaking. Protease treatment was stopped with 3 mM PMSF.

Brain homogenates (10% w/v) from individual voles were prepared in 100 mM Tris/HCl, pH 7.4, containing 2% sarcosyl, incubated for 20 min at room temperature and then digested with proteinase K (50 µg ml<sup>-1</sup>) for 1 h at 37 °C with gentle shaking. Protease treatment was stopped with 3 mM PMSF.

Electrophoresis and Western blotting were performed as described previously (Nonno *et al.*, 2006). Vole PrP<sup>res</sup> was detected with the monoclonal antibody SAF84 (0.8 µg ml<sup>-1</sup>; epitope at aa 163–173 of the sheep PrP sequence), while the monoclonal antibody P4 (0.2 µg ml<sup>-1</sup>, epitope at aa 93–98) was used for sheep PrP<sup>res</sup> because of its higher sensitivity, which was necessary to properly detect PrP<sup>res</sup> in tonsil samples. Both antibodies were incubated for 1 h at room temperature. The membranes were developed with an enhanced chemiluminescence method (SuperSignal Femto, Pierce). Chemiluminescence was detected with the VersaDoc imaging system (Bio-Rad). Apparent molecular mass and glycoform patterns were determined with QuantityOne software (Bio-Rad). For the analysis of SS7B and SS7T PrP<sup>res</sup> content, chemiluminescence signals from the SBH dilution series along with SS7B and SS7T were quantified with QuantityOne software.

**Histopathology, immunohistochemistry and paraffin-embedded tissue (PET) blot.** At post-mortem, each brain was divided into two parts by a sagittal paramedian cut. The smaller portion was immediately frozen and stored at –20 °C for Western blotting. The remaining part was immersed and fixed in 10% formol saline for 4 days. The brains were trimmed at standard coronal levels, decontaminated with formic acid for 1 h, and embedded in paraffin. Sections (6 µm thick each) were cut for haematoxylin and eosin staining, immunohistochemistry and PET blot, randomly mixed and coded for blind pathological assessment.

For the construction of lesion profiles, vacuolar changes were scored in nine grey-matter areas of the brain on haematoxylin and eosin-stained sections, as described previously (Fraser & Dickinson, 1968; Nonno *et al.*, 2006). Vacuolation scores are derived from the examination of at least six voles per group.



PET blot and PrP immunohistochemistry were performed as described previously (Nonno *et al.*, 2006).

For glial fibrillary acidic protein (GFAP) immunolabelling, deparaffinized sections were treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min at room temperature to abolish endogenous peroxidase activity and, after heat-induced antigen retrieval (citrate buffer 0.01 M, pH 6.0, 1 × 5 min in microwave oven, 750 W), incubated 30 min with polyclonal rabbit anti-cow GFAP (1:500, Dako-Cytomation). Immunoreactivity was detected by the avidin-biotin-complex (ABC) method as suggested by the suppliers.

In each immunohistochemistry run, positive and negative control sections were included.

## RESULTS

### High sensitivity of voles to scrapie prions

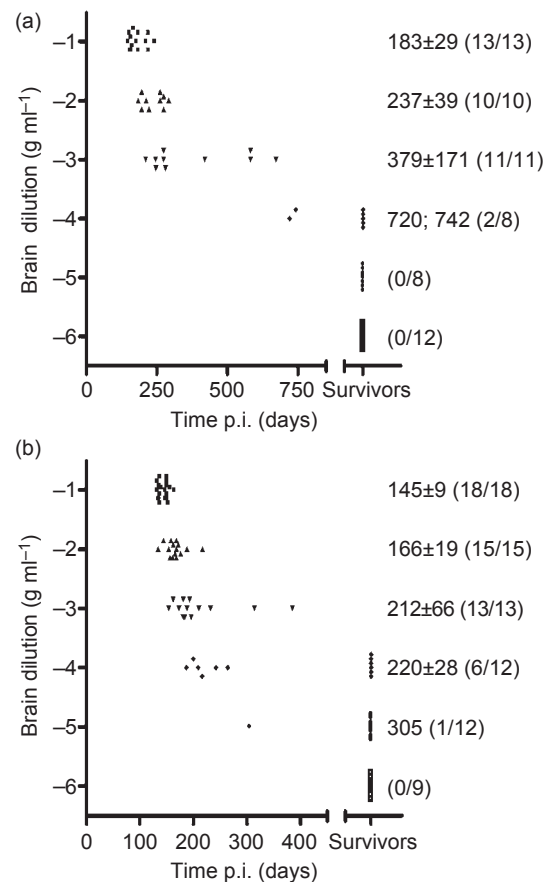
All voles inoculated with 10<sup>-1</sup> dilution of an ARQ/ARQ source of scrapie (SS7B) showed overt clinical signs and succumbed to scrapie with a mean survival time of 188 ± 33 days p.i. (mean ± SD) (Table 1). Increasing survival times were observed with 10<sup>-2</sup> and 10<sup>-3</sup> dilutions, and dilutions greater than 10<sup>-3</sup> showed a decreasing attack rate (Fig. 1a). The infectivity titre of SS7B was 10<sup>5.5</sup> i.c. ID<sub>50</sub> U g<sup>-1</sup>.

Transmission of a ARQ/VRQ sheep scrapie isolate (named SCR1) to voles was rapid and efficient, with a survival time of 145 ± 9 days p.i. upon inoculation of 10<sup>-1</sup> dilution (Table 1) and 100% attack rate up to 10<sup>-3</sup> dilution. The SCR1 isolate contained 10<sup>5.8</sup> i.c. ID<sub>50</sub> U g<sup>-1</sup> (Fig. 1b).

Given the high sensitivity observed, we attempted the transmission from the tonsil (SS7T) of the same ARQ/ARQ scrapie-affected sheep (SS7B) used in titration experiments.

Following inoculation of SS7T, 100% of bank voles developed clinical signs of disease, spongiform change and PrP<sup>Sc</sup> accumulation in the brain. The survival time was 211 ± 39 days p.i. (Table 1). Clinical signs were similar in voles inoculated either with the brain or the tonsil. The first signs of disease were hyperactivity/reactivity followed by the progressive disappearance of the typical hiding behaviour. Overt neurological signs appeared later on, consisting of characteristic head bobbing, accompanied by severe and progressive ataxia of the forelimbs. The bobbing movements of the head increased progressively and may have resulted from visual difficulties. Hunched posture, apathy and pronounced hypoactivity/reactivity preceded sacrifice or death, which occurred 15–20 days after the beginning of neurological signs.

C57Bl/6 mice were much less susceptible than voles to the same inocula from ARQ/ARQ sheep (Table 1). They did not show obvious clinical signs, although some animals were culled after showing hyporeactivity, weight loss and gait abnormalities. Since these signs were not specific and often accompanied by the occurrence of age-related diseases (tumours and skin lesions), it was impossible to recognize evidence of scrapie on clinical grounds. However,



**Fig. 1.** End-point titration of scrapie infectivity in voles. Serial dilutions of SS7B (a) and SCR1 (b) scrapie isolates were inoculated i.c. in voles. Symbols represent individual survival times. The diseased voles were positive for brain PrP<sup>res</sup>. Inoculated voles that were not infected after 900 (a) or 700 days p.i. (b) are plotted in compressed form after the x axes break point. Voles culled with intercurrent disease are not included. The mean survival time (days ± SD) and the number of diseased/inoculated voles are indicated on the right of each chart.

based on PrP<sup>Sc</sup> accumulation and/or spongiform change, four out of six and one out of seven animals, inoculated with SS7B and SS7T, respectively, were identified as positive after very long survival times (Table 1).

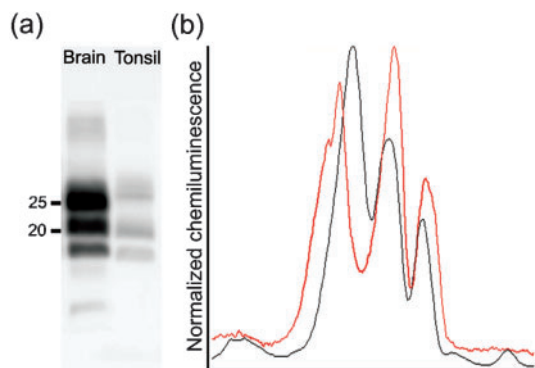
### Brain and tonsil isolates show similar biological properties, despite their discordant molecular phenotype

It has been reported that PrP<sup>res</sup> isolated from lymphoid tissues of sheep with scrapie displays electrophoretic differences compared to brain PrP<sup>res</sup> (Madec *et al.*, 2000). We investigated the molecular properties of PrP<sup>res</sup> from brain (SS7B) and tonsil (SS7T) isolates by Western blotting. In agreement with previous findings (Madec

*et al.*, 2000), we observed that the electrophoretic profile and the glycoform ratio of tonsil PrP<sup>res</sup> were distinct from that of those found in brain (Fig. 2). The apparent molecular mass of diglycosylated PrP<sup>res</sup> was indeed ~1 kDa higher in tonsil, while monoglycosylated and unglycosylated fragments were ~0.5 kDa lower. The glycoform pattern was characterized by the typical di >mono >unglycosylated glycoform pattern in the brain, while in the tonsil monoglycosylated PrP<sup>res</sup> was the most abundant (Fig. 2b). Similar findings were obtained with lymphoid tissues from several sheep with natural or experimental scrapie (R. Nonno, unpublished results).

In spite of these molecular differences, scrapie isolates from brain and tonsil transmitted to voles with similar clinical signs and survival times. Western blot analysis of PrP<sup>res</sup> from the brain of voles of the two groups showed the same molecular pattern (Fig. 3a), reminiscent of that observed in the sheep brain isolate. Lesion profiles were also similar (Fig. 3b).

In order to further compare their biological properties, we thus set up subsequent transmission in voles. Upon second passage, survival time in vole decreased to  $102 \pm 6$  days p.i. for SS7B and  $101 \pm 7$  days p.i. for SS7T (Table 1). Western blot analysis of PrP<sup>res</sup> from the brain of voles of the two groups showed the same molecular profile (Fig. 3c), which was also identical to that observed after primary passage (compare Fig. 3a and c). Lesion profiles on second passages showed some differences compared to primary passage (compare Fig. 3b and d), suggestive of adaptation of these isolates to voles (Kimberlin *et al.*, 1987). Notwithstanding, remarkably similar lesion profiles were obtained from voles inoculated with the second passage of SS7B and SS7T (Fig. 3d).



**Fig. 2.** Different PrP<sup>res</sup> profiling in brain and tonsil homogenates. (a) Western blot analysis of PrP<sup>res</sup> from brain and tonsil homogenates used for bioassay (SS7B and SS7T). PrP<sup>res</sup> was detected with mAb P4. The positions of molecular mass markers (kDa) are indicated. (b) Blot scans from the immunoblot in (a), measured along the lanes of brain (black line) and tonsil (red line). The chemiluminescence signals were normalized taking the maximum value in each scan as 100 %.

### Relative infectivity titres in brain and tonsil correlate with relative PrP<sup>Sc</sup> levels

To quantify the relative amount of PrP<sup>res</sup> in SS7B an SS7T, a dilution curve of a reference brain homogenate (SBH) from the medulla oblongata of a sheep with clinical scrapie was constructed. The amount of PrP<sup>res</sup> in 0.5 mg equivalent of SS7B and SS7T, derived by plotting their chemiluminescence signals on the linear regression of SBH dilutions, was calculated as equivalent to the PrP<sup>res</sup> content of 0.160 and 0.018 mg equivalent of SBH, respectively (Fig. 4). These results show that SS7T contains 8.8-fold less PrP<sup>res</sup> than SS7B.

We then estimated the infectivity titre of tonsil (SS7T) by the incubation time assay, based on the survival times obtained in the end-point titration of the brain of the same sheep (SS7B). The titre estimated in the tonsil homogenate was  $10^{4.8}$  i.c. ID<sub>50</sub> U g<sup>-1</sup> (Fig. 5), i.e. fivefold lower compared with that in the brain (SS7B). This estimate correlates quite well with the relative levels of PrP<sup>res</sup> found in tonsil and brain isolates.

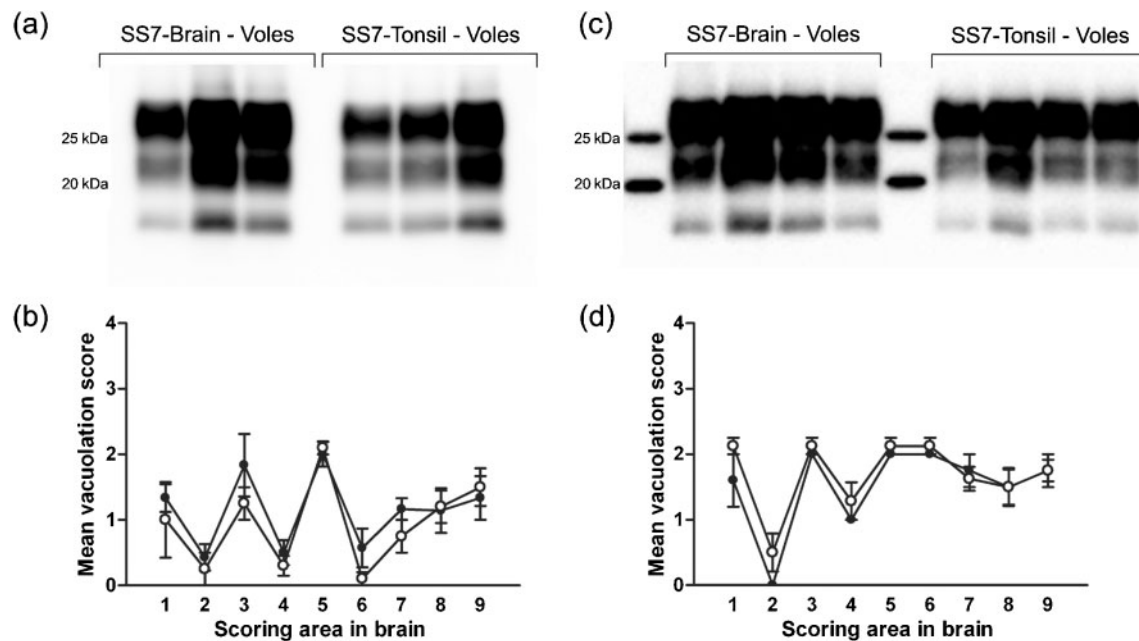
### Bank voles are susceptible to natural Suffolk scrapie isolates

The natural Suffolk scrapie source investigated derives from a single closed flock managed under near commercial conditions and which has had endemic scrapie since 1990. The flock has been intensively monitored over this period and presents a consistent, uniform and distinctive clinicopathological phenotype in ARQ/ARQ genotype sheep (Hunter *et al.*, 1997; Gonzalez *et al.*, 2003), suggesting that infection is maintained in the flock by a single, stable sheep scrapie strain. Scrapie-affected sheep from this flock thus represent a convenient source of infected material for studying and quantifying the tissue distribution of scrapie prions in sheep.

We investigated the susceptibility of voles to the above-mentioned scrapie source by means of two different scrapie isolates from the same flock: SCR6, an isolate collected in 1992 and already reported as being poorly transmissible to mice (Bruce *et al.*, 2002), and SSUK6, a brain pool prepared in 2002.

All inocula gave 100 % transmission rate in voles. Mean survival times of voles inoculated with SCR6 and SSUK6 were  $197 \pm 19$  and  $175 \pm 18$  days p.i., respectively. Upon second passage, survival times decreased to 95–100 days p.i. with both isolates, similarly to what was previously observed with SS7B and SS7T (Table 1). Voles developed clinical disease with features indistinguishable from those of SS7B and SS7T. We thus compared the pathological phenotype induced by these ARQ/ARQ isolates in voles.

Upon second passages of SCR6, SSUK6, SS7B and SS7T, PrP<sup>Sc</sup> was abundant throughout the brain and showed a similar distribution in all groups of voles. Prominent immunolabelling was always observed in the frontal and



**Fig. 3.** Similar molecular and pathological phenotypes in voles after primary and second passages of brain and tonsil isolates. (a) and (c) Western blot analysis of PrP<sup>res</sup> from brain of individual voles after primary (a) or second (c) passage with SS7B and SS7T. PrP<sup>res</sup> was detected with mAb SAF84. The molecular mass of markers is indicated. (b) and (d) Lesion profiles in voles after primary (b) or second (d) passage with SS7B (closed circles) and SS7T (open circles). Brain-scoring positions are medulla (1), cerebellum (2), superior colliculus (3), hypothalamus (4), thalamus (5), hippocampus (6), septum (7), retrosplenial and adjacent motor cortex (8) and cingulate and adjacent motor cortex (9).

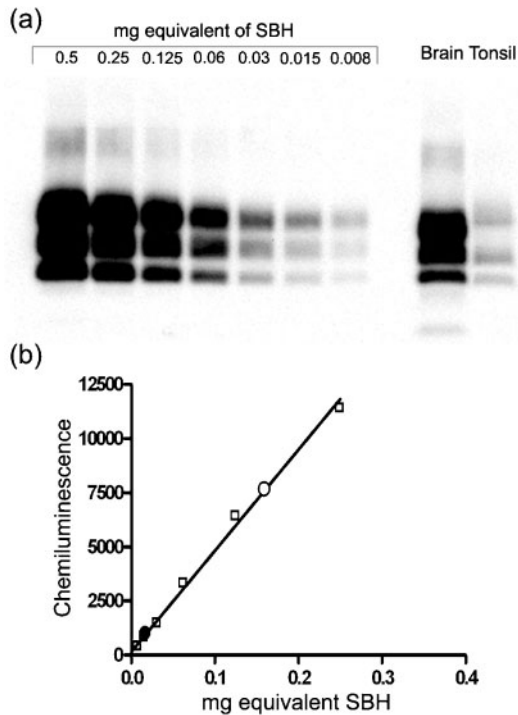
parietal cortices, septal nucleus, hippocampus, thalamus, superior colliculi, geniculate nuclei, medulla oblongata, vestibular nuclei and cerebellar cortex. In all voles, distinctive features of PrP<sup>Sc</sup> deposition were the accumulation as focal deposits in all layers of the hippocampus, the involvement of the stratum lacunosum moleculare and the presence of focal PrP<sup>Sc</sup> deposits in the molecular layer of the dentate gyrus (Fig. 6a, b). In several individual brains, prominent PrP<sup>Sc</sup> aggregates were identified along the alveus by immunohistochemistry and PET blot analysis (Fig. 6a, b). Spongiform neurodegeneration was prominent in all affected voles, accompanied by gliosis and neuronal loss (Fig. 6c). There was evidence of diffuse astrocytosis, revealed by GFAP immunohistochemical staining (Fig. 6d). In several areas, including CA1 tract of the hippocampus, there was a striking correlation between the severity of vacuolation, gliosis, neuronal loss and the extent of PrP<sup>Sc</sup> deposition (Fig. 6). Lesion profiles of SCR6 and SSUK6 (Fig. 7) were remarkably similar to those obtained after second passages of SS7B and SS7T (Fig. 3d).

## DISCUSSION

Despite intensive studies on sheep scrapie, a number of crucial questions remain unanswered, such as the natural mode of transmission, the amount of infectivity which accumulates in edible tissues at different stages of scrapie

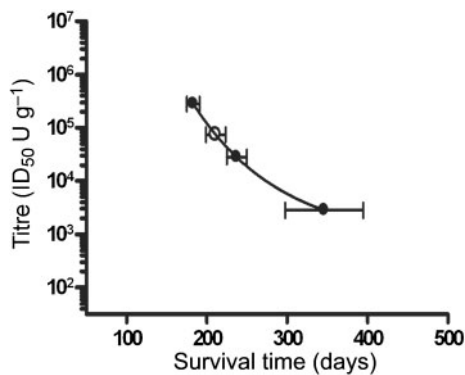
infection and the extent of strain diversity in the field. Studies using the mouse model for bioassay have proved to be useful for recognizing scrapie strain diversity, but the low sensitivity of mice to some scrapie sources and the variable susceptibility to natural scrapie isolates limit the utility of mouse bioassays. In this study, we report that the bank vole represents a sensitive bioassay for the detection and quantification of scrapie infectivity in sheep tissues. The availability of an animal model which allows fast and sensitive replication of scrapie prions will bring new opportunities for the study of strains and for the accurate measurement of infectivity.

Of note, several Italian scrapie isolates which transmitted easily to voles, but not to mice (Piening *et al.*, 2006), including the ones used in the present study (SS7B and SS7T), were previously reported to transmit quite inefficiently to tg338 transgenic mice harbouring the VRQ PrP allele, being the group of isolates with the longest incubation time in tg338 mice (Beringue *et al.*, 2006). Furthermore, the transmission of the same Suffolk scrapie isolate used in the present study was also attempted to tg338 mice, showing a similarly inefficient transmission (H. Laude, Virologie et Immunologie Moléculaires, INRA, Jouy-en-Josas, France, personal communication). In contrast, our results show that voles allow a fast and efficient replication of these ARQ/ARQ isolates, suggesting that this new laboratory model can be useful for the study of a



**Fig. 4.** Quantification of PrP<sup>res</sup> in the tonsil homogenate used for bioassay. (a) Western blot analysis of PrP<sup>res</sup> from brain and tonsil homogenates (0.5 mg equivalent/lane) along with twofold dilutions of a reference brain homogenate (SBH). PrP<sup>res</sup> was detected with mAb P4. (b) Linear regression of chemiluminescence signal measured from the dilution curve of SBH (open squares). Interpolated  $x$  values for SS7B (open circle) and SS7T (closed circle) were 0.16 and 0.018 mg equivalent of SBH, respectively.

scrapie strain harboured by both Italian and UK ARQ/ARQ sheep. However, we cannot exclude that the advantage of voles compared to other models might be limited to some



**Fig. 5.** Determination of prion titre in tonsil by incubation time assay. Non-linear regression analysis prion titre against mean survival time ( $\pm$  SEM) of voles inoculated with serial dilutions of SS7B (closed circles). Interpolated  $y$  value for SS7T (open circle) was  $10^{4.8}$  i.c. ID<sub>50</sub> U g<sup>-1</sup>.

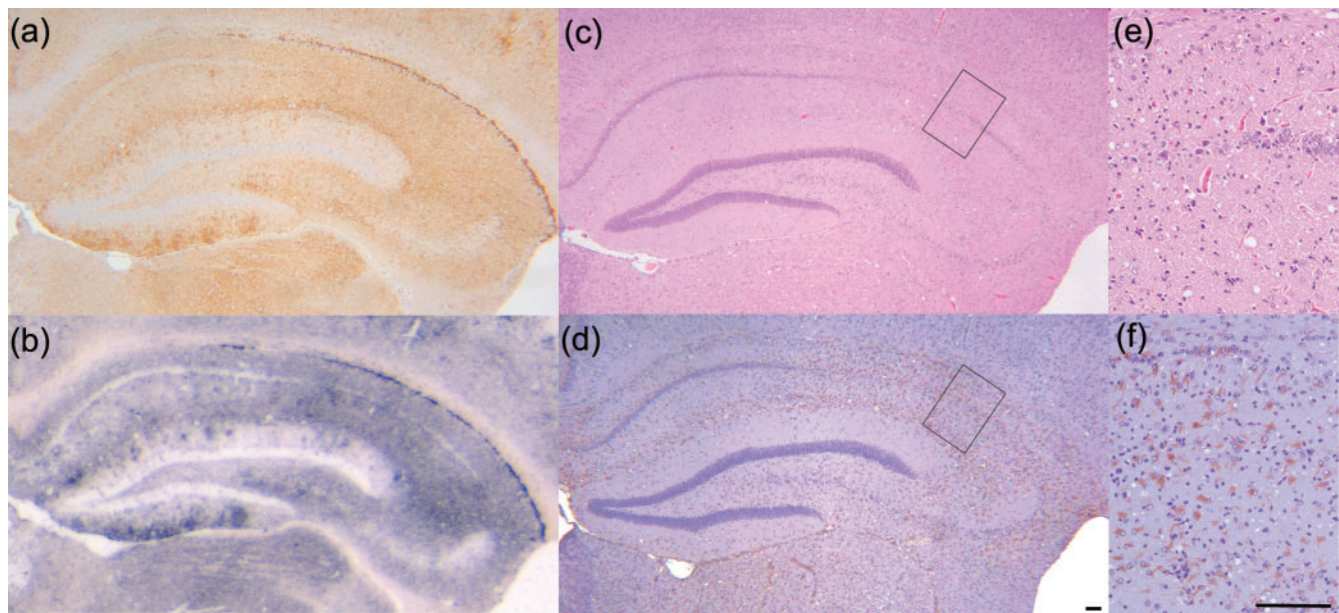
particular scrapie strains. Larger comparative studies are required to investigate this issue.

Regarding the sensitivity of detection of low amounts of sheep scrapie prions, limited data have been published to date from transgenic mouse models expressing ovine PrP. While the abrogation of the species barrier is expected to endow ovine transgenic mouse models with an improved sensitivity compared to wild-type mice, PrP overexpression per se was reported to have no effect on limits of detection (Fischer *et al.*, 1996). Titration experiments performed in transgenic mice expressing human or bovine PrP proved that these models are useful for the detection of low amounts of sporadic Creutzfeldt–Jakob (sCJD) and bovine spongiform encephalopathy (BSE) prions, respectively (Safar *et al.*, 2002, 2005, Buschmann & Groschup, 2005). The only titration experiment in an ovine transgenic mouse model reported up to now shows that tg338 mice expressing the sheep VRQ allele are able to measure  $10^{6.6}$  ID<sub>50</sub> g<sup>-1</sup> in a VRQ/VRQ scrapie isolate (Andréoletti *et al.*, 2004). This is only five times higher than the titre measured in the present study with the ARQ/VRQ SCR1 isolate ( $10^{5.8}$  ID<sub>50</sub> g<sup>-1</sup>). Although the biological variability of scrapie isolates does not allow a direct comparison of titration experiments performed with different isolates, the data obtained with voles suggest that this model may complement ovine transgenic models for bioassay of scrapie prions, particularly for ARQ/ARQ scrapie isolates.

Based on the data obtained by end-point titration, we expect that voles would be able to detect prions in tissues which contain up to 3–4 orders of magnitude less infectivity than the brain. In contrast, wild-type mice were barely susceptible to undiluted brain homogenates. This finding indicates that previous negative attempts to quantify scrapie prions in edible tissues, such as muscles and kidneys, or animal products, such as milk, may have underestimated the infectivity titres. Recently, the use of sensitive diagnostic methods has allowed the detection of PrP<sup>Sc</sup> in tissues such as skeletal muscles, kidneys, salivary glands and mammary glands (Andréoletti *et al.*, 2004; Sisó *et al.*, 2006; Ligios *et al.*, 2005, 2007; Vascellari *et al.*, 2007), and for which there was no previous evidence of scrapie infectivity (Detwiler & Baylis, 2003). Interestingly, PrP<sup>res</sup> quantification in these tissues was reported to be 2–4 log lower than in the brain, suggesting that bioassay in vole should be able to quantify their infectivity. The quantification of infectivity present in such tissues from sheep with pre-clinical and clinical scrapie is of crucial importance for public health reasons, as it will conceivably allow a more appropriate assessment of the risk posed by these products, and for gaining insight into scrapie pathogenesis and the natural mode of sheep-to-sheep transmission.

The quantification of infectivity by end-point titration is time-consuming and requires the use of large numbers of animals, and thus animal models with enhanced sensitivity can also be limited in their use by both practical and ethical issues. In the present study, we investigated the use of



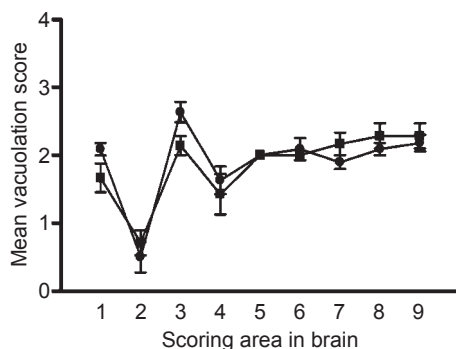


**Fig. 6.** Immunohistochemical and histological analysis of hippocampus from voles infected with vole-passaged scrapie isolate SSUK6. Serial sections of the hippocampus were analysed by immunohistochemistry for PrP (a), PET blot (b), haematoxylin and eosin staining (c) and immunohistochemistry for GFAP (d). (e) and (f) are higher magnifications of the inserts in (c) and (d). Bars, 100  $\mu$ m.

possible alternatives by estimating the levels of infectivity present in the tonsil from a scrapie affected sheep either by incubation time assay, or by PrP<sup>res</sup> quantification.

Incubation time assay was developed several years ago, based on the finding that time intervals between inoculation and onset of illness were inversely proportional to the size of the dose injected by the intracerebral route (Prusiner *et al.*, 1982). It is known, however, that different prion strains can have variable incubation times in the same host, and the relationship between the size of the dose

and incubation time is valid only when comparing inocula containing the same prion strain. Thus, an important prerequisite for the use of incubation time assay to estimate infectious titres in different tissues is that all tissues harbour the same strain. It has been suggested that strains are encoded in the conformation of PrP<sup>Sc</sup> aggregates (Telling *et al.*, 1996) and this, at least in part, can be deduced by the electrophoretic profile of PrP<sup>res</sup> (Bessen & Marsh, 1994). It was previously reported that PrP<sup>res</sup> extracted from lymphoid tissues of sheep with scrapie (Madec *et al.*, 2000) and of human patients with vCJD (Wadsworth *et al.*, 2001) has molecular features different from brain PrP<sup>res</sup>, and these differences can be interpreted as suggestive of the presence of different prion strains in nervous and lymphoreticular tissues. We analysed PrP<sup>res</sup> in tonsil from a scrapie-affected sheep by Western blot, showing that the molecular mass and the glycoform ratio is indeed different from brain PrP<sup>res</sup>, and these findings have been confirmed in several sheep with natural or experimental scrapie (R. Nonno, unpublished results). These results prompted us to compare in voles the biological properties of scrapie prions from brain and tonsil. They showed similar incubation time upon second passage and indistinguishable brain regional distribution of vacuolation upon first and second passages. Furthermore, we found that voles infected with tonsil and brain homogenates accumulate PrP<sup>res</sup> with identical electrophoretic profile. Overall, these results suggest that brain and tonsil harboured the same prion strain showing different glycoprofiles in relation to the different cellular/tissue



**Fig. 7.** Similar pathological phenotypes in voles after second passage of SCR6 and SSUK6 isolates. Lesion profiles in voles infected with SCR6 (closed circles) and SSUK6 (closed square). Brain-scoring positions are as in Fig. 3.

types in which it replicated, and that incubation time assay can be exploited for estimating the infectious titres in lymphoreticular tissues of sheep with scrapie. The infectious titre estimated for the tonsil homogenate was  $\sim 1$  log less than that found in brain, which is consistent with previous findings (Hadlow *et al.*, 1982), indicating that lymphoreticular tissues can contain very high levels of infectivity.

Importantly, we were able to quantify the level of PrP<sup>res</sup> in the tonsil relative to the brain and found that the relative amounts of PrP<sup>res</sup> and infectivity were consistent. This consistency is very promising as it shows that the relationship between brain and tonsil is similar when measured either by PrP<sup>res</sup> or by intracerebral vole ID<sub>50</sub> and indicates that in principle, although endowed with tissue-specific modifications, PrP<sup>res</sup> can be exploited as a useful marker for the quantification of scrapie infectivity.

In order to validate the use of PrP<sup>res</sup> as a quantitative marker of infectivity, it is necessary to perform more detailed studies comparing PrP<sup>res</sup> and infectivity in different tissues, such as kidneys, salivary glands and muscles. Natural sheep scrapie represents an appropriate model for acquiring knowledge on the relationships between PrP<sup>Sc</sup> and infectivity, which may also be extrapolated to other TSEs. The natural Suffolk scrapie source investigated derives from a closed flock maintained since 1980 in which the first case of scrapie occurred in 1990. Thereafter an increase in incidence of scrapie was observed. This flock was subsequently bred to ensure the maintenance of susceptible genotypes and managed to allow the perpetuation of infection. Scrapie isolates from this source do not transmit efficiently to wild-type mice (Bruce *et al.*, 2002) and transmit with long incubation times to tg338 mice (H. Laude, personal communication). In search for an ideal source of sheep with natural scrapie, we investigated the susceptibility of voles to scrapie isolates from this Suffolk flock. The two scrapie isolates studied transmitted very efficiently to voles and induced incubation times and lesion profiles very similar to those observed with Italian ARQ/ARQ scrapie isolates, suggesting that UK and Italian ARQ/ARQ isolates may harbour the same scrapie strain. Furthermore, transmission in tg338 mice of the same isolates studied here also shows that they have similar biological properties (Beringue *et al.*, 2006; H. Laude, personal communication). These findings suggest that voles are a promising animal model for bioassay of non-nervous tissues of Suffolk sheep infected with scrapie and prompt us to exploit this model with the objective to validate PrP<sup>res</sup> as a quantitative marker for scrapie infectivity.

In summary, we report on the sensitivity of bank voles to sheep scrapie, showing that this animal model allows the sensitive detection of prions from two unrelated scrapie sources, one of which is refractory to isolation in conventional mouse models and in at least one over-expressing transgenic line. Furthermore, we investigated

the relationships between PrP<sup>Sc</sup> molecular characteristics, strain and infectious titre in the brain and tonsil of the same scrapie-affected sheep, showing that a PrP<sup>Sc</sup>-based estimate of scrapie infectivity in sheep tissues could in principle be achieved by combining sensitive PrP<sup>res</sup> detection methods and bioassay in voles.

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