Widespread PrP^{Sc} accumulation in muscles of hamsters orally infected with scrapie

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Scrapie, bovine spongiform encephalopathy and chronic wasting disease are orally communicable, transmissible spongiform encephalopathies (TSEs). As zoonotic transmissions of TSE agents may pose a risk to human health, the identification of reservoirs for infectivity in animal tissues and their exclusion from human consumption has become a matter of great importance for consumer protection. In this study, a variety of muscles from hamsters that were orally challenged with scrapie was screened for the presence of a molecular marker for TSE infection, PrP^{Sc} (the pathological isoform of the prion protein PrP). Sensitive western blotting revealed consistent PrP^{Sc} accumulation in skeletal muscles from forelimb and hindlimb, head, back and shoulder, and in tongue. Previously, our animal model has provided substantial baseline information about the peripheral routing of infection in naturally occurring and orally acquired ruminant TSEs. Therefore, the findings described here highlight further the necessity to investigate thoroughly whether muscles of TSE-infected sheep, cattle, elk and deer contain infectious agents.

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in elk and deer, and Creutzfeldt–Jakob disease (CJD) in humans, are fatal neurodegenerative disorders of the central nervous system (CNS). Their causative agent is widely considered to represent a new biological principle of infection. The prion hypothesis (Prusiner, 1982) states that TSE agents (so-called ‘prions’) consist mainly, if not entirely, of a misfolded form of PrP (the prion protein), which is known as PrP^{Sc}. Although the exact molecular structures of TSE agents remain to be elucidated, there is overwhelming evidence that PrP is fundamentally involved in the development of scrapie and related diseases. In particular, PrP^{Sc} consistently accumulates in the CNS of affected individuals, and has also been found in a variety of peripheral tissues from donors

with experimentally induced and naturally occurring TSEs (for a review, see Prusiner, 1998).

Although the aetiology of TSEs is not yet fully understood, the oral route of infection seems to be the, epidemiologically, most relevant pathway for their naturally occurring and accidental transmission within and between species (Diringer *et al.*, 1994). Substantial evidence suggests that several TSEs affecting animals (naturally occurring ovine scrapie, BSE and possibly, CWD) are caused by ingestion of infectious TSE agents (Wilesmith *et al.*, 1991; Kimberlin & Wilesmith, 1994; van Keulen *et al.*, 2000; Schulz-Schaeffer *et al.*, 2000; McBride *et al.*, 2001; Sigurdson *et al.*, 2001). This is also the case for two human TSEs, Kuru and variant CJD (vCJD), of which the former was transmitted by ritualistic cannibalism (Gajdusek, 1977) and the latter is linked to BSE (Bruce *et al.*, 1997; Hill *et al.*, 1997), presumably through consumption of BSE-contaminated foodstuffs. Recently, a possible alimentary transmission of the cervid CWD agent to humans has led to substantial concern, and whether this may pose a potential new TSE risk remains to be elucidated (Belay *et al.*, 2001).

With this background in mind, the identification of reservoirs for TSE infectivity in tissues from cattle and other TSE-susceptible animals that are used for human consumption, and their reliable exclusion from the human food supply, has become a matter of great importance for consumer protection and public health. So far, direct titration of TSE agents is only possible in bioassays, that is, by inoculation of samples into reporter animals. However, when it can be shown to co-purify and to be quantitatively associated with infectivity, as in the animal model used in this study (Beekes *et al.*, 1996; Baldauf *et al.*, 1997), PrP^{Sc} (or its protease-resistant core, PrP^{27–30}) can be used as a molecular marker for the infectious agent.

Muscles from animals provide an important component of human food, and have therefore been examined in several studies for the presence of TSE infectivity or PrP^{Sc}. Until recently, this did not reveal any evidence for significant amounts of TSE agents in this type of tissue (Hadlow *et al.*, 1982; Bradley, 2001). However, a new study by Bosque *et al.* (2002) reported the detection of substantial amounts of infectivity and PrP^{Sc} in hindlimb muscles from mice that had been intracerebrally infected with scrapie. Subsequently, PrP^{Sc} was detected in tongues from hamsters after intracerebral inoculation with several strains of TSE agents (Bartz *et al.*, 2003). These findings emphasize the conclusion by Bosque *et al.* (2002) that “a comprehensive effort to map the distribution of prions in the muscle of infected livestock is needed.”

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Fig. 1 | Highly sensitive western blotting of PrP27–30, the protease-resistant core of PrP^{Sc}, the pathological isoform of the prion protein. Brain homogenates from donor hamsters that were intracerebrally infected with 263K scrapie were digested with proteinase K and serially diluted in sample loading buffer before western blotting. Lane 1, molecular-weight marker; lanes 2–8, samples containing the following amounts of proteinase-K-digested scrapie-infected brain tissue: 1×10^{-6} g (lane 2); 1×10^{-7} g (lane 3); 8×10^{-8} g (lane 4); 6×10^{-8} g (lane 5); 4×10^{-8} g (lane 6); 2×10^{-8} g (lane 7); 1×10^{-8} g (lane 8). Lane 8 corresponds to ~ 1 pg of PrP27–30 or $10\text{--}30 \text{ ID}_{50\text{ i.c.}}$. The film was exposed for 20 min.

Thus, we screened for the presence of PrP^{Sc} in a wide range of muscles from hamsters fed with strain 263K scrapie, an animal model that during the past few years has revealed key pathogenetic features of the spread of infection through the body (Baldauf *et al.*, 1997; Beekes *et al.*, 1998; McBride & Beekes, 1999; Beekes & McBride, 2000; McBride *et al.*, 2001). Similar features have also been observed in naturally occurring sheep scrapie (van Keulen *et al.*, 2000), field cases of BSE (Schulz-Schaeffer *et al.*, 2000) and orally transmitted or naturally occurring CWD (Williams & Miller, 2000; Sigurdson *et al.*, 2001). Using a high-yield purification method, PrP^{Sc} was extracted from muscle tissue in the form of PrP27–30 and visualized immunologically using a highly sensitive western blotting method. With this approach, we found that, in terminally ill scrapie-infected hamsters, all of the examined types of skeletal and other muscles from various parts of the body contained PrP^{Sc}.

RESULTS AND DISCUSSION

To investigate whether, and to what extent, the biochemical marker for TSE infectivity, PrP^{Sc}, accumulates in skeletal, heart and tongue muscles of hamsters after an oral challenge with 263K scrapie, we combined the following approaches: a high-yield purification method for the extraction of this protein in the form of PrP27–30 from tissue samples; deglycosylation of the protein; and sensitive western blotting for its visualization. The latter allows the reliable detection of PrP27–30 in the equivalent of 2×10^{-8} g of diluted, proteinase-K-digested brain homogenate from terminally ill hamsters infected intracerebrally with scrapie (Fig. 1, lane 7). Based on the infectivity titre and content of pathological PrP determined previously in brain homogenates from our model animals (Beekes *et al.*, 1995, 1996), 2×10^{-8} g of scrapie-infected hamster brain homogenate contains $\sim 20\text{--}60$ 50% intracerebral (i.c.) infective doses ($\text{ID}_{50\text{ i.c.}}$). After digestion with proteinase K, this contains ~ 2 pg of PrP27–30.

With these detection thresholds, our screening of tissue samples from orally challenged scrapie-infected hamsters showed that all of the analysed skeletal muscles and tongue specimens from each

animal ($n = 6$) had a consistent, and in most samples substantial, accumulation of PrP^{Sc} (as shown by visualization of the extracted proteinase-K-resistant PrP27–30 by western blotting; Fig. 2A,B).

The immunostaining intensities of skeletal muscles showed variation between different specimens from the same donor, as well as variation for the same muscles from different animals. Varying progression of infection in individual animals and tissues, in addition to unequal samples sizes, may explain this finding. The range of signals shown in Fig. 2A (lanes 2–7), which shows data from one animal, covers the range of PrP27–30 staining intensities observed in all of the samples of skeletal muscles that were blotted in our experiments.

A slightly higher level of PrP^{Sc} appeared to be present in tongue homogenate than in samples from skeletal muscles. The sciatic nerve also showed substantial PrP^{Sc} accumulation (Fig. 2B, lane 6). In the tissues taken from the hamsters orally infected with scrapie that were examined in this study, the heart showed relatively low levels of PrP^{Sc} deposition (Fig. 2E, lane 2), and was found to be contaminated in three out of six animals. Muscle samples from uninfected control hamsters gave consistently negative results (Fig. 2B, lane 7).

To verify that the signals shown in Fig. 2A,B and lane 2 in Fig. 2E represent PrP27–30, aliquots of the corresponding extracts were deglycosylated. After treatment with N-glycosidase F (PNGase F), the bands showed a uniform shift to approximately 19 kDa, the molecular weight expected for the unglycosylated form of hamster PrP27–30 (Fig. 2C,D; Fig. 2E, lane 3). This confirms the identity of the immunolabelled material as PrP27–30.

The staining intensities for PrP27–30 in extracts from muscle samples from scrapie-infected hamsters were comparable with, and frequently higher than, those observed in the positive controls (that is, in muscle tissue from uninfected hamsters, which was spiked with the equivalent of 5×10^{-6} g of homogenized scrapie-infected hamster brain from terminally ill donors; Fig. 2B, lane 3). Therefore, taking into account some loss of PrP^{Sc} and PrP27–30 during the processing of the samples, it can be concluded that the blotted samples of skeletal muscle and tongue (corresponding to 20–50 mg of tissue) contained an amount of PrP^{Sc} that is at least equivalent to that present in 5×10^{-6} g of homogenized scrapie-infected hamster brain. If, in muscles, PrP^{Sc} correlates with infectivity in a similar ratio to that observed previously in the brain and spinal cord (Beekes *et al.*, 1996; Baldauf *et al.*, 1997), this would correspond to an infectivity titre of $\sim 1\text{--}2 \times 10^5 \text{ ID}_{50\text{ i.c.}}$ per gram of tissue (1–2 50% oral infective doses per gram of tissue; Diringer *et al.*, 1998). Bioassay experiments, using intracerebrally and alimentarily administered samples, from terminally ill and preclinically infected hamsters that have been orally challenged with scrapie, should reveal precisely the amount of infectivity and the course of its accumulation in muscle tissue.

Additionally, endpoint and timecourse studies have been set up to identify the exact location of PrP^{Sc} deposition in the muscles of our model animals by immunohistochemistry. This will elucidate whether, and at which timepoint, PrP^{Sc} occurs in association with myocytes (Bosque *et al.*, 2002), neural tissue or other anatomical structures in muscles. These studies are also expected to provide insights into the pathways that mediate the spread of infection to skeletal muscles, heart and tongue. For the animal model used in this study, it is well established that after an oral challenge of hamsters with 263K scrapie, the centripetal spread of infection to the brain and spinal cord involves components of the gut-associated lymphoid tissue and of the enteric nervous system, as well as autonomic fibres of the splanchnic and vagus nerves (Beekes *et al.*,

Fig. 2 | Western blot detection of PrP²⁷⁻³⁰, the protease-resistant core of PrP^{Sc}, the pathological isoform of the prion protein, extracted from muscles and sciatic nerve of terminally ill hamsters that were orally infected with 263K scrapie. (A) Lane 1, molecular-weight marker. Lanes 2–7, muscles from the head (*Musculus (M.) masseter*, lane 2), shoulder (*M. trapezius*, lane 3), forelimb (*M. extensor carpi radialis*, lane 4; *M. triceps brachii*, lane 5) and hindlimb (*M. tibialis cranialis*, lane 6; *M. biceps femoris*, lane 7). Lane 8, proteinase-K-digested brain homogenate from hamsters infected with 263K scrapie, containing 1×10^{-7} g of brain tissue. Lane 9, molecular-weight marker. (B) Lane 1, molecular-weight marker. Lane 2, same sample as in lane 8 of (A). Lane 3, skeletal muscle from an uninfected control hamster, spiked before extraction with 5×10^{-6} g of brain homogenate from hamsters infected with 263K scrapie. Lane 4, muscle from the back (*M. psoas major*). Lane 5, tongue. Lane 6, sciatic nerve. Lane 7, skeletal muscle from an uninfected control hamster. (C) Lanes 1–7, same samples as in (A), but deglycosylated with N-glycosidase F (PNGase F; lanes 2–7). (D) Lanes 1–5, same samples as in lanes 3–7 in (B), but deglycosylated with PNGase F. (E) Lane 1, molecular-weight marker. Lane 2, heart muscle. Lane 3, heart muscle deglycosylated with PNGase F (note molecular-weight marker to the left). The signal at ~35 kDa in (C,D) and lane 3 in (E) was obtained only for deglycosylated samples, and in the mixture of deglycosylation reagents with no tissue extracts (not shown), and is therefore a technical artefact resulting from the deglycosylation procedure. Films were exposed to membranes for 5 min.

1998; McBride & Beekes, 1999; Beekes & McBride, 2000; McBride et al., 2001). Furthermore, after reaching the CNS, the infection may spread centrifugally back into the peripheral nervous system (PNS), thereby further distributing the TSE agent throughout the body. Thus, it is possible that after an oral challenge, muscles are infected through the following pathways: neural pathways, directly or indirectly linked to the ascension of the TSE agent to the brain and spinal cord, including those associated with the lymphoreticular system (LRS); PNS projections mediating centrifugal spread of infection from the CNS; or lymphatic or haematogenous spread after invasion of the LRS. However, which of these theoretical pathways actually mediates the infection of muscle tissue, and whether additional routes of spread should be taken into consideration, remains to be established in more detailed studies.

We detected substantial amounts of PrP^{Sc} in a variety of muscles from hamsters that were orally challenged with scrapie. This confirms and expands the findings of Bosque et al. (2002) in a different

rodent model for scrapie, using the epidemiologically important route of oral transmission, and for a broader spectrum of affected tissues. Our hamster model of oral challenge has been shown previously to provide baseline information about the peripheral routing of infection in naturally occurring and orally acquired ruminant TSEs. The findings described here therefore highlight further the need to investigate thoroughly whether TSE agents are present in the muscle tissue of scrapie-infected sheep, BSE-infected cattle and CWD-infected elk and deer.

However, the peripheral pathogenesis of TSEs needs to be established for each individual combination of host species, strain of agent and route of infection. Thus, for a variety of reasons, the present findings about 263K scrapie in hamsters may not be mirrored in ruminants or humans. First, the levels of infectivity produced in the brain and other tissues of our model animals tend to be higher than in naturally occurring ovine scrapie, BSE and CJD. Second, transmission of disease to non-human primates has not been observed with skeletal muscle or heart isolates from classical CJD cases (Brown et al., 1994), and heart tissue from vCJD cases lacks detectable PrP^{Sc} (Wadsworth et al., 2001). Third, so far, no infectivity has been detected in skeletal muscles and sciatic nerves from cattle with BSE by bioassays in mice and titrations in bovine species (European Commission, 2002).

However, the outcome of the continuing transmission studies in cattle remains to be seen. The same is true for testing in transgenic mice that lack the species barrier to the BSE agent, which may provide a more sensitive test system than wild-type mice. Further exploration of the potential contamination of muscle tissue with TSE agents is needed, not only for scientific reasons but, possibly, also for consumer protection and public health.

METHODS

Animal inoculations and preparation of tissue samples. Outbred Syrian hamsters were fed individual food pellets doused with 100 μ l of a 10% hamster-brain homogenate from donors infected with 263K scrapie, as previously described (Baldauf et al., 1997). The animals were sacrificed at the terminal stage of clinical scrapie (175 ± 10 days after infection, expressed as the mean $\pm$ s.d.; $n = 6$). Heart muscle (apex), lingual muscle (~5 mm from the tip of the tongue), sciatic nerves and seven different skeletal muscles from the hindlimb (*Musculus (M.) biceps femoris*; *M. tibialis cranialis*), the forelimb (*M. triceps brachii* (caput laterale); *M. extensor carpi radialis*), the shoulder (*M. trapezius*), the head (*M. masseter*) and the back (*M. psoas major*) were dissected, and visible nerve fibres were removed from the muscle samples. All samples were then cut into small pieces and stored at -80°C until examination. The mass of the muscle samples ranged from ~40 to ~100 mg, and that of the sciatic nerves from ~5 to ~9 mg. Control samples were taken from uninfected age-matched hamsters. Instruments used for the preparation of samples were carefully cleaned after removal and processing of each specimen, to avoid cross-contamination.

Processing of tissue samples. Samples were washed three times in TBS (10 mM Tris HCl, 133 mM NaCl, pH 7.4) and incubated in a rocking device at 37°C for 3.5 h in 900 μ l of TBS containing 2 mM CaCl_2 and 0.25% (w/v) collagenase A (Roche). For positive controls, muscle tissue from uninfected control donors was spiked by adding 10 μ l of a 0.1% (w/v) 263K-scrapie-infected hamster brain homogenate (10 μ g of brain tissue) from terminally ill intracerebrally infected donors, which contained ~1 ng PrP^{Sc} (1×10^4 ID₅₀/c.; Beekes

et al., 1995, 1996) before collagenase digestion. After ultrasonification, PrP^{Sc} was extracted from the tissue homogenates in the form of PrP27–30, following a previously published protocol; the final pellets were resuspended in 20 µl of distilled water and one-half of each sample was removed, mixed with an equal volume of 2 × sample loading buffer and heated to 100 °C for 5 min, before western blotting to detect PrP27–30 (Beekes *et al.*, 1995). The other half of each sample was deglycosylated using PNGase F (New England Biolabs), in accordance with the manufacturer's instructions, followed by western blotting.

Proteinase-K-digested positive controls, from brain homogenates from terminally ill hamster donors that were intracerebrally infected with 263K scrapie, provided a PrP27–30 standard for the western blot analyses. These were freshly prepared, as described previously (Beekes *et al.*, 1995), and adjusted to various concentrations by serial dilution in sample loading buffer.

Western blot analysis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analyses were performed as described previously (Beekes *et al.*, 1995, 1996), with modifications based on a protocol for sensitive immunodetection, published by Lee *et al.* (2000). After SDS–PAGE, proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore) using a semi-dry blotting system. Membranes were blocked by incubation for 30 min in TBS containing 3% (w/v) non-fat milk powder (NFMP) and 0.05% (w/v) Tween 20 (NFMP–TBST). Blots were incubated overnight at 4 °C in primary antibody solution (monoclonal anti-PrP antibody 3F4 obtained from cultured cells, diluted 1:2,000 in NFMP–TBST). After washing five times for a total period of at least 20 min with NFMP–TBST, blots were incubated in secondary antibody solution (alkaline-phosphatase-conjugated goat anti-mouse IgG (DAKO) diluted 1:5,000 in NFMP–TBST) for 90 min at 20–23 °C. After washing five times with NFMP–TBST, for a total period of at least 1.5 h, membranes were pre-incubated twice for 5 min in buffer solution (100 mM Tris, 100 mM NaCl, pH 9.5) and developed with CDP-Star solution (Tropix; Applied Biosystems) for 5 min, in accordance with the manufacturer's instructions. PrP signals were visualized using X-OMAT AR film (Kodak).

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REFERENCES

Baldauf, E., Beekes, M. & Diringer, H. (1997) Evidence for an alternative direct route of access for the scrapie agent to the brain bypassing the spinal cord. *J. Gen. Virol.*, **78**, 1187–1197.

Bartz, J.C., Kincaid, A.E. & Bessen, R.A. (2003) Rapid prion neuroinvasion following tongue infection. *J. Virol.*, **77**, 583–591.

Beekes, M. & McBride, P.A. (2000) Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie. *Neurosci. Lett.*, **278**, 181–184.

Beekes, M. *et al.* (1995) Western blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method. *J. Gen. Virol.*, **76**, 2567–2576.

Beekes, M., Baldauf, E. & Diringer, H. (1996) Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie. *J. Gen. Virol.*, **77**, 1925–1934.

Beekes, M., McBride, P.A. & Baldauf, E. (1998) Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. *J. Gen. Virol.*, **79**, 601–607.

Belay, E.D. *et al.* (2001) Creutzfeldt–Jakob disease in unusually young patients who consumed venison. *Arch. Neurol.*, **58**, 1673–1678.

Bosque, P.J., Ryou, C., Telling, G., Peretz, D., Legname, G., DeArmond, S.J. & Prusiner, S.B. (2002) Prions in skeletal muscle. *Proc. Natl Acad. Sci. USA*, **99**, 3812–3817.

Bradley, R. (2001) in *Prions: A Challenge for Science, Medicine and Public Health System* (eds Rabenau, H.F., Cinatl, J. & Doerr, H.W.), 105–144. Karger, Basel, Switzerland.

Brown, P., Gibbs, C.J., Rodgers-Johnson, P., Asher, D.M., Sulima, M.P., Bacote, A., Goldfarb, L.G. & Gajdusek, D.C. (1994) Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. *Ann. Neurol.*, **35**, 513–529.

Bruce, M.E. *et al.* (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **389**, 498–501.

Diringer, H., Beekes, M. & Oberdieck, U. (1994) The nature of the scrapie agent: the virus theory. *Ann. NY Acad. Sci.*, **724**, 246–258.

Diringer, H., Roehmel, J. & Beekes, M. (1998) Effect of repeated oral infection of hamsters with scrapie. *J. Gen. Virol.*, **79**, 609–612.

European Commission (2002) Update of the opinion on TSE infectivity distribution in ruminant tissues (http://www.europa.eu.int/comm/food/fs/sc/ssc/outcome_en.html).

Gajdusek, D.C. (1977) Unconventional viruses and the origin and disappearance of kuru. *Science*, **197**, 943–960.

Hadlow, W.J., Kennedy, R.C. & Race, R.E. (1982) Natural infection of Suffolk sheep with scrapie virus. *J. Infect. Dis.*, **146**, 657–664.

Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C., Gowland, I., Collinge, J., Doey, L.J. & Lantos, P. (1997) The same prion strain causes vCJD and BSE. *Nature*, **389**, 448–450.

Kimberlin, R.H. & Wilesmith, J.W. (1994) Bovine spongiform encephalopathy. Epidemiology, low dose exposure and risks. *Ann. NY Acad. Sci.*, **724**, 210–220.

Lee, D.C. *et al.* (2000) Monitoring plasma processing steps with a sensitive western blot assay for the detection of the prion protein. *J. Virol. Methods*, **84**, 77–89.

McBride, P.A. & Beekes, M. (1999) Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. *Neurosci. Lett.*, **265**, 135–138.

McBride, P.A., Schulz-Schaeffer, W.J., Donaldson, M., Bruce, M., Diringer, H., Kretzschmar, H.A. & Beekes, M. (2001) Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves. *J. Virol.*, **75**, 9320–9327.

Prusiner, S.B. (1982) Novel proteinaceous infectious particles cause scrapie. *Science*, **216**, 136–144.

Prusiner, S.B. (1998) Prions. *Proc. Natl Acad. Sci. USA*, **95**, 13363–13383.

Schulz-Schaeffer, W.J., Fatzer, R., Vandevelde, M. & Kretzschmar, H.A. (2000) Detection of PrP^{Sc} in subclinical BSE with the paraffin-embedded tissue (PET) blot. *Arch. Virol. Suppl.*, **16**, 173–180.

Sigurdson, C.J., Spraker, T.R., Miller, M.W., Oesch, B. & Hoover, E.A. (2001) PrP^{CWD} in the myenteric plexus, vagosympathetic trunk and endocrine glands of deer with chronic wasting disease. *J. Gen. Virol.*, **82**, 2327–2334.

van Keulen, L.J., Schreuder, B.E., Vromans, M.E., Langeveld, J.P. & Smits, M.A. (2000) Pathogenesis of natural scrapie in sheep. *Arch. Virol. Suppl.*, **16**, 57–71.

Wadsworth, J.D.F., Joiner, S., Hill, A.F., Campbell, T.A., Desbruslais, M., Luthert, P.J. & Collinge, J. (2001) Tissue distribution of protease resistant prion protein in variant Creutzfeldt–Jakob disease using a highly sensitive immunoblotting assay. *Lancet*, **358**, 171–180.

Wilesmith, J.W., Ryan, J.B. & Atkinson, M.J. (1991) Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet. Rec.*, **128**, 199–203.

Williams, E.S. & Miller, M.W. (2000) Pathogenesis of chronic wasting disease in orally exposed mule deer (*Odocoileus hemionus*): preliminary results. In *Proceedings of the 49th Wildlife Disease Association Conference*, 29.